



Immunoselection and structural evaluation of *Brucella* O-polysaccharide epitopes and their application to the serodiagnosis of bovine brucellosis

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ABSTRACT

Brucellosis is one of the world's most significant zoonosis and is caused by infection with members of the genus *Brucella*. These have a cell wall characteristic of Gram-negative bacteria including lipopolysaccharides and, in the most significant *Brucella* species, O-polysaccharide (OPS). The major economic and health impacts of the disease arise from livestock, in particular ruminants and swine, where the main clinical feature is reproductive failure. The principle source of infection in the general human population is most often via ingestion of unpasteurised dairy products. Serology is the most cost effective means of disease detection but has significant imperfections including false positive serological reactions (FPSRs) due to antibodies that are raised against other Gram-negative bacteria in possession of OPS structures similar to that of *Brucella*. Serology with non-OPS antigens has been largely ineffective and alternative approaches such as bacterial culture, PCR or measurements of cell mediated immunity are impractical, ineffective or unproven.

The OPS from *Brucella* is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyls (D-Rha4NFO) that are variably α -(1→2) and α -(1→3) linked. This structure contains some epitopes that are shared with the OPS from other organisms, notably *Yersinia enterocolitica* O:9, and some that appear to be unique. Previous attempts to harness the unique epitopes using competitive ELISA failed to resolve FPSRs because the unique and common epitopes overlap causing steric hindrance of specific antibody binding.

Oligosaccharides were derived from the OPS of *B. abortus*, *B. melitensis* and *Y. enterocolitica* O:9 by partial acid hydrolysis. These were separated and analysed by chromatography with on-line ESI-QqToF and QqQ. OPS specific antibodies were used to select from this pool of oligosaccharides and those captured were evaluated by graphitised carbon chromatography with on-line ESI-QqQ. On the basis of the mass spectrometry evidence the synthesis of a D-Rha4NFO tetrasaccharide comprised of a single α -(1→3) link flanked on either side by single α -(1→2) links was commissioned. This tetrasaccharide was used to develop an indirect ELISA for the detection of specific antibodies. Equivalent indirect ELISAs were also developed using native OPS and from synthetic penta- and nonasaccharide antigens, received from a collaborating laboratory.

The tetrasaccharide ELISA was the most effective assay for discriminating between bovine FPSRs and true positives. The diagnostic capability of this ELISA was significantly superior ($P < 0.05$) than all others except the pentasaccharide ELISA ($P = 0.159$). The results show that the iELISA developed with the tetrasaccharide may effectively detect antibodies from animals infected with *Brucella* strains with low or high abundance of α -(1→3) links within their OPS and has a significantly improved capability to resolve FPSRs compared to antigens that include common OPS epitopes.

ORIGINALITY DECLARATION

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John McGiven

March 21st 2014

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Abbreviations

$\alpha(1\rightarrow2)$	Alpha linkage from anomeric carbon to carbon 2 on adjacent monosaccharide
$\alpha(1\rightarrow3)$	Alpha linkage from anomeric carbon to carbon 3 on adjacent monosaccharide
Ab	Antibody
ABTS	2,2-azinobis-3-ethylbenzthiazoline-6-sulfonic acid
ACDP	Advisory committee on dangerous pathogens
AFLP	Amplified fragment length polymorphism
Ag	Antigen
AGIDT	Agar gel immunodiffusion test
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt
AUC	Area under curve
B.ab	<i>Brucella abortus</i>
BCA	Bicinchoninic acid
BCV	<i>Brucella</i> containing vacuole
Bm16M/YeO:9	Result from the <i>B. melitensis</i> OPS iELISA divided by that from the <i>Y. enterocolitica</i> O:9 OPS iELISA
BPAT	Buffered antigen plate agglutination test
BSA	Bovine serum albumin
cELISA	competitive ELISA
CFT	Complement Fixation Test
CID	Collision induced dissociation
COSY	Correlation spectroscopy (NMR)
CW	Column wash
DALY	Disability adjusted life years
DC	Direct current
DHB	2,5-Dihydroxybenzoic acid
DIVA	Differentiates Infected from Vaccinated Animals
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dp	Degree of depolymerisation
D-Rha4NFo	4,6-dideoxy-4-formamido-D-mannopyranose
DSn	Diagnostic sensitivity
DSp	Diagnostic specificity
DTH	Delayed type hypersensitivity

EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Standards Authority
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ES	Electrospray
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
ESI-MS/MS	Electrospray ionisation tandem mass spectrometry
FACE	Fluorophore assisted carbohydrate electrophoresis
Fc	Antibody constant region (derived from 'fragment crystallisable')
FITC	Fluorescein isothiocyanate
FPA	Fluorescence Polarisation Assay
FPSRs	False Positive Serological Reactors
Gal	Galactose
Galf	Galactofuranose
Galp	Galactopyranose
GC	Graphitised carbon
GC-MS	Gas Chromatography Mass Spectrometry
GI	Genomic Island
Glc	Glucose
GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
GlcNFo	N-formyl glucosamine
HCl	Hydrochloric acid
HF	Hydrogen fluoride
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
H-VDJ	Antibody heavy chain variable, diversity and joining gene segments
iELISA	indirect ELISA
IFN γ	Interferon γ
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-10	Interleukin-10

Kdo	2-keto,3-deoxyoctulosonic acid
LAL	Limulus Amebocyte Lysate
LC	Liquid chromatography
LPS	Lipopolysaccharide
Lpt	LPS transport pathway
L-VJ	Antibody light chain variable and joining gene segments
m/z	mass to charge ratio
mAb	monoclonal antibody
MALDI	Matrix Assisted Laser Desorption Ionisation
Man	Mannose
MD-2	Myeloid Differentiation factor 2
MHC	Major histocompatibility complex
MHC II	Major histocompatibility complex class II
MLST	Multi-locus Sequencing Typing
MLVA	Multilocus VNTR analysis
MOPS	4-Morpholinepropanesulfonic acid
MS/MS	Tandem mass spectrometry
NBD	Nucleotide Binding Domain
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy (NMR)
OD	Optical Density
OIE	World Organisation for Animal Health
OMP	Outer Membrane Protein
OPD	o-Phenylenediamine dihydrochloride
OPS	O-polysaccharide
oxi-TSM	oxidised TSM antigen
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.05% Tween 20
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PPD	Purified Protein Derivative
Q	Quadrupole
QqQ	Triple quadrupole mass analyser

QqToF	Quadrupole - Time of Flight mass analyser
QuiNAc	N-acetyl Quinovosamine
RBT	Rose Bengal Test
RF	Radio Frequency
rFC	Recombinant factor c
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Units
Rha	Rhamnose
RID	Radial Immunodiffusion
rLPS	rough Lipopolysaccharide
ROC	Receiver Operator Characteristic
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
rt	Room temperature
RV	Reverse Phase
SAT	Serum Agglutination Test
SDA	Serum Dextrose Agar
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide
SEC	Size exclusion chromatography
SFT	Serum flow through
SLD	Soft Laser desorption
sLPS	smooth lipopolysaccharide
SNP	Single nucleotide polymorphism
SP	Strong positive serum
T4SS	Type 4 Secretion System
TCS	Two-component regulatory system
TFA	Trifluoroacetic acid
TLR	Toll Like Receptor
TLR4	Toll Like Receptor 4
TMD	Transmembrane domain
ToF	Time of Flight mass analyser
TR-FRET	Time Resolved Fluorescence Resonance Energy Transfer
TSM antigen	Tetrasaccharide M antigen
Und-PP	undecaprenyl polyisoprenoid lipid carrier

UV	Ultra violet
VLCFAs	Very Long Chain Fatty Acids
VNTR	Variable-Number Tandem Repeat
WHO	World Health Organisation
WP	Weak positive serum
Y	<i>Y. enterocolitica</i> O:9 OPS column selected antibodies
YeO:9	<i>Y. enterocolitica</i> O:9
YI	Youden Index
YxA	<i>Y. enterocolitica</i> O:9 OPS column de-selected then <i>B. abortus</i> S99 OPS column selected antibodies

Chapter 1

INTRODUCTION

1. INTRODUCTION

Brucellosis is one of the world's most significant zoonosis. The WHO estimates that there are more than 500,000 new cases of human infection per year (Pappas et al., 2006) the majority of which occur through contact with infected animals or their dairy products. This estimated minimum is without doubt a significant understatement of the problem. Brucellosis is considered one of seven neglected endemic zoonoses by the World Health Organisation. One reason for its neglect is that the protean nature of clinical signs in humans leads to poor presumptive diagnosis (Araj, 1999), for example symptoms may frequently be mistaken for malaria (Maudlin et al., 2009). Brucellosis is also considered to be a re-emerging disease (Godfroid et al., 2005; Seleem et al., 2010), including within densely populated regions such as found in China (Zhang et al., 2010) and it is certainly a disease that is transboundary. The traditional view is that brucellosis is most prevalent in countries of the Mediterranean, the Middle East, Central and South America and South East Asia. However the disease is also widespread across Central Asia (where global incidence appears to reach a peak in Mongolia) and is undoubtedly present in India, Pakistan and many areas of sub-Saharan Africa (Pappas et al., 2006) where, as in many other places, the disease is under diagnosed and under detected.

Brucellosis is caused by infection with members of the genus *Brucella*. These are non-spore forming gram-negative cocobacillary rods (figure 1.1) with a cell wall characteristic of gram-negative bacteria including lipopolysaccharide (LPS), O-polysaccharide (OPS, giving 'smooth' strains their characteristic appearance), peptidoglycans, and many outer membrane proteins. Transmission of brucellosis between humans is exceedingly rare (Mesner et al., 2007). Thus the global distribution of human brucellosis reflects the incidence of the disease in livestock as these are the principle source of infection. Likewise, a reduction in the prevalence of the disease in animals coincides with a reduction in human infections (Park et al., 2012; Roth et al., 2003) but health education can also play an important role (Jelastopulu et al., 2008).

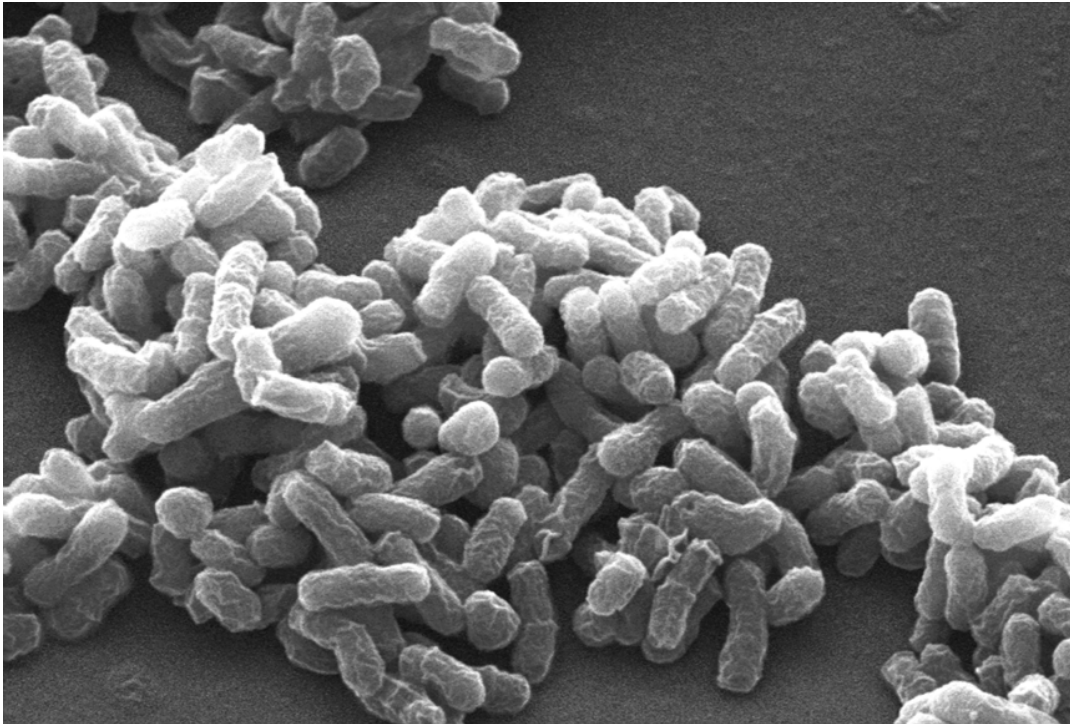


Figure 1.1. *B. abortus* as viewed by electron microscopy
Cells are approximately 0.5-0.7 µm in diameter and 0.6-1.5 µm in length

1.1. History of brucellosis

Brucellosis is an ancient disease. Osteological evidence from the skeletons of those who attempted to flee from the eruption of Mount Vesuvius in 79 AD trace brucellosis back to the time of the Romans (Capasso, 1999; Capasso, 2002). Skeletal evidence for brucellosis has also been found in pre-human remains over 2 million years old (D'Anastasio et al., 2009). Direct molecular evidence, by means of PCR, of the presence of *Brucella* within human remains goes back to the middle ages (Mutolo et al., 2012).

The first identification of the *Brucella* organism itself is credited to the military practitioner Sir David Bruce who, in 1887, reported numerous small coccil organisms (which he named *Micrococcus melitensis*) in the spleens of soldiers with fatal cases of what was then locally called 'Malta Fever' (Bruce, 1887). Ten years later the first diagnostic assay for this disease was developed using the whole cell killed bacteria in a serum agglutination test for the detection of antibodies (Weight, 1897). This test also worked with animal sera and was used to identify specific antibodies in apparently healthy goats. The consequent discovery that such goats were carriers of the disease and shed brucellae in their milk (Zammit, 1905) has been hailed as a significant advance in the study of

epidemiology. Both Bruce and Zammit were members of the Mediterranean Fever Commission (see figure 1.1) that was established to investigate what we now know to be brucellosis. The identification of the source of the disease lead, a few years later, to the elimination of the disease in British military personnel in Malta. The history of the discovery of the first *Brucella* species (*B. melitensis*), the subsequent discovery of the role of animals in the spread of disease and the allocation of credit to those involved is a fascinating and controversial story (Wyatt, 2005).

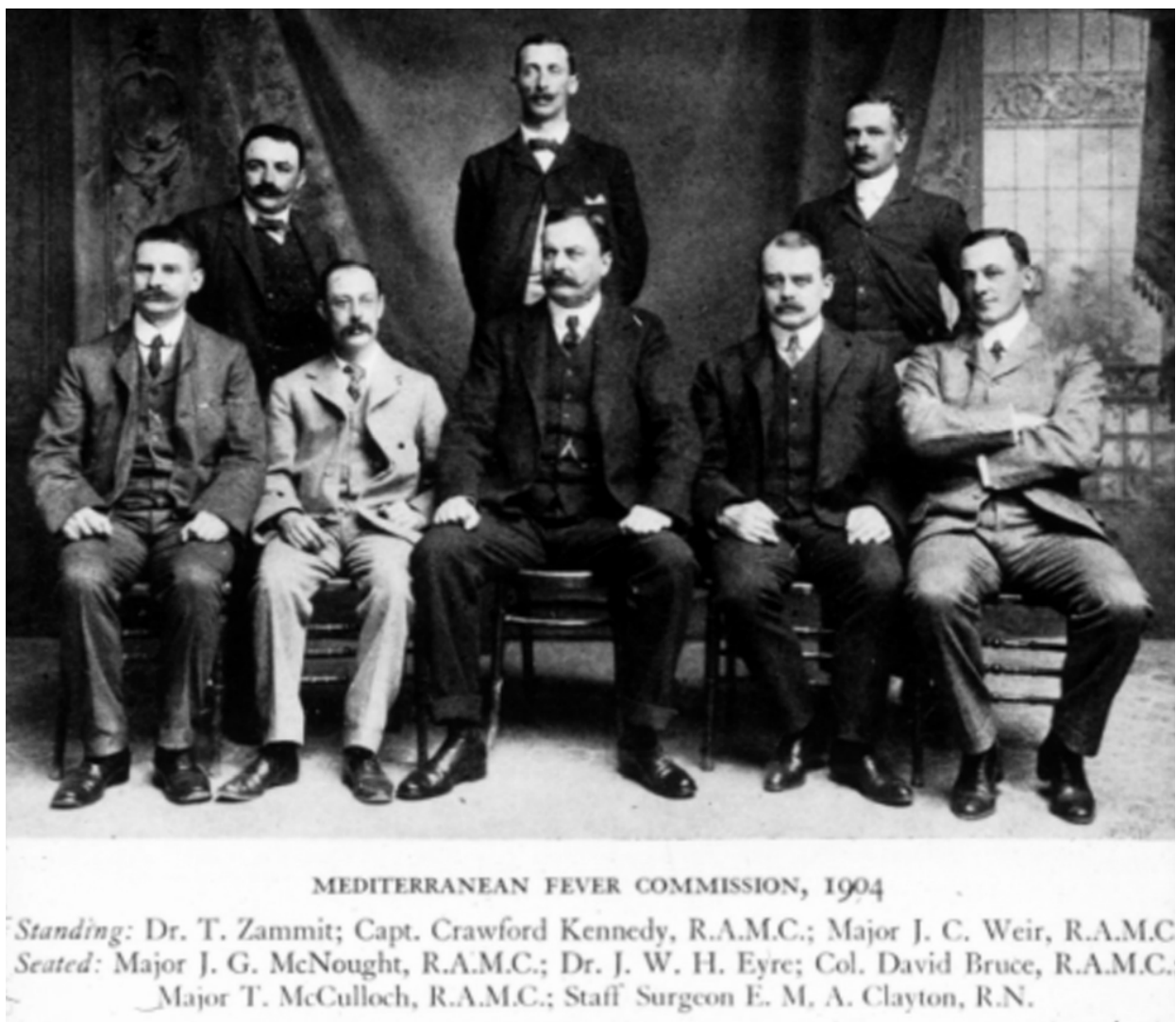


Figure 1.2. Mediterranean Fever Commission

In 1897 a Danish veterinarian isolated and described a bacterial organism which was considered to be a cause of contagious abortion in cattle - Bang's disease (Bang, 1897). This discovery was not connected to that of Bruce ten years previously until 1918 when the similarities between the two

causative organisms were drawn by Alice C Evans (Evans, 1918a). She also suggested the zoonotic capability of the aetiological agent of Bang's disease (Evans, 1918b) which was subsequently confirmed by others including Bevan (Bevan, 1922). The similarities between the organisms were confirmed and the generic name *Brucella*, in honour of Bruce, was proposed (Meyer and Shaw, 1920). A third species, identified as a cause of epizootic abortion in swine in (Traum, 1914) and implicated as a source of brucellosis (Huddleson, 1943) was added to the genus. This completed the discovery of the most pathogenic and zoonotic *Brucella* species.

A fourth species, *B. neotomae*, was isolated from desert wood rats in Utah, USA but has not been found elsewhere (Stoenner and Lackman, 1957). *B. ovis* was identified in rapid succession in both Australia (Simmons and Hall, 1953) and New Zealand (Buddle and Boyes, 1953). It shows a high specificity for sheep and is of negligible to zero zoonotic importance. *B. canis* was first identified as an abortive agent in dogs in the USA (Carmichael and Bruner, 1968) but has subsequently been demonstrated to infect dogs in many other countries.

Although the classical species are often defined in terms of their host preference based on isolation frequencies, the range of hosts infected by a specific *Brucella* species can be enormous. For example, cattle have been described as the primary host for *B. abortus* but it has also been isolated from various orders of mammalian host such as horses, pigs, sheep, goats, Bactrian and dromedary camels, water buffalo, yaks, elk, dogs and humans (Crawford et al., 1990).

1.2. Classification and typing of *Brucella*

1.2.1. Classical biotyping

For many years the taxonomy of *Brucella* was established and invariant and consisted only of the six afore mentioned species. The naturally occurring smooth strains *B. abortus*, *B. melitensis*, *B. suis*, which are responsible for most cases of brucellosis. *B. canis*, *B. ovis*, which are naturally occurring and virulent rough forms and the rare *B. neotomae*. These classical species are so defined mainly on the basis of their primary host - although many strains are capable of infecting other species including humans. The classical smooth strains are sub-divided into biovars on the basis of phenotype: biochemical assays, lysis by phage, growth on dye plates and serotype (Alton et al., 1994; Nielsen and Ewalt, 2010). There are seven biovars of *B. abortus*, five of *B. suis* and three of *B. melitensis*.

The system of biotyping bears some relation to epidemiology but only on a macro scale and provides limited functionality as an epidemiological tool for following and tracing outbreaks of disease. For example, prior to the eradication of bovine brucellosis from Great Britain, Eire, Canada, Australia and New Zealand all *B. abortus* biovars in these areas were predominantly biovar 1. Prior to eradication in the Netherlands, Belgium and France the predominant biovar was 3. The fact that a recent outbreak of *B. abortus* in March 2012 in Belgium and Northern France was biovar 3 therefore came as no surprise.

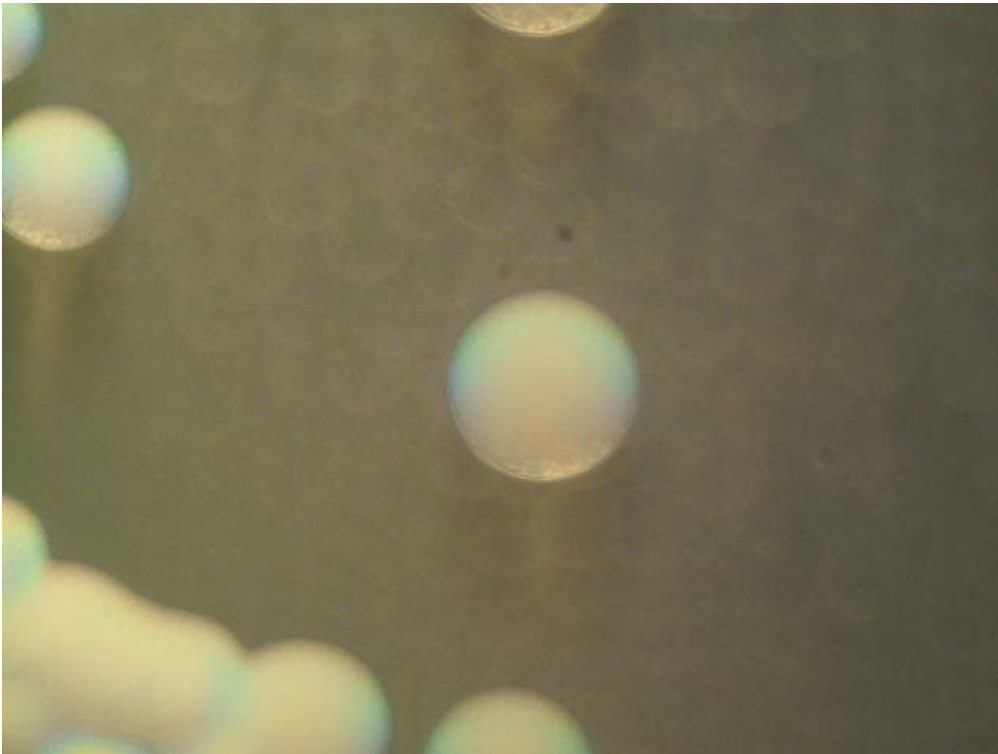


Figure 1.3. A colony of smooth *Brucella* grown on serum dextrose agar

The colony shows the distinctive refraction of light around the edges of the when illuminated by obliquely (Henry's Illumination)

1.2.2. Molecular typing and phylogeny

To biotype strains of *Brucella* requires a high level of skill, experience and access to specialist reagents and facilities. The procedure is also time consuming owing to the need to perpetuate the growth of the stain under study. This increases the burden for biocontainment which requires a high and ongoing investment in facilities and training. Even then the process is not without risk to the operator. Furthermore the discovery of novel strains of *Brucella*, such as those found in marine mammals (Ross et al., 1994), and closely related non-pathogenic organisms challenged and

confounded the existing biotyping scheme (Whatmore, 2009). To address these issues much effort has been put into developing novel classification schemes based upon DNA sequences. Despite the high level of DNA homology between species (Bohlin et al., 2010; VERGER et al., 1985), which from a purely taxonomic point of view suggests that all *Brucella* species should be one with separate subspecies (for example, all *Brucella* species have identical 16S rRNA gene sequences), molecular epidemiology (and taxonomy) has revolutionised the understanding of *Brucella*.

Contemporary research on the development and application of molecular techniques for epidemiological investigation and taxonomic analysis has recently been expertly and comprehensively reviewed in several book chapters (Scholz et al., 2012; Sobral and Wattam, 2012; Whatmore and Gopaul, 2012) but, owing to the progress made, it is worth summarising here.

The genus *Brucella* belongs to the family *Brucellaceae* within the order *Rhizobiales* of the class *Alphaproteobacteria* which is one of the largest and most diverse within the phylum *Proteobacteria*. This phylum contains many species that coexist with and within higher eukaryotes as either symbionts or pathogens in both plants and animals and there seem to be many shared features within the genomes that enable this lifestyle (Batut et al., 2004; Moreno, 1998). From recent genome sequencing analysis it has become evident that *Brucella* share extensive genetic similarity with some plant pathogens and symbionts such as *Agrobacterium* and *Rhizobium*, especially *B. suis*. It seems likely that *Brucella* evolved from an ancestral bacterium that was a soil dwelling associate of plants (Paulsen et al., 2002).

Brucella's closest phylogenetic neighbour is *Ochrobactrum*. Protein profiling by immunoblot revealed a great deal of cross reaction between protein antigens derived from *Brucella* species and *Ochrobactrum anthropi* (Velasco et al., 1998b). A subset of strains proved even more closely related, based on the 16S rRNA gene analysis which were then established as *Ochrobactrum intermedium* to reflect their intermediate position between *O. anthropi* and *Brucella* (Velasco et al., 1998b). *Brucella* and *Ochrobactrum* share more than 97% 16S rRNA gene sequence similarity as well as other phenotypic traits. Under many measures this would be enough to place them within a single genus, which by taxonomic convention would be called *Brucella*. However it is generally felt that in so doing there would be a risk of misinterpretation as to the pathogenicity and transmissibility of strains which may present an unnecessary human and animal health risk and experts have argued against such a move (Ficht, 2010; Whatmore, 2009).

Comparative genomics resolves the *Brucella* strain sequences so far evaluated into species specific clades, with the exception of *B. canis* which falls within the *B. suis* clade. This indicates that at least at this level, the original classification of the classical species is supported by the latest molecular analysis. However, resolving the order of divergence and the subspecies relationships is more challenging and various methods such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and multilocus variable-number tandem repeat (VNTR) analysis (MLVA) (Al Dahouk et al., 2007; Groussaud et al., 2007; Maquart et al., 2009; Whatmore et al., 2005; Whatmore et al., 2006) do not reproduce the biovar relationships as derived from classical typing. When *B. melitensis* strains from human patients were evaluated by MLVA strains isolated from three different regions, West Mediterranean, East Mediterranean and Americana's all clustered on their own distinct branches (Al Dahouk et al., 2007). This suggests that the molecular analysis has more epidemiological meaning than the biotyping methods which did not correlate with geography. In some contrast to *B. melitensis*, *B. abortus* seems to segregate into two main branches, one with biovars 1, 2 and 4 and the other with biovars 3, 5, 6, 7 and 9 however further subdivision fails to reproduce the biovar classifications (Maquart et al., 2009). Due to the opportunity to select both relatively stable and rapidly evolving loci for MLVA analysis this tool has proven extremely useful for both phylogenetic and epidemiological analysis and numerous studies based on MLVA have been reported but few using exactly the same loci. A unified approach would prove extremely useful for those in the field.

Multi-locus sequencing (MLST) approaches (Whatmore et al., 2007) generally define the same species clusters as MLVA, however this technique provides more detailed information on the possible evolutionary pathway of *Brucella*. The data shows a clear divergence of *B. abortus*, *B. melitensis* and *B. neotomae* and then what appears to have been a rapid divergence that resulted in five separate clades, *B. microti*, *B. suis* biovar 5, *B. ovis*, the marine mammal strains and a combined *B. suis* and *B. canis* clade. The MLST method was applied to the two discovered *B. inopinata* and demonstrated their relatively distant relationship from other *Brucella* strains as well as from each other (Tiller et al., 2010a).

Single nucleotide polymorphism (SNP) analysis of 13 whole genomes confirms the overall phylogeny described above (Foster et al., 2009). The discovered SNPs were then applied to 340 diverse isolates which correctly placed each isolate into its molecular clade (Foster et al., 2012). Based on the SNP data the authors speculate that *B. suis* is the most basal lineage and that other *Brucella* species diverged from a *Brucella* ancestor in the last 86,000 to 269,000 years. Whole genome

analysis based on global SNP analysis will rapidly become the standard technique for studying phylogeny and for epidemiology (Scholz and Vergnaud, 2013).

1.2.3. Molecular typing and phylogeny: novel strains

Due to the close genetic relatedness of the *Brucella* species and the uncertain and controversial taxonomic situation the formal description of novel species within the genus has been challenging. It was therefore not until 2007 that the marine mammal strains of *Brucella* were officially recognised as two new species: *B. ceti* and *B. pinnipedialis* with cetaceans and seals respectively as their preferred hosts (Foster et al., 2007). More recent molecular analysis indicates that both new species could be further subdivided up into subclusters (Dawson et al., 2008) one of which seems specific to dolphins leading to the proposal of a novel species (as yet unadopted) named *B. delphini* (Groussaud et al., 2007). Most marine mammal isolates have come from the Atlantic whereas those originating from the Pacific may yet constitute a different species (Maquart et al., 2009). It is not clear what disease, if any, *Brucella* may be causing within their sea mammal hosts as pathology is frequently absent in seropositive animals (Nymo et al., 2011). However pathogenic associations, including abortion, have been made (Miller et al., 1999) and some strains are zoonotic with recorded cases of human brucellosis where strains of marine mammal origin have been isolated (McDonald et al., 2006; Sohn et al., 2003). Experimental infection of cattle with a marine mammal *Brucella* strain resulted in seroconversion and abortion (Rhyan et al., 2001).

Brucella microti was initially isolated from common voles in the Czech Republic (Hubalek et al., 2007) and was published as a novel species a year after the first report (Scholz et al., 2008). A further 12 isolates at least have been discovered, two from common vole, three from soil samples and seven from foxes (Al Dahouk et al., 2010; Scholz et al., 2009). It's persistence in soil suggests a possible environmental reservoir that may hark back to *Brucella*'s ancestral roots. Although *B. microti* shares most of the phenotypic characteristics of the genus *Brucella* it can be readily differentiated by its rapid growth and high metabolic activity – even though it shares 99.4% genetic homology with *B. suis* (Whatmore, 2009). It is highly virulent in mouse models of infection and can replicate within human macrophages (Jiménez de Bagüés et al., 2010) although no cases of human brucellosis have been attributed to *B. microti* to date.

Brucella inopinata ('unexpected') was isolated from a breast implant from a patient in the USA showing clinical signs of brucellosis (De et al., 2008). Using a polyphasic approach (Tindall et al., 2010) this could be clearly defined as a novel *Brucella* species (Scholz et al., 2010) although it has a significantly lower level of DNA sequence similarity in house keeping genes to other *Brucella* and as such it is the most divergent species within the genus. However it shares > 80% DNA relatedness to *B. melitensis* and would therefore belong to the same species if the commonly accepted borderline for species delineation were applied. Another strain, *B. inopinata* BO2, was isolated from a lung biopsy from a patient in Australia (Tiller et al., 2010a). One particularly interesting feature of this strain is that it has a smooth appearance, possesses sLPS but does not react against any anti-*Brucella* OPS specific monoclonal antibodies (Zygmunt et al., 2012). DNA sequence information demonstrates that many of the genes required for the production of typical *Brucella* OPS are missing in this strain but genes suggesting a rhamnose based OPS are present (Wattam et al., 2012). This will be discussed in greater detail later.

Some historically interesting isolates originally attributed to *B. suis*, such as those isolated from rodents in Australia probably require re-classification (Tiller et al., 2010b). This includes *B. suis* biovar 5 for molecular analysis reveals it to be relatively distantly related to other *B. suis* biovars. Atypical strains of *Brucella* have also been isolated from non-human primates in association with cases of still-births (Schlabritz-Loutsevitch et al., 2009) and from African bullfrogs (Eisenberg et al., 2012). The latter are of particular interest as they appear to be motile with a single laterally attached flagellum as has also been described for *O. anthropi*. Genome sequencing confirms the status of both discoveries as members of the genus *Brucella*.

1.3. Impact of brucellosis

Brucellosis is routinely recognised as one of the worlds most important zoonotic diseases and it's heavy impact in many low-income countries has recently been reviewed (McDermott et al., 2013). In some more wealthy countries the disease has been controlled and in some cases eradicated. In middle income countries the disease tends to be present but is monitored and measured. In low income countries the prevalence is typically high and chronically under reported (McDermott and Arimi, 2002). In Africa the prevalence of the disease in ruminants is estimated to be from 8.2% in East Africa to 15.5% in West Africa, 13.2% in North Africa and 14.2% in South Africa. In South Asia the prevalence in ruminants is estimated to be 16.0% (more than 100 million predicted cases per

year). In such areas brucellosis is a neglected disease with little or no control and little or no reporting of cases. In poor countries as a whole, 12% of susceptible livestock animals have recent or current infections reducing production by 8%. On an individual basis the disease causes significant human and animal disease. On a broader basis the impact of brucellosis can be felt economically. For example it was estimated that, on the basis of an individual prevalence in cattle of 4-5%, that the annual loss to the livestock industry in Argentina was US \$60 million, approximately equivalent to US \$1.20 per bovine nationally (Samartino, 2002). In Nigeria, losses have been estimated at an average of US \$3.16 per head of stock. This does not take into account the additional losses due to the costs on human health that arise as a consequence. Attempts to measure the regional economic impact of the human disease have been made using disability adjusted life years (DALYs). This has been done in order to attempt to quantify the benefits from control programmes and justify them (Roth et al., 2003). In this instance, in Mongolia, the control programme implemented was estimated to give a cost-effectiveness of US \$19.1 per DALY averted. As set against the cost effectiveness of other programmes this is considered to be highly rewarding. However, the timescales against which the outcomes are measure are also important. Since the control programme in Mongolia ceased the disease, which was never eradicated, proliferated and prevalence in humans and animals is back to pre-control levels.

These types of quantifications are important as the evaluation of predicted cost benefit ratios are critical in the decision making process when it comes to deciding on a disease control programme and adopting a particular strategy. Within this framework it is critical to consider that the economic and health impact of brucellosis is really only properly understood within the framework of a 'one-health' concept (Plumb et al., 2013). In the past this cost benefit ratio has really only been considered on the basis of benefits to one sector or the other and not in a combined sense. In general successful control of brucellosis appears to provide positive economic outcomes. However more needs to be done in order to quantify these benefits in order to make stronger cases for developing control programmes and reducing the impact of this disease. Innovations, such as new vaccines or more reliable diagnostic assays, will have an impact upon the costs of control programmes and this will in turn affect the economic balance and considerations. Access to funds for disease control is highly competitive and quantifiable justification of benefits is required for a case to be successful. In less wealthy countries dependent upon Western aid programmes funding may often be directed against emerging and threatening diseases, such as avian flu, that present relatively little ongoing downward health and economic pressure compared to embedded and insidious diseases, such as brucellosis, that present less of a threat to the West.

1.3.1. Human brucellosis

The course of human brucellosis has recently been reviewed (Baldi and Giambartolomei, 2013) and the clinical signs and pathogenesis are not typical of that found in livestock. This is perhaps not surprising as humans are not the primary hosts for *Brucella* species and there is no significant human to human transmission. The principle source of infection in the general human population is most often via ingestion of unpasteurised dairy products. Indeed the global map of human brucellosis reflects the prevalence of the disease in animals (Pappas et al., 2006). Animal workers may contract the disease through contact with infected tissues or inhalation of aerosols and laboratory workers are also at high risk (Robichaud et al., 2004).

Human brucellosis is most commonly caused by *B. melitensis* and less so *B. abortus* and then *B. suis*. *B. melitensis*, and *B. suis* biovars 1 and 3 are considered to be the most virulent species followed by *B. abortus* and then the rough species *B. canis* (Corbel, 2006). *B. ovis* is considered non-zoonotic. The relative frequency of the aetiological species is not only due to their virulence in humans but also due to their prevalence in the local animal populations and human behaviour – notably the consumption of raw milk. For example, although *B. suis* biovars 1 and 3 are highly virulent they are relatively infrequently cultured from humans as pig milk is not often consumed. Interestingly *B. suis* biovar 2 appears to be non-pathogenic for humans (Garin-Bastuji et al., 2000; Godfroid et al., 2005).

However, as is the case in other host species, brucellae enter the host via mucus membranes and establish themselves within phagocytic cells, mainly within the reticuloendothelial system. Although brucellosis in humans is rarely fatal it causes significant morbidity. It is primarily a febrile disease that can display a range of protean symptoms that challenge and complicate clinical diagnosis (Franco et al., 2007). The acute fever in humans is frequently associated with bacteraemia as brucellae spread from the lymph nodes that drain the site of incursion and distribute throughout the reticuloendothelial system. This usually occurs within 1 to 6 weeks of exposure but may vary according to the individual, the route of entry, the virulence of the infecting strain and the magnitude of the infectious dose. The maximum temperature of the fever is usually within 38-41°C but may sometimes be substantially higher leading to hyperpyrexia and occasionally death. Typically the temperature is near normal during the early part of the day and then rises sharply. After reaching a peak the temperature falls

rapidly accompanied by profuse sweating. This process gives rise to the term 'undulant fever' and is most frequently observed in untreated cases where the disease has persisted for some time. Failure to correctly diagnose disease frequently leads to initial treatment failure (where any treatment is available) and promotes the tendency of the disease towards persistence and the chronic phase. The recommended treatment is in the form of the concurrent administration of doxycycline and streptomycin. For a completely oral regime doxycycline and rifampicin may be used for a period of six weeks. This alternative is especially useful if subjects become infected with the *B. melitensis* Rev 1 vaccine strain (Blasco and Díaz, 1993) as this acquired streptomycin resistance during the attenuation process. If brucellosis occurs during pregnancy then streptomycin is not recommended but rifampicin is more often used. However, the *B. abortus* vaccine strain RB51 remains virulent for humans and is resistant to rifampicin (Schurig et al., 1991).

In addition to fever, features of the disease in humans include osteoarticular manifestations (most frequently sacroiliitis and spondylitis), hepatomegaly, splenomegaly, lymphadenopathy, genitourinary and neurological complications. Endocarditis is the condition that accounts for most of the mortality attributable to human brucellosis. The mortality rate in untreated cases is between 2-5%. *Brucella* causes little direct effect. The damage to tissue, either bone, joints, components of the central nervous system or cardiac is due to localised inflammatory responses (Baldi and Giambartolomei, 2013). Brucellosis in humans is not clearly associated with reproductive failure and abortion although it is probably statistically linked (Al-Tawfiq and Memish, 2013).

1.3.2. Brucellosis in livestock

The main clinical feature of brucellosis in livestock is reproductive failure which is most evident through abortion. The main route of disease transmission in livestock is through contact with brucellae that have been expelled in large quantities with aborted fetuses (see figure 1.2), foetal membranes, placental material, uterine secretions and contaminated milk. The most common route of infection is via ingestion or inhalation of aerosols. Although *Brucella* may be excreted in semen, the venereal route of infection is relatively minor under natural conditions but may be a significant source of spread via artificial insemination. Vertical transmission may also occur. Infection via the conjunctival sac may also be induced. As a consequence brucellosis may spread rapidly within a herd especially where animals are in close contact.



Figure 1.4. Lambs aborted due to brucellosis

As is also the case in humans, the pathogenesis of brucellosis in livestock has also recently been reviewed (Poester et al., 2013). Invasion of brucellae occurs through the oral and nasal membranes and the digestive tract, although there is little sign of inflammatory activity. After passing through the epithelia the organisms are drained into local lymph nodes such as the retropharyngeal and iliac. Brucellae may replicate within phagosomes in the lymph nodes and from here a bacteraemia will arise which leads to the colonisation of other sites. In susceptible livestock, such as cattle, sheep, goats, and pigs the tissues of the reproductive system are targeted as well as other locations such as the spleen and supramammary lymph nodes. The association between reproductive disorders and brucellosis is the major feature of the disease in the primary hosts: large ruminants (*B. abortus*), small ruminants (*B. melitensis*) and swine (*B. suis*).

It has for some time been suspected that one of the main reasons for the different pathological and clinical manifestations of brucellosis in humans and the primary hosts is the production of erythritol (Williams et al., 1962) by the latter. This is produced in large quantities by placental cells during the last trimester of pregnancy in ruminants and swine (Samartino and Enright, 1996) and is an effective source of carbon and energy for *Brucella* (Rodríguez et al., 2012). More recent work has

demonstrated that the presence of erythritol *B. melitensis* upregulates virulence genes and it acts as a chemoattractant (Petersen et al.). It is also noteworthy that the vaccine strain *B. abortus* S19 is deficient in erythritol utilisation (Williams et al., 1964) although the significance of this has not been fully established. *Brucella* appears to invade the placenta via erythrophagocytic cells and their multiplication leads to an inflammatory response and tissue damage (Carvalho Neta et al., 2008). This in turn compromises exchange between the foetus and the placenta which may lead to abortion which is the principal manifestation of brucellosis in livestock. The most significant clinical feature in males is orchitis which, if it becomes chronic, may lead to fibrosis and permanent infertility.

Bovine brucellosis is caused primarily by *B. abortus* and outbreaks of disease are associated with abortion during the last trimester of gestation, production of weak newborn calves and infertility in cows and bulls (Carvalho et al., 2010). Most cows abort only once, although some may abort a second or a third time (Manthei and Carter, 1950). Even after seemingly normal calvings, vast numbers of brucellae may be shed from infected animals.

The picture of disease incursion therefore varies according to the history of the herd. In a naïve herd perhaps just one or a few abortions may initially occur. However, depending upon the gestation stage of the remaining animals in the herd and the level of exposure, an 'abortion storm' affecting upwards of 50% the pregnant animals will occur. Non-pregnant animals are just as vulnerable to infection and may incubate the bacteria until proliferation during any subsequent pregnancy. Occasionally latently infected cows may give birth to infected calves which may play a role in perpetuating the disease within the herd. A reduction of milk production follows from loss of young and infected cows that are still in milk may be intermittently excreting large numbers of brucellae in this product (Capparelli et al., 2009; Oleary et al., 2006; Sreevatsan et al., 2000). In herds that have been previously exposed, a new incursion of disease will have less extreme consequences as animals which become reinfected are less likely to abort. Some previously exposed animals may also have the necessary immune response to resist infection.

Cattle may be infected by *B. melitensis* (Álvarez et al., 2011; Banai, 2010; Verger et al., 1989) or *B. suis* (Andersen and Pedersen, 1995; Ewalt et al., 1997; Godfroid et al., 2002; Olsen and Hennager, 2010). Infection with either induces antibodies, however these species appear to be less adapted to cattle and have reduced virulence and rates of transmission potentially being self limiting diseases. A similar picture, described below, is observed in small ruminants and pigs where infection with the non-host 'specific' species of *Brucella* may occur but in a seemingly attenuated fashion. Although

host specificities are not 100%, different species of *Brucella* are certainly adapted to a preferred host. The mechanisms that underlie these host preferences are not yet understood.

Although small ruminants are susceptible to some extent to *B. abortus* and *B. suis*, brucellosis is invariably caused by *B. melitensis* and this infection is pathologically and epidemiologically similar to the infection of *B. abortus* in cattle. Reproductive failure is the main clinical feature and transmission of the disease to other animals primarily occurs when brucellae are expelled in large quantities with the placenta, foetal fluids and vaginal discharges that occur after abortion or full term parturition.

The course of brucellosis in pigs is somewhat different from that of ruminants although the routes of infection, transmission and principle clinical outcomes remain the same. The disease is primarily caused by *B. suis* biovars 1, 2 or 3. Pigs may become infected with *B. melitensis* or *B. abortus* but, as observed for other species, such infections are less serious and less sustainable. The disease is more generalised in pigs with some of the additional complications observed, such as joint and bone lesions, appearing with greater frequency than in ruminants. The bacteraemia in pigs also seems to be of longer duration although this may still be intermittent. Abortion may also occur at a much earlier stage of gestation and may be missed such that the first signs of reproductive failure may be a sow's return to oestrus (Megid et al., 2010).

Animal movement and contact that results in ingestion of contaminated material is the main means of transmission of brucellosis between herds and flocks. Clean herds most commonly become infected due to the introduction of pregnant, recently aborted or recently calved animals with brucellosis. In systems where premises are shared, for example grazing areas, then contact between animals from different herds and flocks may spread and perpetuate the disease. The disease may also be introduced via fomites, contaminated food, water and slurry. In outbreaks where epidemiology has revealed no apparent source the emergence of disease following congenital infection has been proposed (Mailles et al., 2012). It has not been ruled out that the disease may re-emerge from within some environmental, non-animal, niche to cause disease. After all, *Brucella* appears to have evolved from soil dwelling ancestors and share many similarities to their phylogenetic neighbours. Re-introduction of disease from wildlife reservoirs is also a risk in many areas.

1.3.3. Brucellosis in wildlife

In many areas wildlife reservoirs of disease present a significant challenge to disease control which necessitates constant vigilance due to the constant threat of re-introduction from this source (Godfroid et al., 2013). This includes the widespread and prevalent existence of *B. suis* in wild boar across Europe (Al Dahouk et al., 2005; Cvetnic et al., 2009; Gregoire et al., 2012; Munoz et al., 2010; Wu et al., 2012) not to mention it's prevalence within the European wild hare population (Gyuranecz et al., 2011), and within North-eastern Australia (Eales et al., 2010), *B. abortus* in North American bison and elk (White et al., 2013), *B. melitensis* in French alpine chamois (Garin-Bastuji et al., 1990b), *B. abortus* and *B. melitensis* in Iberian wild ruminants (Munoz et al., 2010). These reservoirs of potential infection put additional pressure on the interpretation of serologically positive results from surveillance studies in livestock. The isolation of *Brucella* from rare and protected wildlife that share pastures with livestock presents ethical as well as practical challenges to disease control. This adds to the desire to fill the currently vacant niche for a reliable confirmatory diagnostic assay. The pathology of brucellosis in wildlife is considered, generally, to be similar to that observed in livestock (Rhyan, 2013) although the understanding is less advanced.

1.4. Virulence factors (other than OPS)

Brucella is often described as an facultative intracellular parasite, a more accurate description of the lifestyle would be that of a facultatively extracellular intracellular parasite (Moreno and Moriyón, 2002). *Brucella* are easy to propagate *in-vitro* and may also persist for a short time in the environment, stemming from their probable evolution from a soil living ancestor (Audic et al., 2009; Wattam et al., 2014). However, *in-vivo* they maintain a largely intracellular existence within professional and non-professional phagocytic cells. The localisation of infected macrophages in tissues and organs of the reticuloendothelial system such as the lymph nodes, liver and spleen provide the focal points for sustained infection (Moreno and Gorvel, 2004). It is the ability of *Brucella* to survive and propagate within these cells, protected from the action of complement and antibodies, that facilitates their ability to produce chronic and persistent infections (Roop et al., 2004).

The virulence mechanisms of members of the *Brucella* species have only relatively recently been understood. Brucellae do not possess many obvious classical virulence factors such as capsules, adhesins, fimbriae, or excreted toxins (Seleem et al., 2008; Wu et al., 2008). Even the

lipopolysaccharide endotoxin is of unusually low toxicity (Moreno et al., 1981a), although as it turns out this is a key mediator of the invasion process (Barquero-Calvo et al., 2007). Comparative genomics indicates that the brucellae have diverged from a common ancestor with other members of the *alpha-proteobacteria*, such as those within the genera *Bartonella*, *Agrobacterium*, *Rhizobium*, *Sinorhizobium* and *Mesorhizobium*, that inhabit eukaryotic cells (Boussau et al., 2004). In the absence of many classical virulence factors, the study of the host interactions of these relatives has been most informative in unravelling the methods by which brucellae invade and perpetuate within their host (Batut et al., 2004; LeVier et al., 2000).

1.4.1. Host cell invasion

During natural infection the brucellae enter the host via interactions with mucosal barriers and associated epithelial cells. The uptake of brucellae into these cells has been demonstrated *in-vitro* but this occurs to a lesser extent when compared to bacteria that are considered truly invasive (Moreno and Gorvel, 2004). The application of an *in-vitro* lymphoepithelial cell model demonstrated that *B. melitensis* was able to spread rapidly through monolayers containing M-like cells (Paixão et al., 2009). This finding has been reinforced in a detailed study into the role of cellular prion protein (PrPc) during the uptake of *B. abortus* into M cells (Nakato et al., 2012). PrPc has been shown to bind to heat shock protein Hsp60 although the significance of this finding related to cell entry is yet to be confirmed.

Cellular entry of non-opsonised *Brucella* into murine macrophages and human monocytes occurs via lipid rafts through what seems to be an OPS mediated mechanism (Porte et al., 2003) although it is not clear whether the OPS plays an active role or serves to shield Pathogen Associated Molecular Patterns (PAMPs) on the outer membrane. It is notable that although rough mutants of naturally smooth strains enter cells via non-lipid raft mechanisms and are readily killed (Pei and Ficht, 2004), naturally rough strains may enter via lipid rafts (Martín-Martín et al., 2010). Uptake via this route seems to take place without significant activation of the host cell although there is evidence to suggest a role for class A scavenger receptor (SR-A) interaction (Kim et al., 2004). Whereas rough strains are rapidly internalised, as early as one hour post infection, the internalisation of smooth *Brucella* may take up to 24 hrs (Turse et al., 2011). *Brucella* entry is also dependent on the two-component regulator BvrR/BvrS. This regulator has a highly significant impact on the behaviour of *Brucella* and amongst the genes it regulates are those that govern the acylation status of the LPS

lipid A (Manterola et al., 2005) - a major Gram-negative PAMP - as well as outer membrane proteins (Guzmán-Verri et al., 2002; Lamontagne et al., 2007).

Once within cells brucellae reside within a vacuole, known as the *Brucella* Containing Vacuole (BCV), and this undergoes extensive interaction with the endocytosis and secretory pathways. A summary of this process is shown in figure 1.5. This interaction occurs immediately after internalisation yet is very transient (Celli et al., 2003). Early BCVs are enriched in cholesterol and flotilin-1, a protein associated with lipid rafts, phagosome maturation and interaction with endosomes. BCVs go on to lose these early endosomal markers and acquire the late endosomal marker LAMP1. Other markers that lead to interactions with lysosomes are acquired at a much lower rate in BCVs containing live as compared to dead *Brucella* (Starr et al., 2008). Several studies have shown that lysosomal enzymes such as cathepsin D cannot be found on BCVs containing live cells although the evidence suggests that the route to the endoplasmic reticulum (ER) derived replicative niche involves controlled and limited fusion with lysosomes and late endosomes. It is now clear that the type four secretion system (T4SS) is a key virulence factor that effects the favourable passage of the BCV to its ER derived niche (de Jong and Tsolis, 2011). T4SS deletion mutants are highly attenuated and their BCVs undergo extensive interaction with lysosomes and interact less with the endoplasmic reticulum.

1.4.2. The Type 4 Secretion System

The T4SS is one of several systems that bacteria use to transport macromolecules such as proteins and DNA across their cell wall. There are three basic functional T4SS types: those that transfer DNA between bacteria in a process known as conjugation, those that take up DNA in a process known as transformation, and a third type used to secrete proteins (Wallden et al., 2010). This third type are an important virulence factor in many pathogenic bacteria. The T4SS can be further subdivided into IVA and IVB types. The archetypal IVA T4SS found in the plant pathogen *Agrobacterium tumefaciens* consists of 12 genes, VirB1-11 plus VirD4. This is the type found in *Brucella* although it possesses a VirB12 gene rather than VirD4 (O'Callaghan et al., 1999) all of which sit within the *virB* operon. This IVA type is also found in human pathogens such as *Bartonella* and *Rickettsia* species, *Bordetella pertussis*, *Anaplasma phagocytophilum* and *Helicobacter pylori* where it is used to secrete host effectors proteins. Pathogens with IVB T4SSs include *Legionella pneumophila* and *Coxiella burnetii*.

Of the 12 proteins that make up the IVA T4SS, some are located within the periplasm and cytoplasm whereas others form the channel through the membrane through which effectors are passed. This channel is formed of VirB6-10 with 14 copies each of VirB7, 9 and 10, the latter acting as scaffolding which is inserted in both the inner and outer bacterial membranes. The pillus is composed of VirB2 and VirB5 (Terradot and Waksman, 2011).

Although the T4SSs may be similar, the effector proteins secreted vary considerably and, naturally enough, the effects vary (Voth et al., 2012). The T4SS in *Bartonella* is required to modify the route of host cell ingress to ensure the creation of the ivasome that is required for infection. In *Rickettsia* species the T4SS is essential in enabling the invading bacteria to escape the phagosome shortly after uptake into epithelial cells. *A. phagocytophilum* is an obligate intracellular pathogen that invades granulocytes via lipid rafts. The resultant inclusion body lacks late endosomal and lysosomal markers and lysosomal fusion is inhibited, as is IFN γ production. The TS44 appears to be a critical component in this process. *L. pneumophila*, like *Brucella*, replicates within an ER derived vacuole, the derivation of which requires the T4SS. Unlike *Brucella*, the type IVB T4SS of *L. pneumophila* is known to secrete mobile plasmids and over 300 effector proteins have been identified (Hubber and Roy, 2010).

The acidification of the BCV at the early stage of phagosome maturation is a crucial phase in the lifecycle of *Brucella*. The reduction of pH and deprivation of nutrients leads to the engagement of virulence factors such as the type IV secretion system (T4SS) (Boschiroli et al., 2002), and this is critical for the establishment of the replicative niche (Celli, 2006). Although the majority of the *Brucella* cells die during the infective process, once this niche is established, through fusion with the ER approximately 12 hours post infection, it creates something of a safe haven. Once within this niche, smooth *Brucella* may replicate with little disruption to the integrity of the host cell and appear to suppress apoptosis (He et al., 2006). Escape from the host cell and spread of infection may be mediated by subversion of autophagic processes (Starr et al., 2012) or through generation of rough progeny, through dissociation, that are cytotoxic (Turse et al., 2011).

On the intracellular journey towards this niche the BCVs acquire many ER proteins such as lectin chaperones calnexin and calreticulin (Celli et al., 2003). The mechanism by which the fusion of the BCV with the ER takes place remains to be described but appears to occur at ER exit sites via interaction with the Sar/COPII complex. The actions are most probably supported via proteins secreted from the T4SS. Inhibition of the small GTPase Rab2, which is known to play a role in the vesicular trafficking from the Golgi to the ER, prevents BVC-ER fusion (Fugier et al., 2009). It was

subsequently discovered that Rab2 interacts with a protein translocated from the *Brucella* T4SS, RicA (de Barsy et al., 2011). So far, this is the only T4SS secreted protein for which a function has been indicated, as shown in figure 1.5. Otherwise despite some recent developments the effectors secreted by the *Brucella* T4SS and their actions are relatively poorly understood (De Jong et al., 2008; Marchesini et al., 2011). It is clear however that the T4SS is essential to virulence in all *Brucella* species. The BCVs of VirB deficient strains are incapable of sustaining interaction with the ER and eventually fuse with lysosomes and are degraded. Such mutants are also attenuated in the mouse model (Kahl-McDonagh and Ficht, 2006) as well as in goats (Kahl-McDonagh et al., 2006).

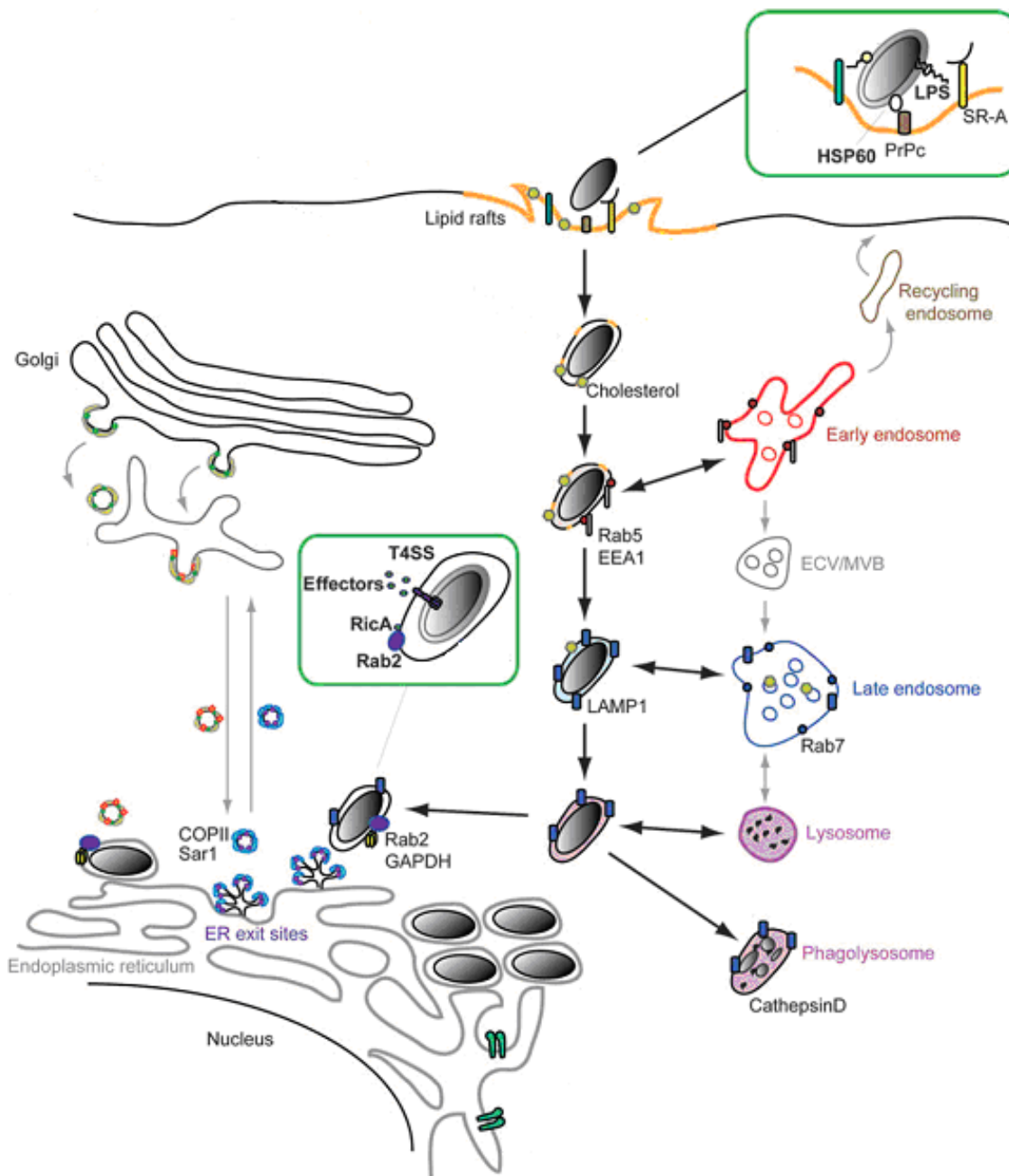


Figure 1.5. The intracellular trafficking pathways of *Brucella*

The intracellular trafficking pathways of *Brucella* within host eukaryotic cells from the point of entry to the establishment of the replicative niche. The top green box indicates the possible reactions between the LPS and HSP60 *Brucella* with the host cell surface and the interplay with class A scavenger receptors (SR-A) and the cellular prion protein (PrPc) within lipid rafts. On their route to the ER the BCVs interact transiently with endosomes and lysosomes acquiring some of the indicative markers of that process. Once located within the proximity of the ER exit sites the secretion of RicA by the T4SS interacts with Rab2 at the interface of the vacuole to facilitate the formation of the ER derived replicative niche. Many BCVs are not successful and those containing dead cells acquire cathepsin D. Image adapted from (von Bargen et al., 2012).

1.4.2.1. Regulation of the T4SS – Quorum Sensing

Expression of the *Brucella* T4SS is closely controlled by several regulatory systems. During macrophage internalisation the *virB* operon is rapidly expressed reaching its maximal activity after approximately five hours post infection. Once replication begins, the expression is suppressed (Sieira et al., 2004). The promoter region of the *virB* operon interacts with a number of different regulators, foremost amongst which is quorum sensing protein VjbR (Delrue et al., 2005).

Quorum sensing (QS) systems allow bacteria to coordinate gene expression according to the local population density. They are an important aid to virulence. Such systems are based on signalling molecules that can transmit messages between individual bacteria that result in modulation of gene expression. Compared to the understanding of quorum sensing in model organism such as *P. aeruginosa* and *V. cholerae* the level of knowledge about such systems employed by *Brucella* are still in their infancy. However, it is clear that QS has a vitally important role in virulence.

Brucellae synthesise a C₁₂ N-acyl homoserinelactone (AHL) quorum sensing molecule (Taminiau et al., 2002). AHL based QS depends on two main components other than the AHL itself, its synthesising enzyme and the cognate transcriptional regulator upon which it acts. Most of these pairs are genetically linked. Three families of AHL synthase have been identified to date, the most common of which is the Lux family. The associated regulators are classed as LuxR and contain a C-terminal DNA binding and an N-terminal AHL binding domain. Most of these regulators are transcriptional activators whereby in the presence of AHL, RNA polymerase is recruited to the target promoter. Other LuxR regulators bind DNA and repress transcription but are released in the presence of AHL.

Five LuxR type transcriptional regulators have been identified within *Brucella* based on DNA sequence assignment. Two of these have been investigated more closely, only one of which, VjbR, has been associated with virulence (Delrue et al., 2005; Weeks et al., 2010), the other being BabR. Despite homology searches, the cognate AHL Lux synthase has not been identified. VjbR is a direct activator of the *virB* operon (Uzureau et al., 2010). Transcription is decreased in the presence of C₁₂-HSL and the ability of *Brucella* to reach its replication niche is impaired. It has been shown that C₁₂-HSL binds VjbR disassociating it from the *virB* operon promoter (Arocena et al., 2010). Unlike what has been observed for other Lux regulators, it therefore seems as if VjbR is a transcriptional activator when bound to the promoter.

During the non-replicative stage when ingested *Brucella* is being trafficked towards its niche, the relatively low levels of C₁₂-HSL would condition the cells for their current environment, such as the production of the T4SS. Having arrived at the safe replicative vacuole; the levels of C₁₂-HSL may increase shutting down systems, such as the T4SS which are now redundant. Throughout its entire intracellular life *Brucella* is contained within vacuoles and it is thus likely that the quorum sensing signal doesn't exceed this space and it may be used to monitor the intracellular confinement state (Carnes et al., 2010). This may be the reason why *Brucella* produces a relatively low level of QS signal.

A search across the available *Brucella* genomes revealed 144 other genes with highly similar promoter sequences to the *virB* operon that may also be regulated by VjbR in this manner (De Jong et al., 2008). VjbR seems to have an effect on many more (Uzureau et al., 2010). *Brucella* also appears to possess AHL degrading enzymes, 'quorum quenchers', that are themselves regulated by VjbR and induced by C₁₂-HSL. Owing to the widespread activity of VjbR, and also BabR, it appears that quorum sensing has a global regulatory influence and is highly significant in directing the passage of *Brucella* through the host cell. As expected *VjbR* deletion mutants are highly attenuated.

1.4.3. Two component regulatory systems

As a facultative intracellular pathogen, *Brucella* must adapt to a wide range of environments. One means by which to achieve this is via two component regulatory systems. These consist of a transmembrane sensor kinase which reacts, via autophosphorylation, to a particular extracellular signal, for example a reduction in pH. The phosphate group is then passed on to the response regulator that triggers a cascade that leads to changes in gene transcription (Casino et al., 2010). Genome analysis of *Brucella* has revealed the existence of potentially 22 sensors and 24 regulators, although only those that are linked pairs are believed to be fully functional (Lavín et al., 2010). Although not the first discovered, the BvrR/BvrS (Sola-Landa et al., 1998) TCS is the best studied. It is well conserved within the α -*Proteobacteria* with homologues found in *Agrobacterium*, *Rhizobium*, and *Bartonella* species. The BvrR/BvrS appears to be critically involved in brucellae making the switch from an extracellular to an intracellular lifestyle.

The BvrR/BvrS regulates over 100 genes including the *virB* operon and the LuxR-type regulator VjbR (Lamontagne et al., 2007; Martinez-Nunez et al., 2010; Viadas et al., 2010). BvrR has been shown to bind directly to the *virB* promoter and it has also been shown to activate the transcription of VjbR. This system has been shown to regulate outer membrane proteins (Guzmán-Verri et al., 2002). Full proteomic analysis of outer membrane fractions demonstrated differences in expression by *BvrR* and *BvrS* mutants for 167 proteins (Lamontagne et al., 2007). An analysis of the full transcriptome identified 127 genes which were found to be differentially expressed including many relating to the outer membrane (Viadas et al., 2010). It also effects the acylation of the lipid A within the LPS which increases the sensitivity of the organism to cationic peptides with a concomitant decrease in virulence (Manterola et al., 2005). Mutants are more sensitive to the bactericidal action of non-immune serum and are also incapable of replicating within macrophages (Grilló et al., 2006; Sola-Landa et al., 1998). There are however, no noted changes to the OPS.

Thus it currently seems as if the BvrR/BvrS TCS acts as a master regulator controlling many of the adaptations of *Brucella* that promote virulence. The severe attenuation of these mutants, coupled with their retention of unaltered OPS structures on the outer membrane, has lead to them being proposed as candidates for live vaccines and the experimental protective efficacy has been shown to be similar to the conventional *B. abortus* S19 vaccine (Grilló et al., 2006).

1.5. Virulence mechanisms: rough LPS and the 'stealthy strategy'

Given the lack of the more obvious virulence factors (Seleem et al., 2008) the pathogenic properties and virulence of brucellae were not well understood until relatively recently. The currently accepted paradigm is that the lack of such factors enables brucellae to evade the immune system and become established before the host mounts a significant immune response. The atypical nature of the LPS endotoxin, lipid A being the toxic component, appears to be a critical element within this so called 'stealthy strategy' (Barquero-Calvo et al., 2007). In contrast to other species, the lipid A of *Brucella* is a weak endotoxin (Moreno et al., 1981a) and thus must surely be related to its not being effectively recognised by Toll Like Receptor 4 (Lapaque et al., 2006b) present on the surface of cells of the innate immune system. Whereas the adaptive response is mediated primarily by lymphocytes, the innate immune response is governed mainly by macrophages and dendritic cells. The two responses are linked as the environment created by the innate response having a governing role in determining

the type of adaptive response. The interaction of the LPS with the innate immune response has a significant bearing on the development of the adaptive response (Vella and McAleer, 2008).

This initial interaction is achieved via a number of recognition receptors that recognise non-self structures that are common within the repertoire of microbial pathogens. These are known as Pathogen Associated Molecular Patterns (PAMPs) and their recognition serves as an early warning system for the host and stimulates the initial responses as well as setting the framework for the adaptive response. Of these, the Toll-Like Receptors (TLRs) are amongst the most important (Takeda and Akira, 2005). There are currently thought to be 13 different TLRs in the mammalian genome (10 identified in humans, 12 in mice), and each serves a different purpose, recognising different PAMPs and triggering a different blend of immune response. The PAMP recognition element of TLRs are horseshoe shaped solenoid structures (Park and Lee, 2013). Their binding draws these complexes into aggregates and this leads to a cascade of intracellular signalling that results in the activation of NF- κ B, IRF3 and the subsequent production of cytokines (Takeda and Akira, 2005).

As a common feature of all Gram negative bacteria, the LPS is a PAMP recognised by the innate immune system via TLR4 (Park and Lee, 2013). LPS binds to CD14, a soluble molecule which breaks up LPS aggregates, formed due to its amphiphilic nature, into monomers that may then be presented to the TLR4-MD2 complex on the host cell surface (Park et al., 2009). It is the lipid A portion of the LPS that is chiefly recognised by the TLR4-MD2 complex as the associated carbohydrates in the core or O-polysaccharide may be highly variable.

The typical lipid A has a di-phosphorylated di-glucosamine backbone to which between 4 to 7 acyl chains, of variable length, are attached. Although generally conserved, even this basic structure may contain a considerable number of permutations. However, CD14 has quite a broad binding spectrum which helps to account for this. MD-2 is smaller than TLR4 yet is the main LPS binding element within the complex. It has two anti-parallel β -sheets that allow it to make its hydrophobic interior accessible to the environment and this is the site for binding to the relatively short, hydrophobic, chains of the lipid A. These acyl chains are inserted deep into the hydrophobic pocket of MD2 with the negatively charged phosphate ions protruding which interact with the TLR4. These interactions lead to a dimerisation of the LPS, MD2 and TLR4 complex (Park et al., 2009) and this stimulates the intracellular signalling pathways.

After TLR4-MD2 dimerisation, subsequent downstream reactions are mediated through the recruitment of toll/interleukin-1 receptor (TIR) domain containing adaptor proteins as shown schematically in figure 1.6.. This includes myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor like (MAL)/TIR domain containing adaptor protein (TIRAP), TIR-domain encoding adaptor protein inducing IFN- β (TRIF) and TRIF related adaptor molecule (TRAM). Dependent upon the subsequent direction of intracellular signalling, stimulation via TLR4 leads to a pro-inflammatory or a type 1 IFN response. The former is mediated through a MyD88 dependent pathway whilst the latter occurs through a TRIF dependent pathway. Both involve induction of NF- κ B although this is delayed in the TRIF pathway which induces type 1 IFNs via IRF-3 (Kawai and Akira, 2006; Takeda and Akira, 2005).

Endotoxins from *Brucella*, and its phylogenetic neighbour *Ochrobactrum anthropi* promote significantly lower levels of pro-inflammatory chemokines and cytokines than the equivalent endotoxins from *Y. enterocolitica* and *E. coli* (Bhattacharjee et al., 2006; Dueñas et al., 2004; Goldstein et al., 1992; Lapaque et al., 2006b). The significance of the delayed onset of the innate immune response is demonstrated by the differential brucellacidal ability of macrophages that are activated pre- or post infection. Pre activated macrophages are significantly more brucellacidal than those activated 24 hours post infection which in turn demonstrated capabilities that were little different from those of naïve macrophages (Barquero-Calvo et al., 2007).

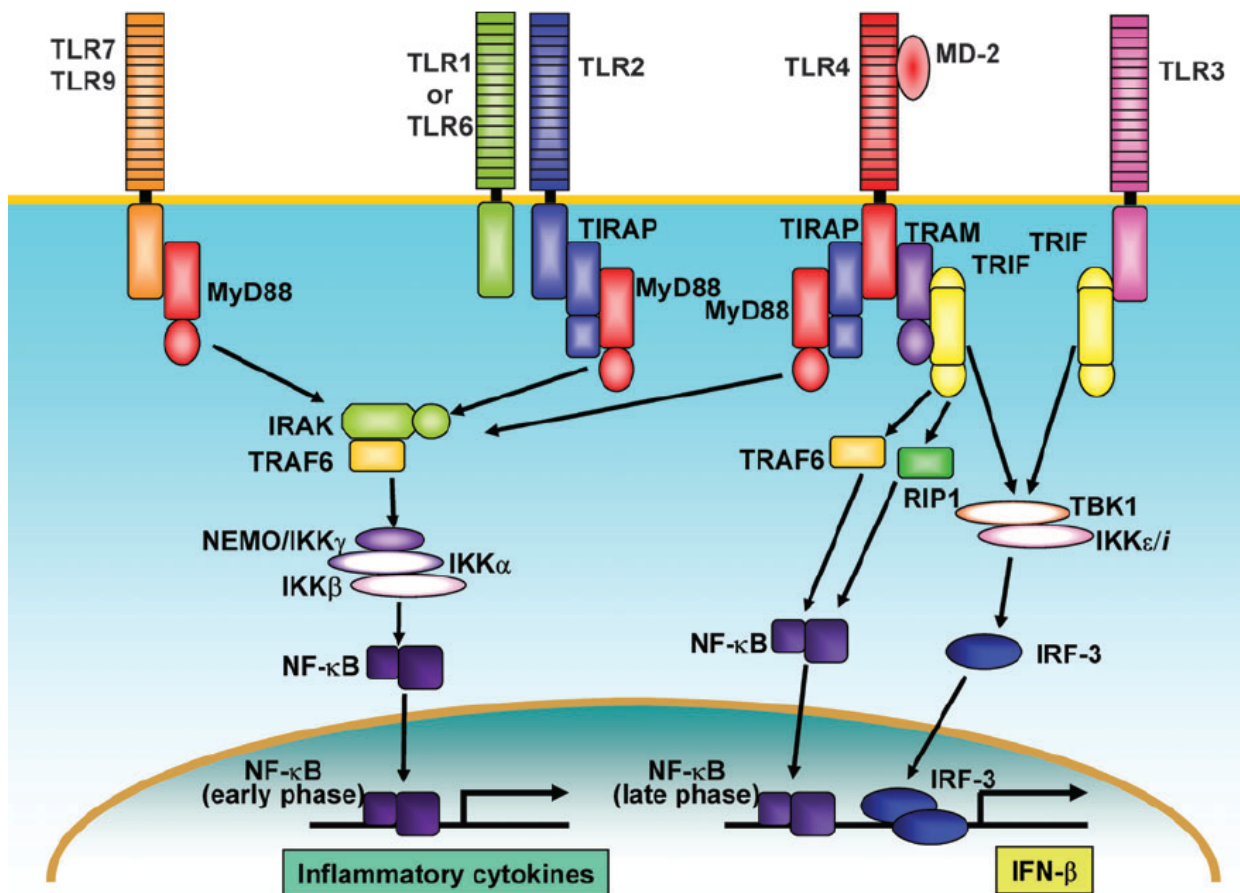


Figure 1.6. Schematic representation of TLR signalling pathways

The recognition of endotoxin by cells of the innate immune system takes place through TLR4 in combination with MD-2. The recognition event triggers intracellular signalling pathways that may take one of two paths. The MyD88 pathway, facilitated by TIRAP, results in the induction of NF-κB and rapid production of inflammatory cytokines. The route is shared by TLRs 1, 2, 6, 7 and 9. The TRIF pathway, facilitated by TRAM, results in a slower induction of NF-κB and the release of IFN-β. Figure reproduced from (Takeda and Akira, 2005).

These properties can be linked to the structure of the LPS. Atypical features of the lipid A from *Brucella* and *O. anthropi* include long fatty acid acyl chains (Barquero-Calvo et al., 2009; Qureshi et al., 1994), as shown in figure 1.7, leading to hydrophobic aggregation and potentially enhanced resistance to cationic peptides. The binding of the lipid chains into MD-2 is a key part of the activation process and the unusually long lipids chains possessed by *Brucella* and *Ochrobactrum* are thought to impede the binding process (Barquero-Calvo et al., 2009).

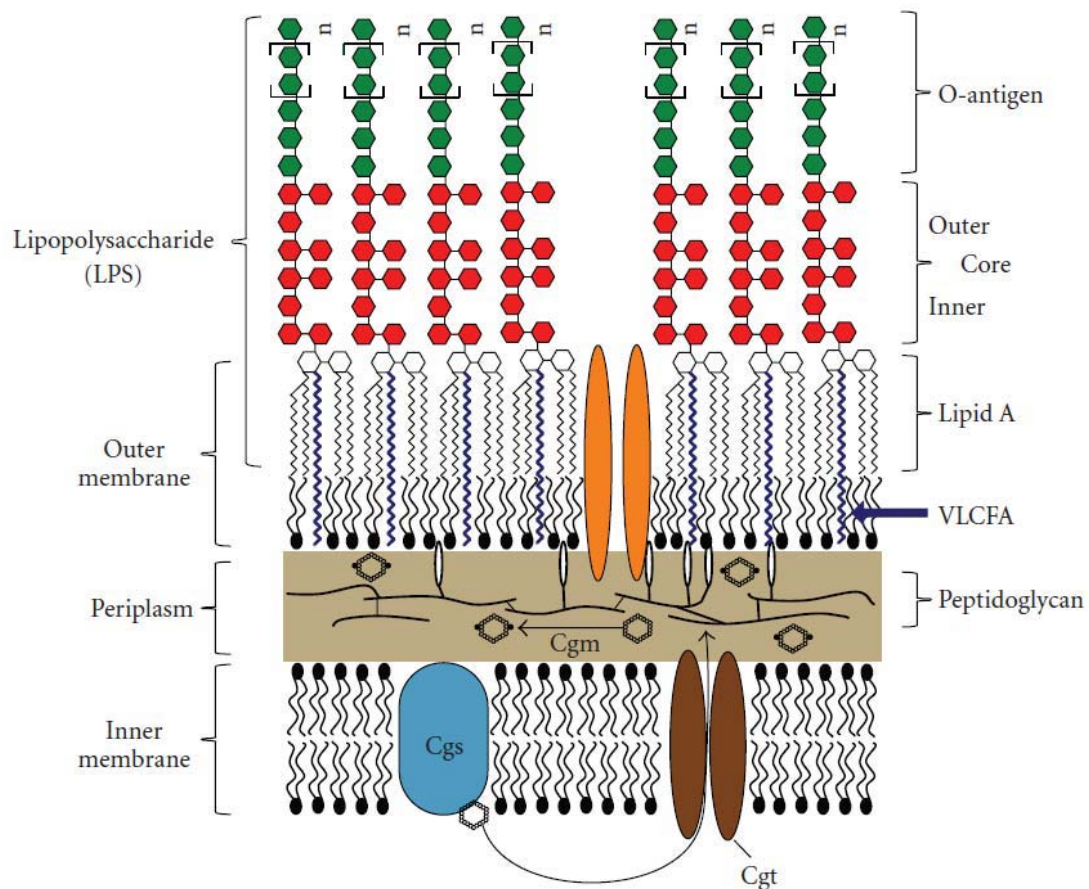


Figure 1.7. Schematic depiction of the *Brucella* cell membrane

This shows the inner membrane which comprises a bilayer of phospholipids, a peptidoglycan within the periplasm and an outer membrane within which the sLPS is embedded. The external surface of the outer membrane contains the lipid A portion of the sLPS and this is anchored into the membrane by very long chain fatty acids (VLCFAs). These are attached to a diaminoglucose backbone (white hexagons) to which the core sugars (red hexagons) are attached. The OPS (green hexagons) extends from the core into the extracellular environment. Image reproduced from (Haag et al., 2010).

Yet, despite their similar yet atypical endotoxin structures, the inflammatory immune response induced by *Brucella* endotoxin is significantly lower than that induced by that of its phylogenetic neighbour (Barquero-Calvo et al., 2009) and its resistance to cationic peptides significantly greater (Velasco et al., 2000). This can be attributed to further structural adaptations that have evolved which appear to have facilitated the change from an ancestral soil based eukaryotic symbiont into stealthy pathogen.

In addition to the structure of the lipid A the entotoxicity of the LPS may be mediated the structure of the core sugars. Typically many of these possess negative charges which assist in the maintenance of the hydrophobic cell barrier and are thus conserved. Consequently they make good targets for cationic peptides such as polymyxin-B and, significantly, modulation of these core characteristics can have a significant impact upon the interaction of the attached lipid A with the TLR4-MD2 complex. The core sugar structure of *Ochrobactrum* (Velasco et al., 1998a) possesses relatively few negative charges but that of *Brucella* appears to possess fewer still, those present due only to Kdo (2-keto,3-deoxyoctulosonic acid) and an increased number of positive charges due to the number of aminated sugars, such as glucosamine, present (Kubler-Kielb and Vinogradov, 2013b). This is reflected in the relative resistance of *Brucella* to cationic peptides (Velasco et al., 2000) and may account in part for its enhanced pathogenic capability.

The unusual positively charged state of the *Brucella* core sugars may also interfere with the multimerisation of the TLR4-MD-2 complex, which is driven in part by the negatively charged phosphate groups on the sugar backbone of the lipid A, and thus reduce the intracellular signal imparted. This theory is supported by the finding that the elimination of a branch of the core, not linked to OPS, in a *wadC* mutant strain lead to a higher induction of proinflammatory cytokines than induced by the parental strain and this was shown to be TLR4 mediated. In particular the LPS from the mutant demonstrated a higher degree of binding to MD2. This is believed to be the first description of a mutation in the core leading to a differential stimulation of the TLR4-MD2 complex (Conde-Álvarez et al., 2012). The mutant also had increased susceptibility to complement C1q and polymyxin B. It is notable that *wadC* homologues are not only found in all stains of *Brucella* sequenced to date but also within *Bartonella* species. Such mutants make potentially attractive vaccine candidates for *Brucella* as they appear protective yet are attenuated in small animal models (Conde-Álvarez et al., 2013). Furthermore the mutations in the core may offer a potential handle for diagnostic methods to differentiate between wild type and vaccine strains.

These findings sit neatly against the newly proposed structure for the core (Kubler-Kielb and Vinogradov, 2013b) within which positively charged glucosamine residues form a mannose containing branch that, given the mutation and attenuation data, appears to be separate from the branch to which the OPS attaches. The process of structural determination indicated that the OPS is not attached to the core via a glucosamine residue (Kubler-Kielb and Vinogradov, 2013b) which suggests that the OPS is attached via intermediate sugars to a pair of Kdo residues from which the mannose and glucosamine branches also arise (Conde-Álvarez et al., 2012).

In addition to evolving means to minimise recognition by the TLR4-MD2 complex *Brucella* has also evolved ways to interfere with the intracellular signalling pathways triggered by this binding event. This is achieved via the synthesis of proteins that interact with and antagonise critical components of the TLR signalling pathway. *Brucella* produces a C-terminal TIR domain containing protein, TcpB (BtpB/Btp1), which has been reported to suppress the TLR2, TLR4 and MyD88 mediated activation of NF- κ B (Salcedo et al., 2013) and inhibit the maturation of dendritic cells (Salcedo et al., 2008). This interacts with TLR4 and TIRAP seemingly by mimicking the former whilst failing to subsequently stimulate MyD88 (Alaidarous et al., 2014). In turn, this inhibition of the MyD88 activation pathway reduces NF- κ B activation (see figure 1.6) and the induction of proinflammatory cytokines resulting, ultimately, in a more favourable environment within which *Brucella* may find its way to its replicative niche.

Biosynthesis of the lipid A – core takes place within the cytoplasm and cytoplasmic surface of the inner cell membrane (Wang and Quinn, 2010) through a process that is relatively conserved in Gram negative bacteria. Once the structure is completed it is transported to the periplasmic surface of the inner cell membrane by the ABC transporter Msba. Additional modifications to the lipid A and core may take place between its initial construction and presentation on the cell surface many of which influence the virulence of the organism (Raetz et al., 2007).

1.6. Biosynthesis of bacterial OPS

Bacterial lipopolysaccharides are the main components of the Gram-negative bacterial surface and the OPS extend into and interact with the extracellular environment. The OPS is attached to the lipid A that is embedded into the outer membrane via the core sugars on the surface of the lipid A (Caroff and Karibian, 2003) and this is the case for *Brucella* (Cardoso et al., 2006). The lipid A and core sugars represent the lipopolysaccharide (LPS) that is termed rough (rLPS) when the OPS is absent, owing to the appearance of bacterial colonies on culture plates, and smooth (sLPS) when the OPS is present. The components of this macromolecule are synthesised somewhat independently, attached and transported to the cell surface.

The biosynthesis of the OPS also occurs at the cytoplasmic surface of the inner cell membrane. This takes place according to three recognised mechanisms: the Wzx/Wzy, ABC transporter or synthase pathways (Wang et al., 2010), although only one example of the latter is known (Keenleyside and Whitfield, 1996). The pathways all share many features. All begin with a similar process whereby the OPS is assembled on undecaprenyl (Und-PP), an polyisoprenoid lipid carrier. This is mediated by the protein WecA leading to the reaction of an N-acetamido sugar (frequently GlcNAc) with Und-PP to form Und-P-GlcNAc to which additional sugars of the OPS are added. Each pathway results in the presentation of complete OPS at the periplasmic face of the inner membrane where the process converges with the synthetic pathway of the lipid A-core. The OPS is ligated to a sugar in the outer core via an O-glycosidic bond by the action of the membrane protein WaaL. This protein appears to have low stringency for the Und-PP-OPS structure but is more specific with regards to the lipid A – core (Han et al., 2012). In examples known to date this conjugation occurs exclusively through a β -linkage. The process by which the complete sLPS is transported to the outer surface of the outer membrane is relatively less understood and has been dubbed the Lpt (LPS transport pathway). All pathways of OPS production lead to a modal distribution of OPS length centred on a chain length with highest abundance with reducing frequency of shorter or longer species.

The pathways differ in the manner in which the OPS is polymerised once attached to the Und-PP and how this structure is transported to the periplasmic face of the inner membrane. In the Wzx/Wzy pathway the OPS is constructed in individual repeating units on the Und-PP which are exported across the inner membrane by the flippase Wzx. These oligosaccharide units are added to the OPS to create the repeating structure by the polymerase Wzy and chain length is controlled by Wzz. This is the most common system in Gram-negative bacteria and is used to create OPS structures comprised of repeating units which contain different nucleotide sugars. To date only one example of the Synthase pathway has been found (Keenleyside and Whitfield, 1996) and consequently it is the least well understood.

1.6.1. Biosynthesis of bacterial OPS by the ABC transporter system

The ABC transporter system is found in Gram-negative bacteria which have simpler OPS structures that are homopolymers or polymers of disaccharide repeats. This group includes *Brucella*, *Vibrio cholerae*, *Klebsiella pneumoniae* and most of the *Yersinia enterocolitica* (Gonzalez et al., 2008; Wang et al., 2010). This process was recently reviewed (Greenfield and Whitfield, 2012) drawing

largely from the understanding of the generation of the variably α 1-2 or α 1-3 linked mannose homopolymer produced by *E. coli* O8, O9 and O9a as well as identical structures produced by *Klebsiella pneumoniae* O5 (identical to *E. coli* O8) and O3 (identical to *E. coli* O9), see figures 1.8 and 1.9. The review was completed by the study of a second ABC transporter system exemplified by *Klebsiella pneumoniae* O2a which is a polymer of repeating disaccharide galatose in furanose and pyranose forms.

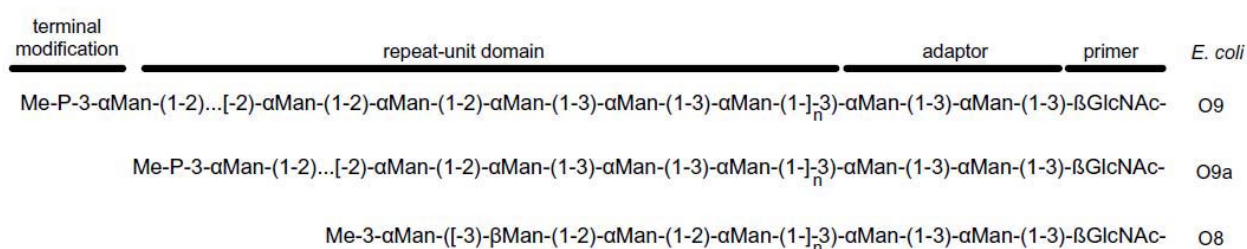


Figure 1.8. Structure of polymannose OPS from *E. coli* O8, O9 and O9a (Greenfield and Whitfield, 2012)

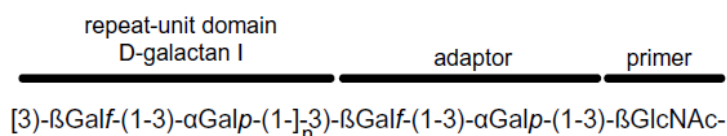


Figure 1.9. Structure of galactose OPS from *Klebsiella pneumoniae* O2a (Greenfield and Whitfield, 2012)

These OPS structures are synthesised entirely within the cytoplasm on the Und-PP and once assembled transported across the inner membrane by ABC transporters comprised of two nucleotide binding domains (encoded by the *wzt* gene) and two transmembrane domains (encoded by the *wzm* gene). A schematic representation of this process is shown in figure 1.10. Although the OPS structures are typically simpler than those synthesised by the Wzx/Wzy pathway due to the limited repertoire of nucleotides within the polymer, additional complexity may be generated by variations of glycosidic linkage. And these linkages may occur in a regular and repeating manner giving rise to specific structural units.

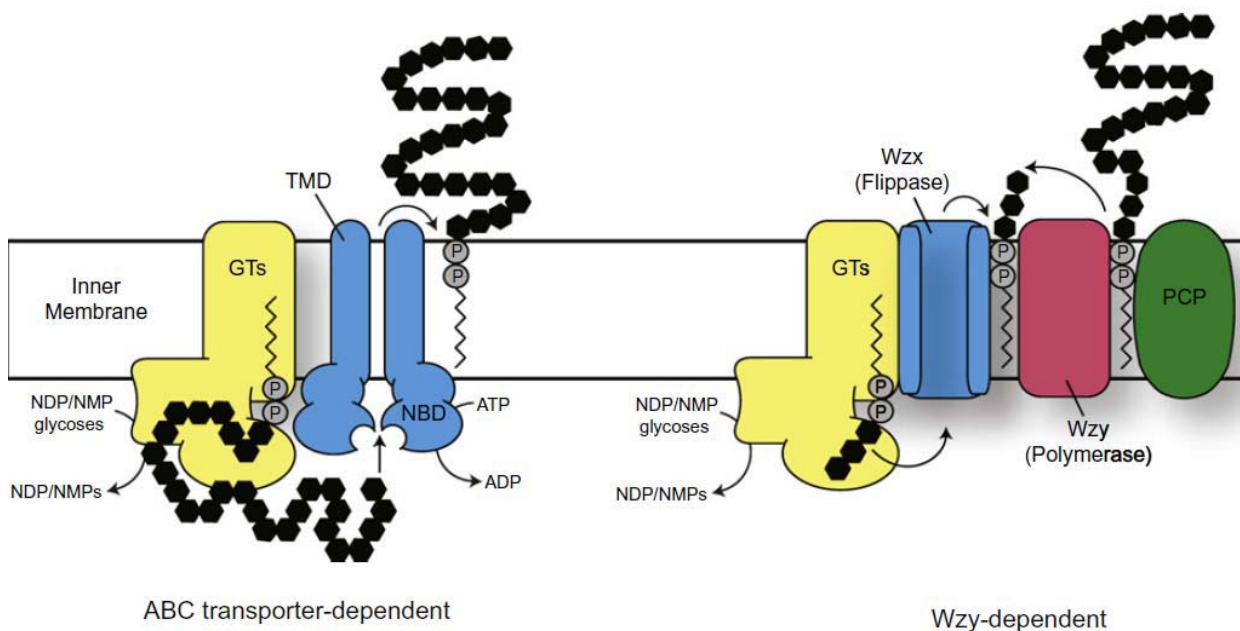


Figure 1.10. Synthesis of OPS by the ABC and Wzy-dependent pathways

OPS extension occurs from the Und-PP entirely at the cytoplasmic face in the ABC dependent pathway though the addition of sugars to the non-reducing terminal of the forming OPS by glycosyltransferases. The OPS is transported into the periplasm by two NBDs (nucleotide binding domains, encoded by *wzt*) and two TMDs (transmembrane domains, encoded by *wzm*). The synthesis of the oligosaccharide repeat units occurs at the cytoplasmic face in the Wzx/Wzy-dependent pathway. These are transported across the inner membrane by the Wzx flippase to the Wzy which polymerises the repeat units to form the OPS. Image reproduced from (Greenfield and Whitfield, 2012).

1.6.2. OPS chain length regulation in the ABC transporter pathway

The chain length of OPS produced by the ABC transporter pathway is regulated by one of two known mechanisms. In some cases, such as in *E. coli* O8, O9 and O9a, and the equivalent identical *Klebsiella pneumoniae* serotypes (Sugiyama et al., 1997), the OPS is capped through modification of the non-reducing terminus by WbdD and this modification prevents further chain extension (Clarke et al., 2004). In *E. coli* O9 and O9a (and *K. pneumoniae* O3) this terminal modification is a methyl-phosphate (Kubler-Kielb et al., 2012) and the WbdD a dual kinase-methyltransferase. The modification on *E. coli* O8 (and *K. pneumoniae* O5) is a methyl group and the WbdD is a methyltransferase (Vinogradov et al., 2002). These studies precisely demonstrate that the terminal modification is made to the final mannose of the repeating unit (see below) and thus explains the observation of a resolved ladder of sLPS species on SDS-PAGE - whereas increments of a single nucleotide sugar would generate an unresolved smear.

A detailed study of the WbdD from *E. coli* O9 and O9a demonstrates that it has some substrate specificity, whereby it preferentially bound and modified an α -(1→2) mannose disaccharide in preference to α -(1→3, 4 or 6) disaccharide or mannose monosaccharide (Hagelueken et al., 2012). These findings confirmed earlier work (Clarke et al., 2011) although it remains unclear exactly which α -(1→2) mannose in the repeating unit becomes modified. The capping process is also an integral part of the exportation of the OPS across the inner membrane as the capped OPS is subsequently and specifically recognised by the C-terminal domain of Wzt (Clarke et al., 2004). Without the capping the polymer accumulates within the cytoplasm. Even with the polymerisation of the OPS regulated by WbdD there is a modal distribution of size. This distribution would appear to be regulated, in a stochastic fashion, by the relative expression and efficiency of the WbdA driven mannose polymerisation and WbdD modification proteins (Clarke et al., 2009).

The second ABC transporter chain termination mechanism is found in Gram-negative bacteria where the OPS is not capped by a terminal modification. This process is best understood in, and exemplified by the study of, *Klebsiella pneumoniae* 02a which, as described above, possess an OPS polymer of repeating disaccharide galatose in furanose and pyranose forms. As for the capped OPS produced by *E. coli* O8, O9, and O9a the OPS from *Klebsiella pneumoniae* 02a is of variable length resulting in a modal distribution with differences that equal the size of the repeating unit. The OPS is exported to the periplasmic face of the inner membrane without a terminal modification by the ABC Wzm/Wzt transporter. It appears that the Wzm/Wzt transporter in such systems act with low stringency and, in contrast to ABC systems with terminally modified OPS, Wzt does not contain a substrate binding domain. In examples studied to date it seems possible that the recognition requirement is the rather generic Und-PP with attached N-acetamido sugar (Kos et al., 2009). This suggests that export of the OPS can be initiated at any point once OPS synthesis has begun. This linkage of synthesis and transport appears to be responsible for the regulation of the length of the OPS in a stochastic fashion owing to the relative speed with which the OPS is synthesised versus the rate at which it is exported. This hypothesis is supported by the experimental observation in *Klebsiella pneumoniae* 02a that the joint over expression of Wzm and Wzt leads to reduction in OPS chain length (Kos et al., 2009).

1.6.3. Prototypical structure of ABC transporter system derived OPS

Although the OPS structures derived from the ABC transporter system are typically simpler than those synthesised by the Wzx/Wzy pathway due to the limited repertoire of sugar nucleotides within the polymer, internal complexity may be generated by variations of glycosidic linkage. These linkages may also occur in a regular pattern giving rise to repeating oligosaccharide sequences within the homopolymers. Additional OPS structural complexity is also provided by a region of the polymer, referred to as the 'adapter' that exists between the N-acetamido glucose 'primer' and the repeating units. These regions are exemplified by the prototype OPS structures of *E. coli* O9, O9a, and O8 and summarised in a recent review (Greenfield and Whitfield, 2012). In these structures the GlcNAc primer is followed by an α 1-3 linked mannose disaccharide primer which in turn is followed by additional mannoses which are variably α (1→2), β -(1→2) or α (1→3) linked in a regular repeating fashion to form the repeat unit domain. In serotype O:8 this is a trisaccharide repeat with one α -(1→2) link, one α -(1→3) link and one β -(1→2) link (Jansson et al., 1985). In serotype O:9 there is a tetrasaccharide repeat with two α -(1→2) links and two α (1→3) links (Prehm et al., 1976). Serotype O:9a is the same as O9 but with an additional α (1→2) link to form a pentasaccharide repeating unit (Parolis et al., 1986). As described above, each of these structures also possesses a terminal modification, therefore there are four elements to each structure: the primer, adapter, repeat unit and terminal modification (Greenfield and Whitfield, 2012).

As also described above, the synthesis of *E. coli* O8, O9 and O9a OPS is initiated by the addition of the primer to the Und-PP by WecA. The remaining structures are synthesised by WbdA, WbdB, WbdC and WbdD. As has been described above, WbdD creates the terminal modifications and the remaining Wbd proteins are mannosyltransferases. WbdC initiates the process by linking the first mannose to C₃ of the GlcNAc primer (Kido et al., 1995). WbdA and WbdB continue the polymerisation process to the point of terminal modification. There is currently a paradigm shift in the understanding of the means by which this occurs. For example, in the context of the *E. coli* O9 OPS it had previously considered probable that one mannosyltransferase was responsible for the creation of α -(1→2) links (WbdA) and the other for the creation of α -(1→3) links (WbdB) (Kido et al., 1995). This model was challenged by the finding that WbdA from *E. coli* O9a contained two domains, one within the N-terminal half which appeared to polymerise α -(1→2) links and the other in the C-terminal half that appeared to polymerise α -(1→3) links and that WbdB and WbdC were still essential (Kido et al., 1998) for OPS synthesis. The understanding of WbdA was developed by the finding that it is solely

responsible for the serotype of *E. coli* O9 or O9a and that a single amino acid change in the WbdA of *E. coli* O9 creates an O9a serotype – an additional α -(1→2) linked mannose in the repeating unit.

When the bifunctional WbdA from *E. coli* O9a was compared to the trifunctional WbdA from *E. coli* O8 (capable of polymerising tetrasaccharide repeats containing one α -(1→2) link, one α -(1→3) link and one β -(1→2)) (Greenfield et al., 2012a) the proteins were found to contain two and three separate and functionally active domains respectively (Greenfield et al., 2012b). These studies also confirmed that the role of the WbdC and WbdB were restricted to the addition of the first and second mannoses to the GlcNAc primer respectively. This model of OPS polymerisation in the ABC transporter system therefore predicts that such polymers may contain structured repeating units, that just one enzyme is responsible for the polymerisation of the whole of a repeating unit and that the repeats are added without any additional mannosyltransferase. This is some departure from the conventional understanding that different linkage types are necessarily created by different mannosyltransferases.

The OPS produced by *Klebsiella pneumoniae* O1, O2a and O2a,c although lacking terminal modifications adds an additional level of complexity to the prototype OPS structure as described above. Like those structures described so far each includes a primer and a disaccharide adapter followed by a repeating unit. The repeating unit, known as D-galactan I, is a disaccharide of galactopyranose (Galp) and galactofuranose (Galf) linked in alternating α and β -(1→3) fashion (Whitfield et al., 1992). This basic structure is the O2a serotype. The additional complexity arises in serotypes O1 and O2ac. Although each possess OPS of the O2a type, each also possess OPS containing an additional serotype specific disaccharide repeating unit. In serotype O1 this is similar to the initial repeating unit but both galactoses are now in the pyranose form. In serotype O2a,c the additional repeating unit is also a disaccharide comprised of Galf and GlcNAc (Vinogradov et al., 2002). Therefore these OPS chains, although they lack terminal modification, have an additional repeating unit which is added after polymerisation of the initial D-galactan I repeating unit. Synthesis of serotype O2a is undertaken by the transferases WecA (adapter), WbbO which is thought to add one Galp and one Galf residue to GlcNAc to create the primer, and WbbM which extends the adapter (Guan et al., 2001). The current model predicts that the WbbN is an α -(1→3) Galp transferase and that further polymerisation with the Galf occurs through the action of WbbO. However, this model is not entirely satisfactory not least of all because it assigns no role for WbbN, a putative glycosyltransferase, which is required for synthesis of D-galactan I (Kos et al., 2009). It is possible that it is WbbN that extends the OPS with addition of Galf rather than WbbO although at the current time this remains a hypothesis without examination (Kos and Whitfield, 2010).

1.7. *Brucella* OPS

1.7.1. *Brucella* OPS: genetics, structure and linkage

Although the study of the *E. coli* O8, O9, O9a and *K. pneumonia* O2a, O1 and O2a,c OPS gives some valuable insights into OPS production by the ABC transporter system, in general it must be borne in mind that excessive generalisations and extrapolation are speculative. For example, *Vibrio Cholera* serotype O1 possesses two serotypes, Inaba and Ogawa, differentiated by an O-methyl terminal modification of the perosamine homopolymer in Ogawa (Ito et al., 1994). However this modification is not required for export of OPS (Stroeher et al., 1992) and thus fails to fit neatly into each of the export processes described above. However, despite the demonstrable need to be cautious it is of interest to place the synthesis and structure of *Brucella* OPS against the background provided by the study of the ABC transporter OPS prototypes.

The *Brucella* OPS is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyls (N-formyl perosamine, D-Rha4NFO), figure 1.11., that are variably α -(1→2) and α -(1→3) linked (Bundle et al., 1987a; Bundle et al., 1987b, c; Bundle and Perry, 1985; Caroff et al., 1984b; Meikle et al., 1989). The proportion of α -(1→3) linkages varies between 0 and 20% (Meikle et al., 1989; Zaccheus et al., 2013) dependent upon biovar. For example, the *Brucella suis* biovar 2 reference strain (Thomsen) has been demonstrated to be devoid of α -(1→3) linkages in its OPS (Zaccheus et al., 2013). An exception to this is the OPS from *Brucella inopinata* BO2 which is distantly related to the classical *Brucella* species and has a completely different OPS structure (Wattam et al., 2012). The structure of the *Brucella* OPS has been the recipient of a very recent reinvestigation that has added some fine detail as to the specific arrangement and sequence of the α -(1→2) and α -(1→3) links in different prototype structures (Kubler-Kielb and Vinogradov). These structures, in particular the linkage types, were derived using Nuclear Magnetic Resonance (NMR) techniques, a summary of which is provided in section 1.16. The more recent investigation of the OPS also made significant use of mass spectrometry, a technique which underpinned much of this current study. A basic introduction to the more relevant aspects of mass spectrometry is presented in section 1.15.

Strains of smooth *Brucella* have been serotyped using cross absorbed antibodies for many years (Alton et al., 1994; Wilson and Miles, 1932) but it was not until the structural elucidation of the OPS that the basis for this typing was understood (Meikle et al., 1989). *Brucella* are serotyped in this manner into either A dominant, M dominant or mixed A and M dominance (Nielsen and Ewalt, 2010)

on the basis of the proportion of α -(1→2) and α -(1→3) links within their OPS. The A dominant OPS is described as 98% α -(1→2) and 2% α -(1→3) linked (Caroff et al., 1984b; Meikle et al., 1989), or in the case of *B. suis* biovar 2 100% α -(1→2) linked (Zaccheus et al., 2013), whereas the M dominant OPS is described as 80% α -(1→2) and 20% α -(1→3) linked in a regular 4 to 1 repeating unit (Bundle et al., 1987b). Stains with mixed A and M dominance fall somewhere between 2 to 20% of α -(1→3) links.

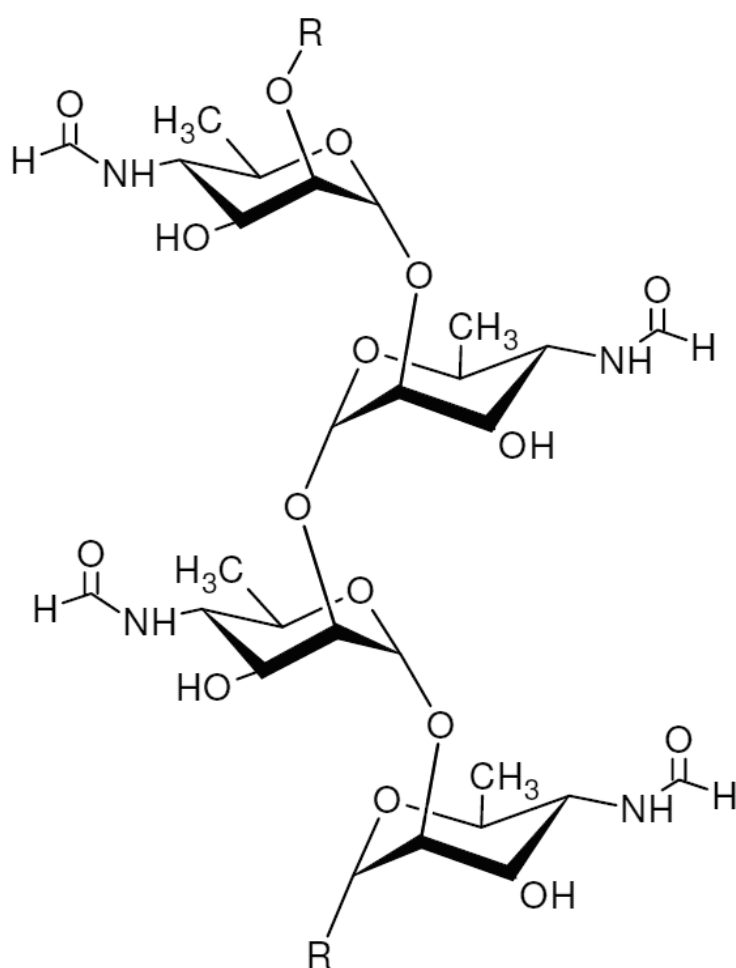


Figure 1.11. An α (1→2) linked D-Rha4NFO (*Brucella* perosamine) polysaccharide

R represents additional D-Rna4NFO as would make up the repeating portion of the OPS. The OPS from *Brucella* also contains α (1→3) with frequency from 0–20% dependent upon strain. Image reproduced from (Cardoso et al., 2006).

The OPS from *Brucella* is generated via the ABC transporter pathway as determined by the discovery of *wzm* and *wzt* genes (Godfroid et al., 2000), and the OPS is not terminally modified. It is therefore of interest to compare the structure and genetics of the *Brucella* OPS with those of the *K. pneumoniae*

and *E. coli* serotypes described above. The network of genes responsible for synthesis and presentation of *Brucella* OPS have been described (Gonzalez et al., 2008) and lie within two genomic regions (see figure 1.12.). The *wbk* region contains many of the genes responsible for OPS synthesis (Godfroid et al., 1998) including those required for N-formyl perosamine synthesis (*per*, *gmd*, *wbkC*), ABC transporters (*wzm* and *wzt*), *wbkD* and *wbkD* genes responsible for synthesis of an NAc-Quinovosamine (*wbkD*) and it's attachment to Und-PP (*wbkF*), genes responsible for the production and polymerisation of mannose (*manA*, *manB* and *manC*), mannosyltransferases (*wbkE* and *wbkA*). Two additional glycosyltransferases (*wboA* and *wboB/wbdA*) critical for the surface presentation of OPS have also been identified in a separate genomic region (Rajashekara et al., 2008) designated *wbo*.

The impact of many of these genes on the phenotype and virulence of *B. melitensis* strain 16M has been studied using transposon mutagenesis (Gonzalez et al., 2008). Several phenotypes were generated. The *wzm* and *wa*** mutants both had a rough phenotype but both produced internal OPS which gave rise to anti-OPS antibodies when the strains were inoculated into mice. This observation is consistent with a failure to synthesise Wzm transporter protein that would lead to accumulation of internal OPS. The *wa*** gene is separate from the OPS synthesis genes but has homology to the WaaX protein from *E. coli* that is an LPS core galactosyltransferase (Heinrichs et al., 1998). The failure to synthesise a complete core could lead to the failure to attach OPS if the particular structure missing was part of the branch that links to the OPS or forms the recognition site for Waal. Consistent with this theory, the rLPS from the *wa*** had an altered migration pattern on SDS-PAGE (Gonzalez et al., 2008). The same study also generated a rough mutant via transposon disruption of the *pgm* gene which also had an altered rLPS SDS migration pattern. The *pgm* gene seems to be required for the generation of glucose and galactose and it's mutation and phenotype has been described elsewhere (Ugalde et al., 2000). The rough *pgm* mutant has also been reported to synthesise OPS internally but in this case not reported to induce anti-OPS antibodies when inoculated into mice (Ugalde et al., 2003). The rough vaccine strain *B. abortus* RB51 has, as one of its mutations, an insertion sequence in the *wboA* gene (Vemulapalli et al., 2000). Complementation of this gene restored the capability of the stain to synthesise OPS, however the rough phenotype was maintained as, it seems, at least one other mutation had occurred that inhibits presentation. The *wboA* complemented RB51 induced anti-OPS antibodies in mice and gave greater protection against challenge with virulent *Brucella* than RB51. The *wzm* and *wa*** mutants described above also gave greater protection in mouse challenge studies than did other mutants that did not give rise to anti-OPS antibodies. The added protective value of the rough phenotype that produces internal OPS is the basis for the development of the

Brucella *pgm* mutant vaccine strain but independent studies demonstrated only weak protection (Gonzalez et al., 2008).

Smooth *Brucella* strains may spontaneously dissociate from smooth to rough *in-vitro*, with some evidence that this also occurs *in-vivo* (Turse et al., 2011). Three mutations found in such dissociated strains have been discovered, one in the *manB_{core}* gene (Turse et al., 2011), another a the deletion of the glycosyltransferase gene *wbkA* (Mancilla et al., 2012) and also the deletion of Genomic Island (GI) 2 which contains the glycosyltransferase genes *wboA* and *wboA* (Mancilla et al., 2010). This deletion also appears to have taken place in *B. ovis* and would account for its naturally occurring rough phenotype despite the retention of the *wbk* region (Tsolis et al., 2009). *B. ovis* also possess a frame shift in the *wbkF* gene (Zygmunt et al., 2009) although in the absence of GI2 this is inconsequential. *B. canis* carries GI2 but also a deletion that overlaps genes *wbkF* and *wbkD* (Zygmunt et al., 2009) which explains the lack of NAc-Quinovosamine in this species (Bowser et al., 1974) and its rough phenotype. The synthesis and subsequent attachment of NAc-Quinovosamine to Und-PP is the initiating step in OPS synthesis.

The roles of many of the genes required for *Brucella* OPS synthesis, transfer and attachment to the lipid A - core have been allocated according to the general model of the ABC transporter pathway. The precise role of the glycosyltransferase genes *wboA*, *wboA*, *wbkA* and *wbkE* in terms of the specific sugar nucleotides polymerised and the linkages created has not been defined. Transposon mutagenesis in any one of these genes results in a rough phenotype (Gonzalez et al., 2008) as determined by phage typing, crystal violet tests and SDS page of purified LPS. Analysis of the LPS using monoclonal antibodies (mAbs) to the OPS and the outer and inner core from each of the mutants demonstrated a lack of reactivity to the OPS with a corresponding increase in reactivity to the core. None of the mice infected with these mutants produced any anti-OPS antibodies suggesting that there is no internal cellular OPS – unlike the *wzm* and *wa*** mutants – and each evoked low protection against challenge against virulent *Brucella* in mice. A summary of gene function is shown in figure 1.13.

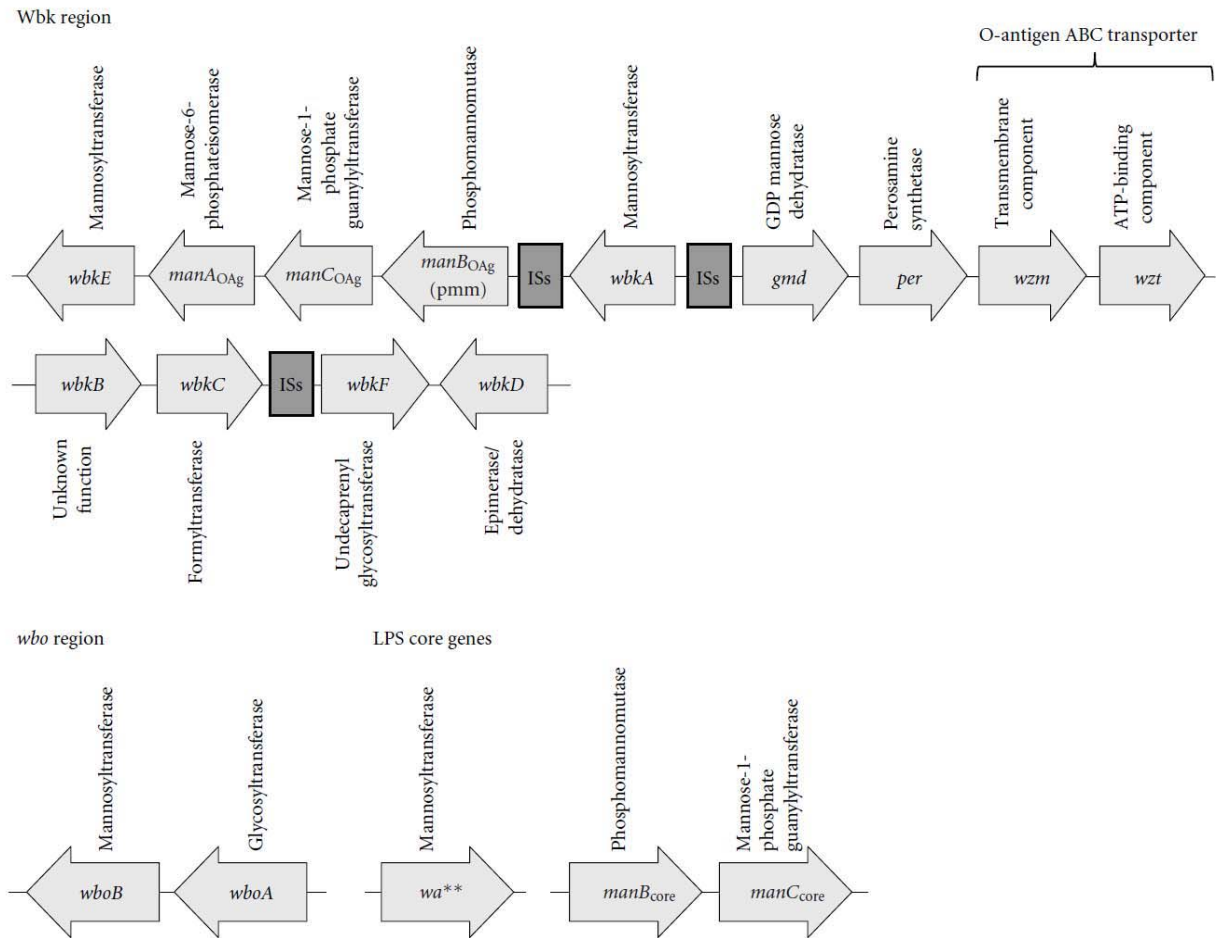


Figure 1.12. The major genetic regions of *Brucella* OPS synthesis and significant core genes

The *wbk* region contains genes required for the synthesis of D-Rha4Nfo (*gmd*, *per* and *wbkC*), the ABC transporters (*wzm* and *wzt*), two glycosyltransferases (*wbkA* and *wbkE*) as well as insertion sequences. The *wbo* region contains two glycosyltransferases related to the formation of smooth LPS (*wboB* and *wboA*). Genes *wa***, *manB_{core}* and *manC_{core}* genes relating to synthesis of the core are found separately from the OPS synthesis genes. Image reproduced from (Haag et al., 2010).

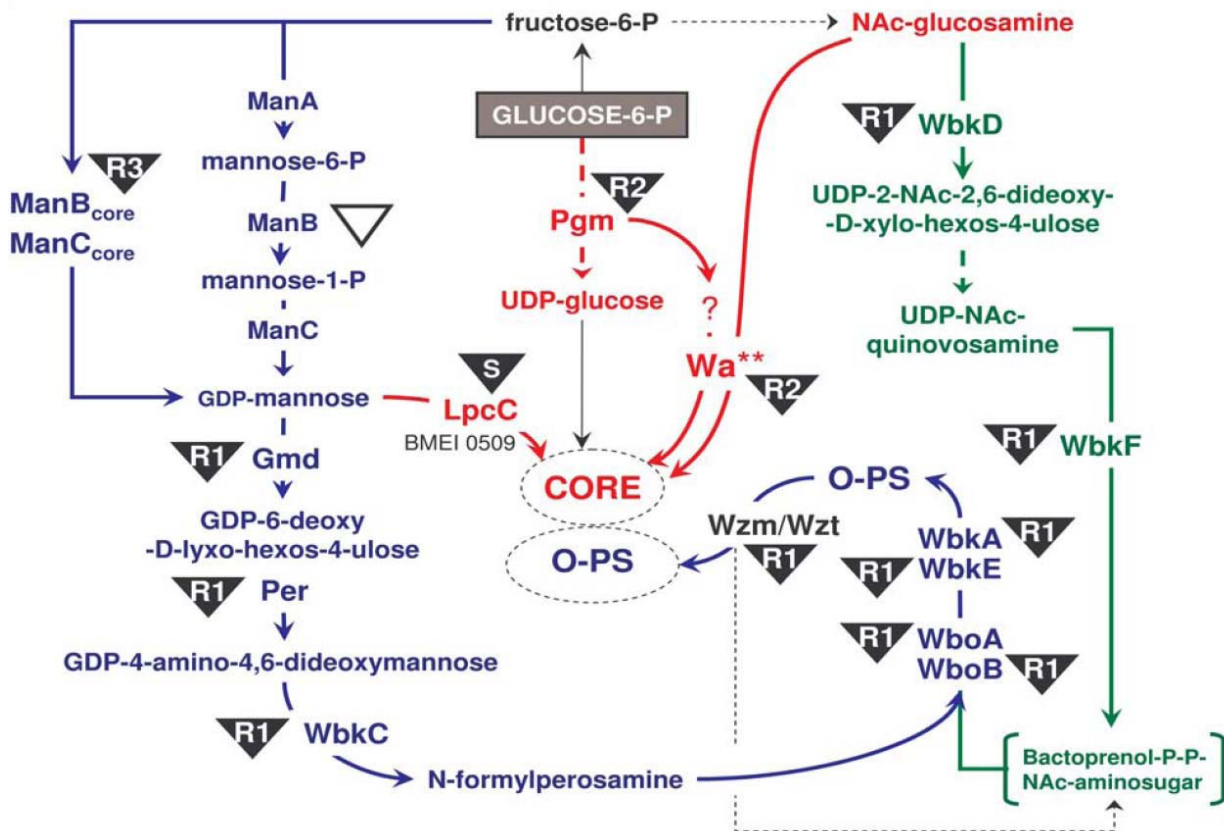


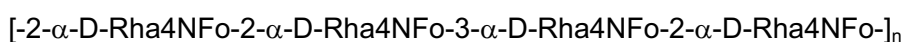
Figure 1.13. Proposed genetic pathway for OPS and core synthesis

The figure above describes the genetic pathway for D-Rha4NFo (N-formylperosamine) synthesis and polymerisation (blue), and other precursor sugars and genes contributing to the core (red), a possible pathway depicting the initiation and priming of the OPS is shown on the right (green). Image reproduced from (Gonzalez et al., 2008).

In order to try and delineate the role of *wboA*, *wboB*, *wbkA* and *wbkE* these genes have been evaluated by PCR-RFLP on several strains representing different species, biovars and OPS types of *Brucella* (Clockaert et al., 2000; Zygmunt et al., 2009). The overall conclusion is that these genes are highly conserved and there are few RFLP patterns. Any patterns that do exist bear no relation to the observed A, M or mixed A and M OPS serotype which in turn is a consequence of the proportion of α -(1→3) links present in the OPS (Meikle et al., 1989). The prevailing assumption at the time was that one of these enzymes is a specific α -(1→3) glycosyltransferase. It was therefore hypothesised that the proportion of linkage types within the *Brucella* OPS, and hence serotype, is due to variations in transcription and or efficiency between these enzymes given that no serotype specific sequence differences have been found. Analysis of the amino acid sequence of these four genes as found in *B. melitensis* strain 16M and *B. suis* biovars 1 and 2 (respectively possessing high, low and zero proportions of α -(1→3) links in the OPS) demonstrated no significant differences. No significant

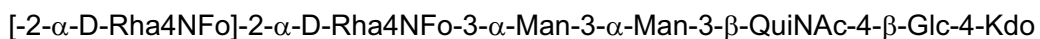
differences were found in the DNA sequences up to 300 base pairs upstream into the putative promoter regions of the genes either (Zaccheus et al., 2013).

It is interesting to review the existing hypothesis for linkage determination in light of recent structural analysis of the *Brucella* OPS and core and the developing understanding of OPS synthesis (as reviewed above). The recent structural analysis of *Brucella* OPS (Kubler-Kielb and Vinogradov) and core – OPS (Kubler-Kielb and Vinogradov, 2013b) indicates that the α -(1→3) linkages are contained within a tetrasaccharide repeat of N-formyl perosamine (D-Rha4NFO) with 3 α -(1→2) linkages:



This repeating sequence is reported to occur on the non-reducing end of a region consisting of exclusively α -(1→2) linked D-Rha4NFO residues. This differs from the model of a pentasaccharide repeat containing a 4 to 1 ratio of α -(1→2) to α -(1→3) linkages previously put forward (Bundle et al., 1987b). Both models contain the same overall proportion of linkages within the OPS but the more recent places the additional α -(1→2) linkages within a homopolymer at the reducing terminal. This new model adds a new layer of depth and structure. The new analysis also showed that variable levels of linkage type are a result of different proportions of tetrasaccharide repeat versus α -(1→2) homopolymer with the repeat always occurring at the non-reducing terminus. The analysis also found a mix of OPS polymers within a single strain with not just variable length but where some consisted solely of the α -(1→2) homopolymer.

Related work (Kubler-Kielb and Vinogradov, 2013b) on the structure of the core polysaccharide of *Brucella* also identified non-perosamine elements at the reducing terminal of the OPS as shown below.



Based upon this new knowledge the *Brucella* OPS structure can be categorised using the system defined by the study of *E. coli* O8, O9 and O9a and *K. pneumonia* O1, O2a and O2a,c described above (Greenfield and Whitfield, 2012). In this scheme the *Brucella* OPS has a QuiNAc 'primer', a disaccharide mannose 'adapter', a first (exclusively α -(1→2) linked) 'repeat-unit' domain, a second tetrasaccharide 'repeat-unit' domain but with no terminal modifications. This structural organisation is

particularly similar to that of *K. pneumoniae* serotypes O1 and O2a,c as the OPS from each serotype may possess both, or just the single, repeat unit domains and there are no terminal modifications (Vinogradov et al., 2002). There is therefore a well studied precedent for the newly proposed structure of the *Brucella* OPS.

The export of OPS to the periplasmic surface of the inner membrane in *K. pneumoniae* serotypes O1, O2a and O2a,c is controlled by the Wzm/Wzt ABC transporter as described above. Increasing the expression of these transporters results in shortened OPS (Kos et al., 2009). It has been shown, in *Salmonella typhimurium* for example, that a reduction in OPS chain length may result in a reduction of virulence (Murray et al., 2003; Murray et al., 2006). The recent study of the *Brucella* OPS structure found differences in the chain lengths between the species and strains evaluated (Kubler-Kielb and Vinogradov). It would be interesting to determine if these differences were due to differences in the efficiency and transcription of *wzm* and *wzt*. It would also be interesting to determine if such differences had an impact on virulence in *Brucella* strains and also if the shorter chains were less likely to possess the tetrasaccharide repeat and therefore influence the serotype. Unfortunately the description of the strains used, and thus their serotypes, in the recent study are unclear. However, the paper drives an interesting hypothesis, especially in light of the recent finding that the OPS from *B. suis* bv 2 is devoid of α -(1→3) links (Zaccheus et al., 2013). This biotype, despite its close association with humans, is rarely zoonotic (Godfroid et al., 2005). Following the *K. pneumoniae* OPS analogy, *B. suis* bv 2 bears comparison to serotype O2a in which the additional repeat unit domains, responsible for the O1 and O2a,c phenotypes, are absent.

The newly resolved structure of the *Brucella* OPS suggests that glycosyltransferase enzymes would be required for the addition of mannose to QuiNAc, mannose to mannose, D-Rha4NFO to mannose, D-Rha4NFO to D-Rha4NFO in an α -(1→2) and an α -(1→3) fashion. This implies potentially five different glycosyltransferases, although some may have multiple domains and functions similar to the bifunctional WbbO that adds Galp and then Galf to the GlcNAc primer on *K. pneumoniae* O1 (Guan et al., 2001). Or to the multidomain bifunctional WbdA of *E. coli* O9a (trifunctional in *E. coli* O8) that creates both α -(1→2) and α -(1→3) linked mannose in a regular fashion whereas WbdB also acts on mannose but only links in an α -(1→3) fashion (Greenfield et al., 2012b). It is tempting to believe that the regularity of the repeating tetrasaccharide of the *Brucella* OPS could only be created by the action of a single multidomain glycosyltransferase that was capable of both α -(1→2) and α -(1→3) linkage types. A separate glycosyltransferase enzyme would then be responsible for the exclusively α -(1→2) linked domain. However, the means by which this process is regulated remains unclear and

furthermore, the deletion of the hypothetical multidomain, bifunctional D-Rha4NFO transferase should still lead to a smooth phenotype as well as reaction to and induction of anti-OPS antibodies.

1.7.2. Acquisition of *Brucella* OPS genes

There is strong evidence that the genes responsible for the production of *Brucella* OPS have been acquired by horizontal transfer. Sequencing of the *wbk* gene cluster reveals that it has a significantly lower GC content at 50.0% than the 57.2% average for the genome (Godfroid et al., 2000; Wattam et al., 2009). It also contains a mobility sequence, IS711, and encodes for a significant virulence determinant. This gene cluster therefore falls neatly into the definition of a Pathogenicity Island (Mancilla, 2012). The glycosyltransferases *wboA* and *wboB*, which are also necessary for the production of OPS, are part of GI2 which also has a significantly lower than average GC content of 51.3% and the IS711 sequence (Mancilla et al., 2010). Further evidence for horizontal gene acquisition is provided by the interesting finding that the novel strain *B. inopinata* BO2, which appears to be an early diverging relative of the classical *Brucella* species, not only does not possess the *wbo* or *wbk* gene clusters (Wattam et al., 2012) but also appears to synthesise an OPS that is the same as the phylogenically related *Ochrobactrum anthropi* (Velasco et al., 1996). This evidence clearly suggests that the typical *Brucella* OPS was not acquired by a vertical evolutionary process. A similar acquisition process may also explain the existence of an OPS of such structural similarity in *Yersinia enterocolitica* serotype O:9. Many of the genes involved in the synthesis of this structure such as *per*, *wzm* and *wzt* bear a high degree of amino acid sequence identity to the equivalent genes from *B. melitensis* (Skurnik et al., 2007). The *wbk* region in *Brucella* is not a continuous unit (Mancilla et al., 2012), so although *Y. enterocolitica* O:9 has homologues for N-formyl perosamine synthesis and transportation, it doesn't carry homologues for the *Brucella* OPS glycosyltransferases (including *wboA* and *wboB* of GI2) and so unfortunately provides no clues as to their precise functions.

1.7.3. Role of *Brucella* OPS in virulence

The induction of antibodies is a key biological property of the OPS but there are also other properties that make this structure a major virulence factor. It has been known for many years that spontaneous rough mutants such as those created by dissociation events and those created by targeted mutations in the OPS pathway are attenuated (Moriyon et al., 2004).

It has been described above how the *Brucella* core oligosaccharide plays a role in the resistance to complement mediated killing by normal serum and resistance against bactericidal cationic peptides but this capability appears enhanced by the ligation of OPS. This is demonstrated by the properties of rough *B. melitensis* mutants compared to the parental strain (Gonzalez et al., 2008). These results concur with earlier and very similar findings for *B. abortus* (Allen et al., 1998; Corbeil et al., 1988; Eisenschenk et al., 1999) as well as more specific studies on cationic peptides (Martínez de Tejada et al., 1995). Both the OPS and the core components of the LPS thus appear to play a role in the inhibition of binding of complement component C1q to the lipid A, in an antibody independent fashion, to prevent the classical pathway of complement activation (Tan et al., 2011). The binding of C1q leads to the activation of C1 and the subsequent generation of anaphylatoxins C3a, C4a and C5a as well as the opsonins C3b and iC3b. *Brucella* can also activate complement via the lectin binding pathway and, once again, rough variants are more susceptible to this action (Fernandez-Prada et al., 2001).

In addition to the role of *Brucella* OPS in resistance to extracellular immune mediators it also has a role in the modulation of the host's cellular immune response. Pathogenic brucellae survive and propagate within their phagocyte niche by modifying, escaping and resisting the phagocytic pathways and eventually residing within the endoplasmic reticulum (Pizarro-Cerda et al., 1998; Starr et al., 2008) from whereupon they escape the cell to complete their lifecycle (Starr et al., 2012). The means and route by which *Brucella* cells enter the host phagocyte determines many of the subsequent processes and consequently affects the ability of the *Brucella* cells to avoid destruction. Virulent brucellae appear to enter phagocytes via lipid rafts (Kim et al., 2004; Watarai et al., 2002) in a process that appears to avoid host cell activation (Pei et al., 2008), inhibits fusion with lysosomes (Naroeni and Porte, 2002) and is favourable to the internal survival of the invading organism (Hartlova et al., 2010; Vieira et al., 2010). The reduced activation of the host cell may be due to the interaction of the LPS with lipid rafts in a manner that is receptor independent (Ciesielski et al., 2012) although it is recognised that eukaryotic receptors such as TLR4 are also enriched in these regions (Ciesielski et al., 2013).

There is significant evidence that the OPS plays an important role in mediating the entry of brucellae into phagocytes via lipid rafts (Pei et al., 2008; Porte et al., 2003). This data helps to explain observations that rough phenotypes induce more cytokines and chemokines than smooth and that they have different intracellular trafficking routes (Rittig et al., 2003). This work also showed that

rough phenotypes were taken into human monocytes in greater number and greater efficiency than smooth types but that only the latter replicated within the cells. Similar observations relating to the intracellular survival of smooth and rough phenotypes and their activation of host cells have also been made in murine cells (Godfroid et al., 1998; Jiménez de Bagüés et al., 2004). However, these observations must be tempered by the fact that they were made using comparisons of engineered rough strains and their parental smooth strains. Investigation into the interaction of the naturally rough and pathogenic species *B. ovis* and *B. canis* indicates that their entry into macrophages is also mediated through lipid rafts with a similar level of host cell activation as observed due to infection by smooth strains (Martín-Martín et al., 2010). It is notable that entry process in *B. canis* and *B. ovis* are dependent upon outer membrane proteins Omp22 and 25d (Martín-Martín et al., 2008). The generation of rough mutants may have altered bacterial cell surface components other than the OPS, as has been observed in other species (Bengoechea et al., 2004), and this may have had some bearing on the experimental data obtained from comparative studies. Despite this uncertainty, the results described are relevant to understanding the reduced protective efficacy obtained with the application of rough vaccines derived from naturally smooth *B. abortus* and *B. melitensis* strains (Gonzalez et al., 2008; Moriyon et al., 2004). These rough strains may be over attenuated.

It has also been demonstrated that sLPS from *B. abortus* resists destruction within phagolysosomes within which it accumulates and is recycled to the host cell surface whereupon it aggregates with MHC II (Forestier et al., 1999a; Forestier et al., 1999b) to form aggregate macrodomains and suppresses T-cell stimulation of the infected cell. Although the association did not affect the ability of the MHC to process or upload peptides the sLPS did affect their capability to activate T-cells possibly due to impeding the interaction with the TCR (Forestier et al., 2000). Further analysis demonstrated that the LPS macrodomains significantly restructure the membrane and sequester most of the surface MHC II within dense and rigid 'macro-domain' structures that are less functional (Lapaque et al., 2006a). Purified *B. abortus* and *B. melitensis* sLPS induced the MHC II associated macrodomains whereas purified rough LPS created macrodomains but these were not associated with MHC II. Purified sLPS from *Yersinia enterocolitica* O:9 did not form macrodomains and was found located within intracellular vesicles. Given the structural similarities of the OPS from *Brucella* and *Y. enterocolitica* O:9 it seems clear that the lipid A – core is primarily responsible for the passage of the *Brucella* sLPS to the host cell surface but that the OPS plays an important role in the sequestration of the MHC II and subsequent reduction in T-cell activation.

Intracellular processing of *Brucella* is clearly of considerable, if not paramount, importance in the lifecycle of the pathogen, it's 'stealthy strategy' and the progression of disease. This processing is linked to the activation state of the phagosome, most particularly the macrophage, and the point of entry into the host cell (as described above). Activation of macrophages with IFN γ is known to be a critical component of the effective immune response (Jiang and Baldwin, 1993; Murphy et al., 2001) and is most effective *in-vitro* if added prior to infection of cells in order to prime or pre-activate the cells. The timing of phagocyte activation appears to be critical as once established, brucellae become difficult to eliminate from the cell even following activation. *Brucella* cells that have been coated in opsonising antibodies, such as IgG, are recognised by receptors on phagocytes leading to their enhanced engulfment and entry into the Fc receptor dependent pathway rather than entry via lipid rafts (Bellaire et al., 2005).

1.7.4. Role of anti-*Brucella* OPS antibodies in the host immune response

The opsonisation of microbes coated in IgG via the macrophage Fc γ R1 receptor (CD64) results in efficient phagocytosis which is usually accompanied by enhanced intracellular killing (Swanson and Hoppe, 2004). However this process may lead to a decrease in the inflammatory response as macrophages transit from being classically activated via, for example, TLR pathways and IFN γ towards regulatory cells that secrete IL-10 and dampen the inflammatory response (Mosser and Edwards, 2008). This antibody dependent anti-inflammatory process may exacerbate infection as macrophages lose their microbiocidal effect and this is exploited by numerous pathogens, possibly including *Brucella* (Halstead et al., 2010). Macrophages present numerous phenotypes between these two extremes and it seems that very high levels of opsonising IgG is required to induce the macrophage to secrete IL-10. Suppression of inflammation in such situations may be useful to limit tissue damage and allow the humoral response to bring the infection to a close (Gallo et al., 2010).

Opsonised *Brucella* replicate in a modified late endosome whereas non-opsonised *Brucella* replicate in vesicles that appear to possess components of the endoplasmic reticulum. However, irrespective of opsonisation it seems that virulent brucellae are able to delay the fusion of phagosomes with lysosomes, resist destruction eventually replicating and persisting within the host cell (Arenas et al., 2000). But there is evidence that opsonised brucellae have much suppressed replication within macrophages (Gross et al., 1998) and that macrophage activation by IFN γ is more effective when brucellae have been opsonised (Eze et al., 2000; Gross et al., 1998). The results from such studies

are also affected by the titre and isotype of opsonising antibodies as well as the virulence of the investigated strain of *Brucella* (Elzer et al., 1994). The interplay between IFN γ stimulation, antibody opsonisation, intracellular processing and replication, and the progression of brucellosis is complex and intertwined (Ritchie et al., 2012). Virulent brucellae appear to be able to modify their intracellular environment but the precise means by which this occurs is still the subject of a great deal of research.

Given the lack of knowledge about the influence of opsonisation in disease progression it is not surprising that the role of antibodies during infection also remains enigmatic and contentious. *Brucella* OPS induces high titres of specific antibodies in the vast majority of infected hosts and thus the protective, or otherwise, role of antibodies during brucellosis can be traced back primarily to this structure. As described above (Halstead et al., 2010) the production of antibodies leading to opsonisation and phagocytosis of pathogens combined with alterations in the inflammatory balance may create favourable conditions for specific pathogens. It is undoubtedly true that *Brucella*, opsonised or not, may replicate within phagocytic cells such as macrophages which in turn act as foci for further infection (Starr et al., 2012).

On the other hand there is a large body of evidence that shows that passive transfer of anti-*Brucella* antibodies confers significant protection against challenge to the recipient whether polyclonal (Corbeil et al., 1988; Winter et al., 1989) or monoclonal anti-OPS antibodies (Cloeckaert et al., 1992a; Jacques et al., 1992; Laurent et al., 2004; Limet et al., 1989b; Montaraz et al., 1986; Phillips et al., 1989; Vizcaíno and Fernández-Lago, 1994; Winter et al., 1989) albeit that the titre and isotype of the transferred antibodies seems significant (Corbeil et al., 1988) as well as the stage of infection (Hoffmann and Houle, 1995). Furthermore, vaccination with OPS, conjugated or otherwise, has also been shown to induce significant protection to subsequent challenge (Jacques et al., 1991; Lord et al., 1998; Winter et al., 1988). The seemingly protective role of internalised non-LPS conjugated OPS as produced by *pgm* (Ugalde et al., 2003), *wa*** and *wzm* mutants (Gonzalez et al., 2008) and *wboA* complemented *B. abortus* RB51 (Vemulapalli et al., 2000) may be due to the role of anti-OPS antibodies that may be induced. When all the data is taken into consideration it is clear that, one way or another, the OPS is a critical component in the pathogenic lifecycle of *Brucella* and the host response.

1.7.5. Antibody epitope mapping of the *Brucella* OPS: A and M dominant serotypes

The OPS from *Brucella* is the humorally immunodominant molecule and hyper-immunisation of mice with killed whole *Brucella* cells results in the generation of high titres of anti-OPS antibodies (Phillips et al., 1989) and corresponding B-cells that may be fused to generate hybridomas for the production of anti-OPS mAbs. These have greatly facilitated the knowledge of the structure of the OPS in tandem with non-immunological methods of resolution. As described above, the *Brucella* OPS is an unbranched homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranosyls (N-formyl perosamine, D-Rha4NFO) that are variably α -(1 $\rightarrow$ 2) and α -(1 $\rightarrow$ 3) linked and the ratios of these linkages confers the dominance and serotype, A, M or mixed A and M, of a smooth *Brucella* strain (Meikle et al., 1989). These serotypes have classically been determined by the use of pre-absorbed hyperimmune sera (Alton et al., 1994) but have also given rise to numerous monoclonal antibodies of similar but more distinct specificities.

There have been many published studies on the development of mAbs against *Brucella* OPS although those evaluated in light of the developing structural information shed most light on the mapping of epitopes that lie within the molecule. In a first example (Douglas and Palmer, 1988) immunisation of BALB/c mice with killed whole *B. abortus* and *B. melitensis* cells generated anti-OPS mAbs of four different specificities as determined by iELISA against sLPS and whole cells of: *B. abortus* (A dominant strain), *B. melitensis* (M dominant strain) and *Y. enterocolitica* O:9 (exclusively α 1 $\rightarrow$ 2 linked perosamines). These mAb specificities were: those that reacted against the A dominant sLPS and cells only (these also reacted to the *Y. enterocolitica* O:9 cells), those that reacted to the M dominant sLPS cells only, those that reacted to all sLPS cells and those that reacted to all *Brucella* cells and sLPS (both A and M dominant) but not to the *Y. enterocolitica* O:9 cells. Such mAb specificities were designated as A, M, C/Y and C respectively. These specificities were subsequently supported using additional techniques such as Western blot and immunoprecipitation (Palmer and Douglas, 1989).

A contemporary study identified similar mAb specificities following immunisation into BALB/c mice of killed cells of *Y. enterocolitica* O:9 and *B. abortus* (A dominant strain 1119-3) (Bundle et al., 1984) and M dominant *B. melitensis* strain 16M (Bundle et al., 1989a). Similarly C, C/Y, A and M specificities of mAbs were generated as defined by iELISA binding profiles against sLPS from each of the three immunising strains described above. The results were given as end-point titres, which were defined as the highest dilution reaching an optical density of 0.1 after 60 minutes substrate

development. Although the mAbs have been defined categorically, the quantitative results show a range of specificities even within each category. However, the results clearly show M type specificity where the endpoint titres against the *B. melitensis* 16M sLPS antigen are up to 1000 times greater than against the *B. abortus* S1119-3 sLPS which is itself 100 times greater than the titre against the *Y. enterocolitica* O:9 sLPS. The A type specificity is exemplified by the mAb with 50 and 10 times higher endpoint titre against *B. abortus* S1119-3 and *Y. enterocolitica* O:9 sLPS respectively than against *B. melitensis* 16M sLPS. The C/Y type specificity mAb has equal endpoint titres against all antigens. The C specific mAb has a near equal endpoint titre against the *Brucella* antigens which is 1000 times higher than the titre against the *Y. enterocolitica* O:9 sLPS. What is particularly interesting about this mAb binding study is the additional generation of data from competitive (c) ELISAs.

Analysis of mAb affinity and specificity by cELISA was performed by passively absorbing the mAbs to the surface of an ELISA plate and adding a biotinylated *B. melitensis* 16M OPS conjugate in solution. After binding and washing, substrate was developed via the use of streptavidin horse radish peroxidase (HRP) conjugate. The concentrations of mAb and *B. melitensis* 16M OPS biotin conjugate required to achieve an optical density of 1.0 was noted and used for the competitive assay. Different concentrations of OPS from *B. abortus* S1119-3, *Y. enterocolitica* O:9 and *B. melitensis* 16M were added to the ELISAs and the concentration of OPS that resulted in 50% inhibition of the optical density was recorded and used for subsequent analysis. This data supported the previous interpretations of specificity, based on iELISA, for the A, M and C/Y specific mAbs. The cELISA data for the mAbs defined as C specific in iELISA showed that a 400 fold greater concentration of *B. abortus* S1119-3 OPS was required for 50% inhibition compared to the concentration of *B. melitensis* 16M OPS required. The concentration of *Y. enterocolitica* O:9 OPS required for 50% inhibition was a further 12 to 15 time greater than the concentration of *B. abortus* S1119-3 OPS required. In this format the specificity of these mAbs appeared to be more 'M' like rather than demonstrating equal binding to both types of *Brucella* OPS – A and M – without binding to the *Y. enterocolitica* O:9 OPS. Thus there was less evidence for C specific specificity in the cELISA format than there was in the iELISA format.

1.7.5.1. The application of synthetic oligosaccharides to epitope mapping studies

This cELISA format was taken further by the application of synthetic oligosaccharides as competitive inhibitors in order to give a clearer picture as to which specific structure the mAbs are binding to

(Bundle et al., 1989a). Each oligosaccharide was a homopolymer of 4,6-dideoxy-4-formamido-D-mannose and varied by length and glycosidic linkage. There were seven oligosaccharides tested in total, three of which were exclusively α -(1→2) linked and contained three, four or five monosaccharides. One was a disaccharide with an α -(1→3) link. Another was a trisaccharide with terminal α -(1→2) and reducing end α -(1→3) link. The remaining two were both pentasaccharides containing 3 α -(1→2) links and a single α -(1→3) link at either the reducing or terminal end. Each oligosaccharide was closed with an O-methyl group at the reducing terminus.

For cELISA with the immobilised A specific mAb, the concentration of oligosaccharide required for 50% inhibition was lowest for the exclusively α -(1→2) pentasaccharide, higher for exclusively α -(1→2) linked tetrasaccharide and higher again for the exclusively α -(1→2) linked trisaccharide (results for the oligosaccharides containing α -(1→3) links were not provided). The results for the C/Y specific mAb demonstrated that the exclusively α -(1→2) pentasaccharide and tetrasaccharide gave equal inhibition but the concentration required was higher for the exclusively α -(1→2) linked trisaccharide. These results suggest that the epitope for the mAb with C/Y specificity is the α -(1→2) linked tetrasaccharide, adding another saccharide and another link does not improve binding whereas losing a α -(1→2) linked saccharide reduces binding affinity. The results also suggest that the structure of the epitope for the A specific mAbs is at least an α -(1→2) linked pentasaccharide as a reduction in length leads to a loss of affinity.

The original interpretation of the described data for the binding of the synthetic oligosaccharides to the A and C/Y specific mAbs was considered to be supportive of the contemporary putative structure of the *Brucella* A and M OPS types (Bundle et al., 1987b; Meikle et al., 1989) and that of *Y. enterocolitica* O:9 (Caroff et al., 1984a). This analysis described the M type of OPS of being a repeating pentasaccharide unit comprised of four α -(1→2) and one α -(1→3) linked 4,6-dideoxy-4-formamido-D-mannopyranosy units. It was therefore easy to visualise from this data that a mAb with a specificity to an α -(1→2) linked tetrasaccharide would bind to the M type OPS whereas a mAb with greater specificity to a α -(1→2) linked pentasaccharide would not, after all - the repeating pentasaccharide unit within the M type OPS contained one α -(1→3) link.

However, the *Brucella* OPS structure has recently been revised (Kubler-Kielb and Vinogradov) and the M type OPS is now reported to contain a repeating tetrasaccharide unit comprised of three α -(1→2) and one α -(1→3) linked 4,6-dideoxy-4-formamido-D-mannopyranosy units. How does this fit

with the epitopes described by the oligosaccharide binding data? Closer scrutiny demonstrates that the oligosaccharide binding data has a superior fit to the newly proposed structure (Kubler-Kielb and Vinogradov, 2013a). OPS comprised of a repeating pentasaccharide unit as described above would, in fact, contain an α -(1→2) linked pentasaccharide when one considers that the individual pentasaccharide only contains four links, each of which may be α -(1→2) according to the 1987 model. Thus, a mAb with specificity to such a structure (which in the original interpretation is described as A specific) should, in fact, bind to the '1987' M type OPS and should not demonstrate specificity for the A type OPS. The revised '2013' M type OPS model does not contain a series of four continuous α -(1→2) links within its repeating unit and would therefore bind less effectively to mAbs of the A type specificity. Thus, the data from the oligosaccharide binding study is more supportive of the revised 2013 OPS structure (Kubler-Kielb and Vinogradov, 2013b) and, to some extent, predicts it.

Although the more recent structural determination describes a tetrasaccharide rather than a pentasaccharide repeating unit, both agree on the ratio of α -(1→2) and α -(1→3) links. This ratio is four to one. In the earlier descriptions this occurs through their relative frequency in the repeating unit whereas the recent description predicts an α -(1→2) repeating polymer of variable length at the reducing end (Kubler-Kielb and Vinogradov). In M type OPS this sequence is relatively short but it may be long enough to bind some A specific mAbs albeit with a lower relative efficiency.

The prediction of the structure of the epitope to which the M specific mAbs bind is less certain than the understanding of the epitope to which the A specific mAbs bind. It seems evident from the *Brucella* biovar binding profiles of the mAbs, and what is known about the structure of the OPS, that the α -(1→3) linkage and its relative abundance plays a critical role. This is borne out, to some extent, by the mAb and oligosaccharide binding data (Bundle et al., 1989a). Three of the six remaining mAbs that are neither A nor C/Y specific, and which are M or C specific (as described above, based on the cELISA results with the native OPS all appear to be M specific) do not react against any of the four oligosaccharides that contained an α -(1→3) linkage at the concentrations applied. Two of the three mAbs that did react reacted only with the pentasaccharide with an α -(1→3) linkage at the reducing end. The final mAb bound to all four of these oligosaccharides, most effectively to the trisaccharide (with a terminal α -(1→3) linkage), then the pentasaccharide with the terminal α -(1→3) linkage, next to the pentasaccharide with the reducing end α -(1→3) linkage and less effectively to the α -(1→3) linked disaccharide. Unfortunately there were no oligosaccharides synthesised containing an α -(1→3) link flanked on either side by α -(1→2) linkages either as a pentasaccharide or a

tetrasaccharide. Indeed, no α -(1→3) tetrasaccharide was synthesised. None of these mAbs bound to the oligosaccharides without an α -(1→3) linkage.

Despite the inclusion of an α -(1→3) linkage seeming critical to the M specific mAb epitope, for five out of six mAbs none of the synthesised oligosaccharides appeared to represent the fully functional epitope structure. The epitopes required may be larger than those synthesised and may indeed require more than one repeating unit in order to create the full antibody binding site, as has been seen to be the case in the binding of mAbs to the O-antigen of *Shigella flexneri* (Vulliez-Le Normand et al., 2008). However, the study at hand demonstrated that disaccharides may be sufficient for antibody binding. It has been shown on studies of the structurally related OPS from *Vibrio cholerae* O1 that mAbs may bind a single monosaccharide with high affinity and specificity (Villeneuve et al., 2000). It is therefore also possible the *Brucella* M type OPS mAbs do bind with maximum affinity to a single repeating unit, or part of it. Given the absent or partial binding of the anti-M mAbs to the synthesised oligosaccharides it is a reasonable hypothesis that a more functional M specific epitope would be one containing an α -(1→3) link flanked on either side by α -(1→2) linkages. Given that the data from the oligosaccharide binding study also demonstrated that the A specific mAbs bound to an α -(1→2) linked trisaccharide (with less effectiveness compared the to equivalent tetrasaccharide) a specific M epitope antigen may need to contain no continuous α -(1→2) linkages. Therefore a reasonable hypothesis for such a structure would be a 4,6-dideoxy-4-formamido-D-mannose tetrasaccharide with a central α -(1→3) link flanked on either side by single α -(1→2) linkages. A schematic representation of the OPS structures and the proposed internal epitopes is presented in figure 1.14. Figure 1.15 presents putative structures for the discrete A, C/Y and M epitopes.

It is also notable that the structural models for *Brucella* OPS provide substantial underpinning to the observed specificities of the A, M and C/Y specific mAbs. There is no such structural feature (or features) that reasonably account for the existence of the C specific mAb(s).

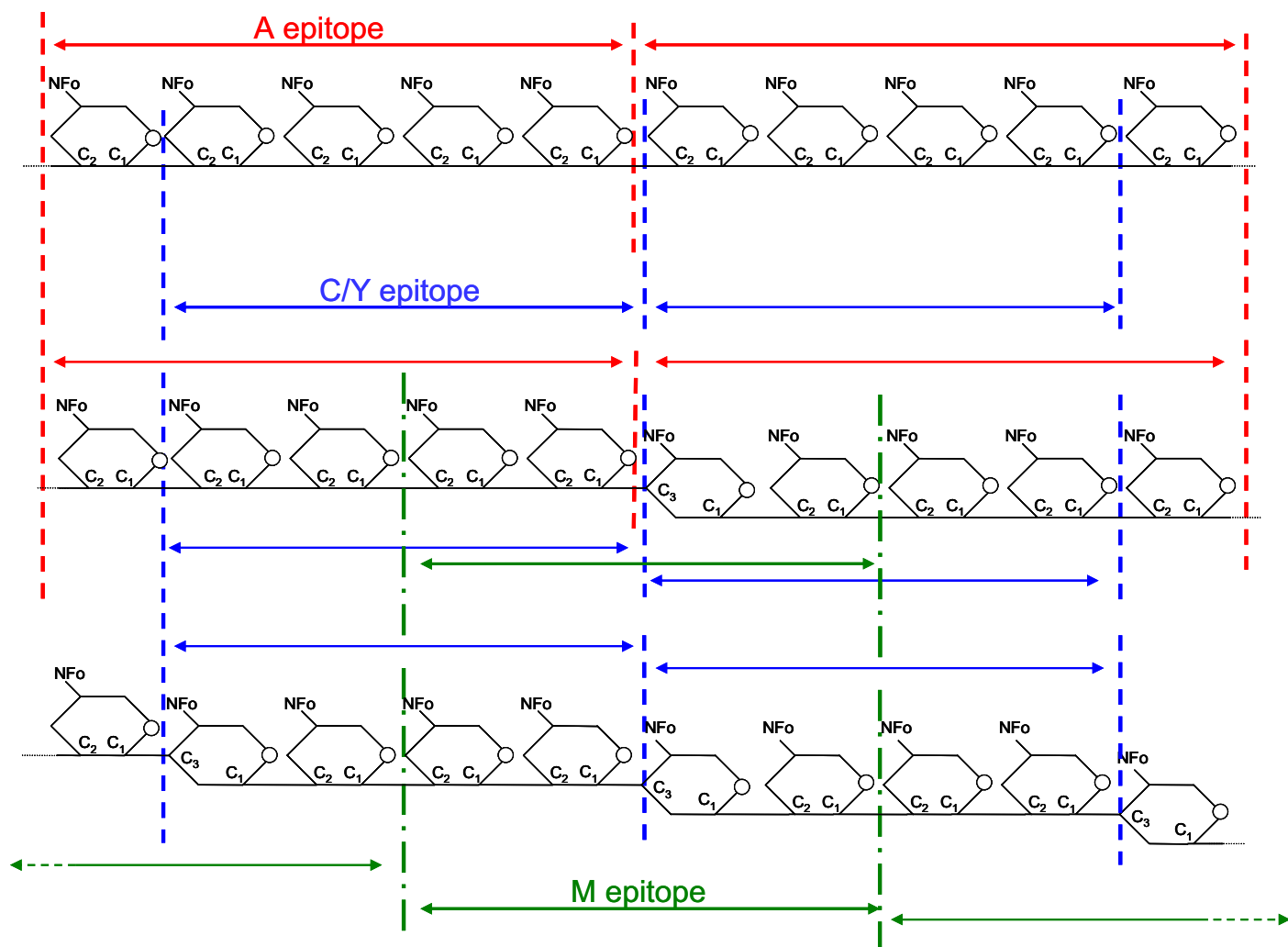


Figure 1.14. A schematic representation of D-Rha4Nfo OPS, glycosidic linkage and epitopes

The figure above is a schematic representation of the structure three of types of D-Rha4Nfo homopolymers (which are shown as hexagons representing the pyranosyl ring with the positions of carbons 1, 2, and 3, the N-formyl group and the oxygen between carbons 1 and 5 shown). The top polymer is exclusively $\alpha(1\rightarrow2)$ linked, as found in *Y. enterocolitica* O:9 OPS. The middle polymer is predominantly $\alpha(1\rightarrow2)$ linked with an occasional $\alpha(1\rightarrow3)$ link (as shown centrally) as found in *Brucella* A dominant OPS. The bottom polymer is a tetrasaccharide repeat of 3 $\alpha(1\rightarrow2)$ and 1 $\alpha(1\rightarrow3)$ linkages as found in *Brucella* M dominant OPS. The red lines indicate an A epitope as would be found in *Y. enterocolitica* O:9 and *Brucella* A dominant OPS. This is formed of five or more D-Rha4Nfo units joined exclusively by $\alpha(1\rightarrow2)$ links. This structure, and therefore epitope is not found in the *Brucella* M dominant OPS. Owing to the repetitive nature of the polymer, these A epitopes overlap each other. They also overlap the C/Y epitopes (common to all *Brucella* and *Y. enterocolitica* O:9 OPS) which are formed of four or less D-Rha4Nfo units joined exclusively by $\alpha(1\rightarrow2)$ links. These may also overlap each other and, because they are found on the *Brucella* M OPS they may also overlap with the M epitopes which are found with high frequency in *Brucella* M dominant OPS but also in *Brucella* A dominant OPS with lower frequency. Because the M epitopes contain an $\alpha(1\rightarrow3)$ link they are not found in *Y. enterocolitica* O:9 OPS.

All this data supports the central importance of the linkage type within the OPS for mAb binding. This was recently reinforced by some additional evaluation of mAb binding to synthetic homologues of *Brucella* OPS (Guiard et al., 2013) where the inclusion of an α -(1→3) linkage into the 4,6-dideoxy-4-formamido-D-mannose polymer allowed an M specific mAb to bind to it. When the structure was a pentaasaccharide containing a single central α -(1→3) link it bound anti-A and anti-M mAbs equally. When this lengthened by additional α -(1→2) linked units flanking the central α -(1→3) link the binding of the anti-M mAb was unchanged whereas the binding of the anti-A mAb increased tenfold. This supports the conclusions from the earlier oligosaccharide binding data but also demonstrates that the pentasaccharide, which contains two contiguous α -(1→2) links, is sufficient for the binding of anti-A specific antibodies, albeit suboptimally.

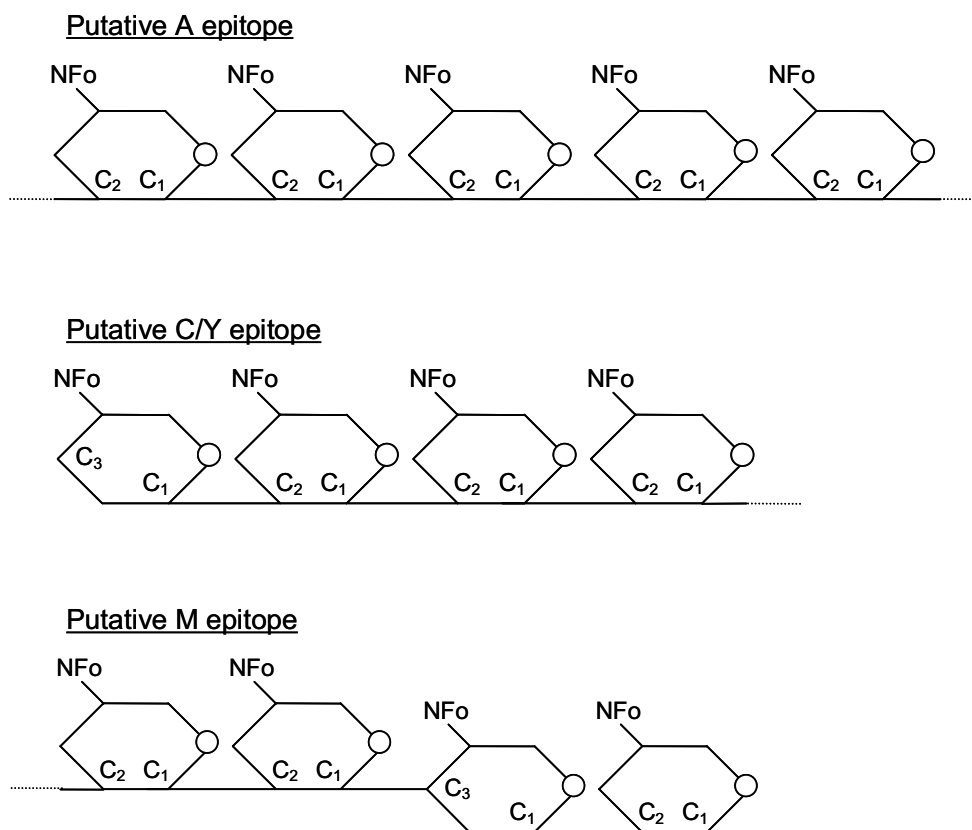


Figure 1.15. Putative structures for *Brucella* OPS epitopes

On the basis of the data derived from mAb binding to native OPS and synthetic oligosaccharides and the structural data derived from Nuclear Magnetic Resonance and Mass spectrometry putative structures for the A, C/Y and M epitopes may be proposed. These structures are not absolute because antibody binding is based on affinity and therefore, in practise, quantitative rather than qualitative. Decreasing or increasing the D-Rha4NFo units or repositioning the glycosidic linkage types will result in quantitative changes to antibody binding. The putative M epitope described has not previously been synthesised.

As well as evaluation by mAbs the method for biotyping *Brucella* strains into A and M types through the use of absorbed monospecific polyclonal sera suggests that the α -(1→3) linkage can also be recognised with some degree of specificity by polyclonal sera. What has not previously been evaluated is the degree to which non-absorbed polyclonal sera may specifically bind to this structure in any measureable manner.

There have been several other distinct examples of anti-*Brucella* mAb production (Bundesen et al., 1985; Greiser-Wilke et al., 1985; Holman et al., 1983; Limet et al., 1987; Portanti et al., 2006; Rojas et al., 1994; Schurig et al., 1984) each of which have used, at least for the initial inoculation, crude inactivated *Brucella* cells. Each study has resulted in the production of mAbs against the sLPS, in particular the OPS (further evidence for the humoral immunodominance of this antigen) and the mAbs produced have fallen into the A, M and C/Y specificities with some investigations also suggesting a C specificity. Several of these mAb production projects have lead to competitive ELISAs for the serodiagnosis of *Brucella* and some of these are commercially available and routinely used (Greiser-Wilke et al., 1985; Perrett et al., 2010). Some of the mAbs have been used as valuable research tools (Gonzalez et al., 2008; Rojas et al., 1994) and others for the evaluation of passive immunity (Holman et al., 1983; Montaraz et al., 1986).

1.8. Generation of anti-*Brucella* OPS mAbs – a case study

A particular series of mAbs have been extensively investigated in their own right (Laurent et al., 2004; Limet et al., 1987) and these provide an informative case study which demonstrates their value and limitations as tools. These were raised against a number of different *Brucella* species and strains that includes the A type OPS carrying *B. abortus* biovar 3 (Limet et al., 1987), M type OPS carrying *B. melitensis* biovar 1 and *B. abortus* biovar 4 as well as the A type OPS carrying *B. abortus* biovar 1 (Limet et al., 1989a) and the rough strains *B. abortus* 45/20 and *B. melitensis* B115 (Cloeckaert et al., 1990). Their specificities were evaluated by iELISA, agglutination assays and Western blot (Cloeckaert et al., 1993; Garin-Bastuji et al., 1990a) the results of which appeared to reaffirm the previously observed OPS specificities: A, M, C/Y and C. Interestingly, mAbs induced by the rough *B. melitensis* B115 induced anti-OPS antibodies of C and M specificity. Although this strain has no surface OPS, this molecule is present in the cytoplasm (Cloeckaert et al., 1992c) and the mAb binding studies suggest that it doesn't contain epitopes in common with the OPS of *Y. enterocolitica*

O:9 OPS. The protective efficacy of passive transfer was also evaluated for mAbs of differing OPS epitope specificity (Cloekaert et al., 1992a; Jacques et al., 1992; Limet et al., 1989b). These studies demonstrated that protection against challenge by either A or M dominant strains of *Brucella* can be achieved via the passive transfer of mAbs that have A, M or C specificity. However, with increased challenge doses and the use of more susceptible mouse models it seems that mAbs of A and C specificity can induce protection against A and M dominant strains but that mAbs of M specificity do not induce protection against A dominant strains (Cloekaert et al., 1992a). The affinity and isotypes of antibodies also plays a role and because of the difficulty to control for these effects the interpretation of the results should be tempered with some caution.

The specificities of the mAbs generated were reinvestigated by iELISA and the conventional A, M, C and C/Y specificities subdivided into A, M, C (M>A), C (M=A), C/Y (M>A), C/Y (M=A) and C/Y (A>M) (Weynants et al., 1997). Several of these specificities are probably likely to represent a more relaxed specificity to certain epitopes that are already at least partly understood – such as the contribution of the α -(1→3) linkage to M epitope and most likely also to the C (M>A) epitope. Yet there appears to be no existing structural rationale for the 'C/Y (M>A)' epitope or for the 'C (M=A)' epitope (itself a redefining of the conventional C epitope). The report also describes the existence of a mAb with specificity to *Brucella* A type OPS with minimal binding to either *Brucella* M type OPS or the OPS from *Y. enterocolitica* O:9 and the authors accept that these binding profiles have no known structural basis. The recent re-evaluation of the structure of *Brucella* OPS (Kubler-Kielb and Vinogradov) does nothing to aid the interpretation of this mAb binding data or the assignment of such specificities.

The study also included data from cELISAs developed by directly labelling the mAbs with HRP and adding them to the wells of sLPS coated microtitre ELISA plates. The mAbs were evaluated against both A and M dominant sLPS and the degree to which their binding was inhibited by mAbs of other specificities were evaluated by measuring the concentration at which these, unlabelled mAbs, caused defined inhibition of OD. The results demonstrated that so long as a mAb was able to bind to the particular OPS type in iELISA it was capable of competing with other binding mAbs even when the mAbs had different epitopes and specificities. As described by the authors, this apparent paradox is explained by the nature of the repeating polymer. Within the OPS the epitopes overlap each other as, for example, the epitope for an M specific mAb may contain one or more α -(1→2) linkages as well as an α -(1→3) linkage. These α -(1→2) linkages may themselves be the target of mAbs with C/Y specificity that bind to relatively short sections of contiguously α -(1→2) linked residues.

One of the *Brucella* OPS specific mAbs, C specific 12G12, was used to develop a cELISA for the serodiagnosis of brucellosis (Weynants et al., 1996a). The cELISA used sLPS from *B. melitensis* vaccine strain Rev1, which possesses M dominant OPS. HRP conjugated mAb 12G12 was co-incubated in the wells of these coated ELISA plates along with diluted test serum. The anti-OPS titre of the test sera was determined by the degree to which the HRP driven substrate colour change was inhibited. This inhibition being the result of competition between the serum antibodies and the HRP conjugated mAb for binding to the OPS. The aspiration was that that the assay would only be sensitive to inhibition by antibodies of the same epitope specificity as the mAb and therefore only sensitive to inhibition by *Brucella* specific antibodies. However, due to the overlapping nature of the antibody epitopes, antibodies with specificities other than C, could not only bind the OPS but inhibit binding of the C specific mAb due to steric hindrance. Such antibodies would include those with M or C/Y specificities, that latter population capable of being generated by infection with *Y. enterocolitica* O:9.

This proved to be the case when the cELISA was evaluated against sera from cattle experimentally infected with *Y. enterocolitica* O:9 and when tested with FPSR sera from the field. Although the cELISA did appear to be more specific than the conventional serodiagnostic assays (RBT and CFT) it did not provide a clear solution to the issue of false positive serological reactions (FPSRs) especially as it appeared to be less diagnostically sensitive than the RBT (although similar to the CFT) (Weynants et al., 1996a). Data similar to that generated from the cELISA developed with the 12G12 mAb has been reported for similar cELISAs developed elsewhere (McGiven et al., 2008) and also for blocking ELISAs where the labelled mAb is added after the incubation and washing of the serum (Kittelberger et al., 1998).

In addition to evaluation by iELISA and cELISA, the mAb specificities were also investigated by flow cytometry (Cloekaert et al., 1998). These results confirmed much of the previously defined mAb specificities but did present some interesting anomalies that were acknowledged by the authors. For example, the mAbs defined as C/Y (A>M) and C/Y (M>A) both appeared to bind equally to all strains whether presenting A or M type OPS. An A specific mAb that did not bind to the surface of *Y. enterocolitica* O:9 in iELISA did so in flow cytometry to a degree equal to that of the binding to the *Brucella* strains. The observations from the flow cytometry are a better fit with the structural information on the OPS. The data also complements earlier studies of protection against *Brucella* challenge by the passive transfer of mAbs (Cloekaert et al., 1992a) in which a mAb previously

defined, by iELISA, as binding preferentially to A type OPS gave good protection against mice challenged with either A or M dominant *Brucella* stains.

These observations prompted the authors to consider that antibody binding during flow cytometry, in suspension, was a better indication of *in-vivo* antibody binding properties than iELISA. Applying this same rationale to the iELISA and cELISA binding studies of others (Bundle et al., 1989a) would provide a case for accepting the specificities defined by cELISA over those defined by iELISA. In that study, mAbs with the appearance of C specificity on iELISA appeared to be M specific by cELISA. The flow cytometry study (Cloeckert et al., 1998) however seems to support the designation of a C specificity to some of the mAbs. Two of the three C specific mAbs studied showed no reaction against *B. suis* biovar 2 strains and it is now known that this OPS doesn't possess any α -(1→3) links (Zaccheus et al., 2013). It is therefore quite probable that this linkage type is an integral component of the epitope for these C specific mAbs. The structure of the epitope for the remaining C specific mAb '12G12' remains difficult to rationalise. Confirmation of the regular sequencing of the α -(1→3) link within the *Brucella* OPS (Kubler-Kielb and Vinogradov) diminishes the potential structural configurations that would have been possible had the linkage occurred randomly. However, the discovery of the non-perosamine elements of the OPS (Kubler-Kielb and Vinogradov, 2013b) does facilitate speculation that the reducing end of the OPS may be a source of the C specific epitope.

1.8.1. Discrete non-overlapping OPS mimitopes

The conclusion that the failure of cELISAs employing *Brucella* specific mAbs to eliminate FPSRs was due to steric hindrance prompted passing comment on the use of specific short oligosaccharides, in particular a synthetic C epitope, for serodiagnosis (Weynants et al., 1997). However as the structure of the C epitope was (and still is) unknown synthesis was not an option. Synthesis of the M epitope was not considered, presumably because the authors considered that assays based on such antigens would only detect sera from individuals infected with M dominant strains of *Brucella*. If true such assays would be of very restricted value given the epidemiological circumstances, residual bovine brucellosis due to *B. abortus* biovars 1 and 3 (A dominant) infection, of the region funding the research (Northern Europe). The research instead followed a path towards the screening of random peptide libraries with the aim of finding peptide mimitopes for OPS epitopes. This approach met with modest success with the selection of peptides from two out of four mAbs used, one A specific and one M specific, which generated a mild anti-sLPS response in some peptide immunised mice (De

Bolle et al., 1999; Mertens et al., 2001). This process was revised with the use of a DNA delivery system to synthesise freshly selected peptides *in-vivo* (Beninati et al., 2009). On this occasion the study was able to discover immunogenic sLPS peptide mimics from two other mAbs including the C specific 12G12. Although some, but not all, of the peptide encoding DNA immunised mice induced moderate anti-sLPS titres the immunisations raised a far higher titre of peptide specific but not sLPS reactive antibodies. This possibly reflects the ability of the peptides to adopt a variety of configurations not all of which are desirable and may be one of the reasons why research in this particular area has not continued. Despite earlier published comments (Weynants et al., 1997), it is intriguing as to why none of the published data relating to the peptides includes any evaluation as serodiagnostic antigens. The impression given is that once the concept of peptide mimics of OPS structures was considered their potential as T-cell recruiting vaccine candidates became the focus of research.

The OPS mimicking properties of anti-idiotope antibodies have also been evaluated (Evans et al., 1994; Young et al., 1999). This approach is not dissimilar to that of the selection of peptide mimics as in both processes the structure of the final antigen is unknown at the outset, it is formed of amino acids and is selected by existing antibodies to OPS. The anti-idiotype antibodies were generated by immunising mice with mAbs of C/Y specificity (Bundle et al., 1984). Some of these were shown by cELISA with biotinylated OPS to bind specifically to the paratope of the anti-*Brucella* mAb. Despite this, immunisation of mice with the anti-idiotypic mAbs did not yield antibodies that were reactive with the original antigen. Although there was a good association between the anti-*Brucella* mAb and its anti-idiotope Fab the interaction did not form an intimate fit within the cleft of the original mAb. Therefore neither of the amino acid based approaches to create, via mimicry, discrete specific epitopes from the *Brucella* OPS was successful. Therefore the aim of developing such epitopes that are useful for diagnosis has been unreachd.

1.8.2. Anti-*Brucella* OPS mAb structure and relation to specificity and function

The amino acid sequence, 3D structure, binding potential and protective efficacy of five anti-*Brucella* OPS mAbs described in earlier studies (Clockaert et al., 1998; Weynants et al., 1997) has also been evaluated (Laurent et al., 2004). The mAb 12G12, previously described as C (M=A), appeared in this set of iELISAs to bind with greater efficiency to the M OPS than to the A type OPS with dose response curves similar to that of the 12B12 mAb (previously described as having a C (M>A)

specificity) albeit with reduced overall titre. The difference in titre between the reaction to the M and A type OPS was approximately 10 fold which is in line with the difference in the proportion of α -(1→3) linkage within the two antigens. Thus the authors accept that both these mAbs bind to epitopes containing the α -(1→3) linkage. These mAbs therefore appear to be of M specificity. The binding of the A specific mAb 2C8C4, previously described as not binding to the OPS from *Y. enterocolitica* O:9, did bind to this antigen by iELISA in this most recent study. Thus much of the mAb binding data that was incongruous when evaluated alongside the structural studies has been gradually refuted.

One remaining outlier in this regard is the M specific mAb 6B3 that doesn't appear to bind the A type (or *Y. enterocolitica*) OPS at all. The authors speculate that the epitope to which this mAb binds may be related to a specific modification on the terminal perosamine akin to the structural basis for the Inaba and Ogawa serotypes of *Vibrio cholerae* O:1 (Villeneuve et al., 2000; Wang et al., 1998) whereupon the terminal residue of the Ogawa serotype is modified by an O-methyl substituent. At the time of publication this type of modification in *Brucella* M type OPS seemed possible, if not probable. However, the absence of any such modifications in the recent structural evaluation of the OPS by NMR (Kubler-Kielb and Vinogradov) makes this theory quite improbable.

The evaluation of the protective efficacy, mediated by passive transfer, of these mAbs against challenge by *Brucella* stains with either A or M type OPS demonstrated that the specificity of the mAb was a significant factor in protection. The 12B12 mAb gave good protection against challenge by *Brucella* with A or M type OPS which matches the iELISA observation that this mAb binds to both types of OPS (albeit to a different degree).

Analysis of the amino acid sequences of the five mAbs demonstrated that they all contained mutations arising from the affinity maturation process in addition to variability that is obtained through recombination of the H-VDJ and L-VJ regions. Although of different clonal origin the mAbs 12B12 and 12G12 possess some identical substitutions within the complementarity determining regions. Overall there is a high level of sequence identity between the antibodies especially for mAbs with similar OPS epitope specificities. Computer generated 3D shapes of the mAbs, based on their sequence within the antibody framework, demonstrated two basic configurations. The three mAbs believed to bind oligosaccharides with an α -(1→3) linkage were predicted by the software to contain a cavity within their paratope whereas those that bind epitopes made of contiguous α -(1→2) linkages were predicted to possess grooves along their surface. This corresponds to the predicted shape of the particular oligosaccharide epitopes and is similar to findings elsewhere (Villeneuve et al., 2000).

Developing a detailed understanding of the *Brucella* OPS has been an important research objective given the role of this molecule in bacterial virulence, pathogenesis, immunity, vaccination, protection, and diagnosis. As has been described above, infection and immunisation with smooth *Brucella* strains gives rise to a high level of anti-OPS antibodies. The OPS dominates the humoral immune response to such an extent that in order to generate mAbs to other cell surface constituents one would be well advised to immunise with rough strains of *Brucella* (CloECKaert et al., 1990). Even then, any OPS present – even if internal – may give rise to a significant antibody response (CloECKaert et al., 1993).

1.9. Detection of polyclonal anti-*Brucella* OPS antibodies for diagnosis of brucellosis

The classical and contemporary serodiagnostic assays for animal brucellosis have been the subject of several recent reviews (Gall and Nielsen, 2004; Godfroid et al., 2010; Nielsen, 2002; Nielsen and Yu, 2010; Poester et al., 2010) and are well described and standardised (Davidson et al., 1969; Garin-Bastuji and Blasco, 2009; McGiven et al., 2011; Nielsen and Ewalt, 2010; Olsen, 2010) with efforts made towards the international harmonisation of serological results (McGiven et al., 2006). Many of these tests are recognised by the World Organisation for Animal Health (still known by its French acronym 'OIE', Office International des Epizooties) and are prescribed for use in the international trade of animals and for the official certification of brucellosis free status (as determined by the standards set in the OIE Terrestrial Animal Health Code). The qualities of these diagnostic tests are underpinned by the validation standards set by the OIE (Jacobson, 1998; Wright, 1998). There have been numerous publications describing the validation of these serodiagnostic assays including meta-analysis of these (Greiner et al., 2009). The diagnostic performance of these assays has also recently been the subject of reviews by the European Food Standards Agency (EFSA, 2006, 2009). All this scrutiny, control, standardisation, and evaluation does not mean that these tests are perfect and in the case of brucellosis, as with many other diseases, there are numerous imperfections (McGiven, 2013).

The SAT, Acidified agglutination assays, CFT, indirect and competitive ELISAs (based on the sLPS antigen) and the FPA are the most recognised and accepted methods for the serodiagnosis of brucellosis due to infection with *B. abortus*, *melitensis* and *suis*. All the assays use antigens that are rich in OPS and this is no coincidence as this molecule dominates the humoral immune response.

This is demonstrated by the preponderance of anti-OPS antibodies produced during mAb production studies and the application of rough strains to produce mAbs to non-OPS antigens (Cloeckert et al., 1990). Hyperimmune sera produced for the classification of strains into A or M types by agglutination do not agglutinate rough strains suggesting an absence of sufficient antibodies to antigens other than the OPS (and sera used for the agglutination of rough strains do not agglutinate smooth strains). The most sensitive assays for serodiagnosis of brucellosis contain abundant OPS. For example the FPA antigen is purified OPS and in the case of the recognised iELISA the antigen is the sLPS. Despite much attention, no universally accepted alternative antigen is in routine use as a serodiagnostic agent (McGiven, 2013).

Despite the imperfections of serology, which will be considered in more detail later, it is the method of choice to aid in the initial presumptive diagnosis of brucellosis (McGiven, 2013). The diagnostic material is relatively easily accessible, the tests are relatively cheap, available and sensitive. Alongside many other crucial components (Blasco, 2010) the currently available serodiagnostic assays have been shown to be capable of enabling the sustained eradication of brucellosis. The situation in Great Britain is a good example of this where, after a sustained campaign of control and eradication lasting for approximately 40 years, England, Scotland and Wales were declared officially free of disease in 1985. In the context of a surveillance or eradication programme for animal brucellosis the fact that serological assays do not demonstrate active infection but exposure at some previous time point is not a significant handicap. Demonstration of exposure is sufficient evidence that some form of action and intervention is justified in order to minimise risks of disease incursion and distribution. The consequences of incorrect inactivity would far out way the consequences of incorrect activity.

This type of process is clearly very different to what would be undertaken in human cases where finding signs of active infection is much more important so as to guide treatment options. In this case evaluation of the relative titres of antibody isotype, especially IgM and IgG would be of interest as would some form of direct detection of bacteria or bacterial DNA (Franco et al., 2007).

Serological testing of cattle in Great Britain is performed using a combination of tests in order to maximise cost effectiveness and diagnostic quality. This includes the CFT, SAT, RBT, iELISA and cELISA (MacMillan et al., 1990; McGiven, 2003). All antigens used and tests performed are done so according to the methods described in the OIE Manual for Standards and Vaccines (Nielsen and Ewalt, 2010). The antigens are either crude cell or sLPS preparations. The discovery of serologically

positive animals necessitates considerable field intervention by the relevant veterinary agency in order to restrict and trace animal movements, consider slaughter, culture and retesting. Thus positive serology incurs great costs to the surveillance programme.

1.9.1. Classical serology: Serum Agglutination Test

The first serodiagnostic assay for brucellosis was reported in the late 19th century (Weight, 1897) which was a simple agglutination assay whereby killed *Brucella* cells were added to diluted serum and the degree to which these cells agglutinated at the bottom of the test tube was observed (agglutination being indicative of an anti-*Brucella* antibody titre). This test, described as the Serum (or Tube) Agglutination Test is still in use in many laboratories today (figure 1.16). At neutral pH the most actively agglutinating antibody isotype is IgM and therefore it may be relatively more prone to agglutination by cross reacting antibodies and have reduced diagnostic specificity (Lamb et al., 1979; McGiven, 2003). The use of chelating agents such as EDTA and EGTA may lead to some improvements in specificity (Nielsen et al., 1994), but in any event the assay is not listed by the OIE as a prescribed assay for serodiagnosis of brucellosis.

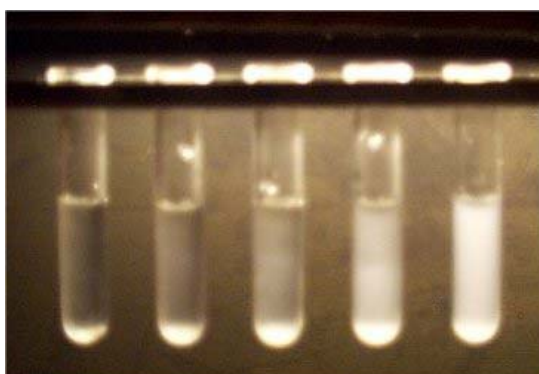


Figure 1.16. Serum Agglutination Test (SAT)

The image shows a titration series of positive serum where anti-*Brucella* antibodies become diluted (from left to right) leading to a reduction and finally absence of antigen agglutination.

1.9.2. Classical Serology: acidified agglutination tests

Improvements to agglutination assays were made by decreasing the pH of the buffer to ameliorate the influence of IgM over other antibody isotypes and by staining the *Brucella* cells to aid in the visual interpretation of rapid agglutination on a slide or tile. The Rose-bengal test (RBT) was developed in the 1960s (Morgan et al., 1969; Nicoletti, 1967) and applied the Rose-Bengal stain so that the cells

were easily visualised (figure 1.17) and the assay was buffered at pH 3.65. The buffered antigen plate agglutination test (BPAT) developed subsequently (Angus and Barton, 1984) fulfils a similar role. Both tests are commercially available, cheap to buy and very simple and rapid to perform. They may be well standardised, dependent upon the source, and both are recognised as prescribed tests by the OIE, and have good diagnostic sensitivity. Although both are often described as being less prone to the interactions of IgM antibodies than the SAT this has been demonstrated not always to be the case (Allan et al., 1976), and this does not make them invulnerable to false positive results. In fact, the good sensitivity makes them prone to the detection of antibodies that have been raised as a consequence of infection with gram negative bacteria in possession of OPS epitopes similar to those of *Brucella* either in cattle (Pouillot et al., 1997) or swine (McGiven et al., 2012).



Figure 1.17. Rose Bengal test (RBT)

The image shows two negative sera (left, 1 and 2) and two positive sera (right, 3 and 4) after mixing with RBT antigen and rocking for 4 minutes. Agglutination is clearly visible with the positive sera.

1.9.3. Classical serology: Complement Fixation Test

The complement fixation test was developed in the 1960s (Hill, 1963; Jones et al., 1963; MacKinnon, 1963) and has often been cited as the 'Gold Standard' serological assay. Although antibodies of IgG₁ and IgG₂ isotypes are capable of fixing bovine complement only isotype IgG₁ can fix guinea pig complement as used in the CFT (McGuire et al., 1979). There have been reports that the CFT was sensitive to IgM (Allan et al., 1976) but this seems to have been firmly refuted (Nielsen and Duncan, 1987). There is some evidence to suggest that the specific detection of antibodies of IgG₁ isotype may endow assays with improved diagnostic characteristics (Butler et al., 1986; Lamb et al., 1979; Nielsen et al., 1984), certainly when compared to those that are susceptible to the detection of IgM. The CFT is frequently used within diagnostic laboratories as a confirmatory assay. This is due to its supposedly superior diagnostic attributes, especially compared to the RBT and the SAT - although

this is not necessarily the case (EFSA, 2006). From a more pragmatic perspective it may be applied as a confirmatory assay because it is a far harder assay to routinely operate to a high quality than the agglutination assays (Gall and Nielsen, 2004). Like the SAT and the RBT, the CFT is also susceptible to FPSRs (Godfroid et al., 2002) due to infection with *Y. enterocolitica* O:9.

The SAT, RBT and CFT assays are all vulnerable to prozone effects whereby, if the antibody concentration within the serum is high enough the assays may give false negative effects. Within the agglutination assays it is thought that the occurrence of too many antibodies saturates the surface of the *Brucella* cells and prevents the cross linking of cells that is essential to the agglutination process. In the CFT prozones are considered to be due to high levels of anti-*Brucella* IgG₂ antibodies and this class does not activate guinea pig complement. These antibodies inhibit the binding of the complement activating antibodies IgG₁, and to a lesser extent IgM (McNaught et al., 1977). In all cases, a positive result will not be observed unless the samples are diluted. Therefore unless this is done there is a higher risk of obtaining false negative results. This is an important consideration if any of these tests are selected for a mass screening programme as the routine testing of extra dilutions adds significantly to the costs.

1.9.4. Alternative serology: Gel Immunodiffusion Assays

Agar immunodiffusion and precipitation assays have also been developed whereby the interaction between antibodies in the serum, added to one well, with antigen from *Brucella*, added in a second well, at the diffusion interface creates a visible line of precipitation within the gel in a positive sample. These assays, the Agar Gel Immunodiffusion Test (AGIDT) or Radial Immuno diffusion (RID) test, have been described as having superior diagnostic specificity than other serological tests. This includes a lower sensitivity to serum from animals presenting FPSRs in other assays (Muñoz et al., 2005) and animals with serological titres due to vaccination with smooth strains *B. abortus* S19 or *B. melitensis* Rev 1 (Cherwonogrodzky and Nielsen, 1988; Diaz-Aparicio et al., 1993; Jones et al., 1980). However the same reports, and others (Lord et al., 1997) also show that these assays have lower diagnostic sensitivity than other serodiagnostic methods including some of the classical tests. Given that the antigen primarily used in these assays, the 'Native Hapten' has now been determined to consist of a polysaccharide identical in structure to that of the repeating section of the *Brucella* OPS (Aragon et al., 1996) it is difficult to envisage a means by which the AGIDT or RID test eliminates many undesirable reactions by a mechanism other than reduced sensitivity. Like the SAT,

these gel diffusion assays are described within the OIE Manual for Diagnostic Tests and Vaccines but not as a prescribed test for international trade.

1.9.5. Contemporary serology: sLPS indirect ELISA

Serodiagnosis may also be performed by Enzyme Linked Immunosorbent Assays (ELISA) as first described in the 1970s (Carlsson et al., 1976; Hurvell et al., 1979). These methods employed lipopolysaccharides extracted by a hot phenol water process as the diagnostic antigens and reported that the ELISAs were 10 to 100 fold more analytically sensitive than tube agglutination assays commonly used at the time. In a sense this work encapsulates the iELISA methods that are still in place and commonly used within diagnostic laboratories. The antigen that is almost exclusively used for routine serodiagnosis by ELISA is a preparation of smooth LPS derived from a hot phenol extraction process (Nielsen and Ewalt, 2010; Westphal et al., 1952) and the indirect ELISA format has a very high analytical sensitivity (McGiven et al., 2011; McGiven et al., 2006). In this format, in many cases an anti-bovine IgG₁ conjugate is also used, the iELISA has been used very successfully in many laboratories and in many studies (EFSA, 2006; Gall and Nielsen, 2004; McGiven, 2003; Muñoz et al., 2005) and variants of this assay are globally available on a commercial basis for human and livestock serodiagnosis. A schematic representation of the iELISA is shown in figure 1.18.

1.9.6. Contemporary serology: sLPS competitive ELISA

Competitive (c) ELISAs, such as the type already described above (Nielsen et al., 1995; Perrett et al., 2010; Weynants et al., 1996a) follow a fairly regular format with an sLPS antigen coated to the ELISA plate against which a mouse mAb specific to the OPS binds. The simultaneous addition of test sera, in the case of positive samples, results in competition between serum antibodies and the mAb. The result of the assay is visualised by the detection of the mAb after unbound antibodies have been washed from the plate. The mAb may be directly conjugated with HRP or a second incubation step using an HRP conjugated anti-mouse antibody may be used. When substrate is subsequently added the colour change is indicative of the quantity of mAb present. Inhibition of colour development signifies that competition with serum antibodies has taken place and is therefore related to the antibody titre of the sample. The cELISAs have some practical benefits such as being independent of the host species antibody used and, dependent upon whether the mAb is directly

conjugated, the method may also require less procedural input. A schematic representation of the cELISA is shown in figure 1.18.

These cELISAs also incorporate an increased element of antibody avidity selection compared to the iELISAs due to the competitive nature of the reaction between the mAbs and serum antibodies. They are also more specific in measuring the anti-OPS antibody response than the typical iELISAs which may also detect antibodies to the core and lipid A components of the sLPS (Barrio et al., 2009), although such antibodies may create some element of steric interference with those that bind the OPS. It is the element of avidity selection that has been given to account for the improved diagnostic specificity of the cELISA against antibodies raised due to vaccination with smooth *Brucella* strains (Aguirre et al., 2002; Biancifiori et al., 2000; Marin et al., 1999; Nielsen et al., 1995; Samartino et al., 1999a) and infection with Gram-negative bacteria in possession of OPS epitopes similar to those found in *Brucella* (Muñoz et al., 2005; Nielsen et al., 2004c). The increased specificity does, at least on occasion, come at a cost of decreased sensitivity (Marin et al., 1999; Muñoz et al., 2005; Nielsen et al., 2004a; Samartino et al., 1999a) especially when compared to iELISA. Furthermore, the attributes put forward for the cELISA regarding the reduction of FPSRs are not universally held (Godfroid et al., 2002; McGiven et al., 2008; McGiven et al., 2012; Weynants et al., 1996a).

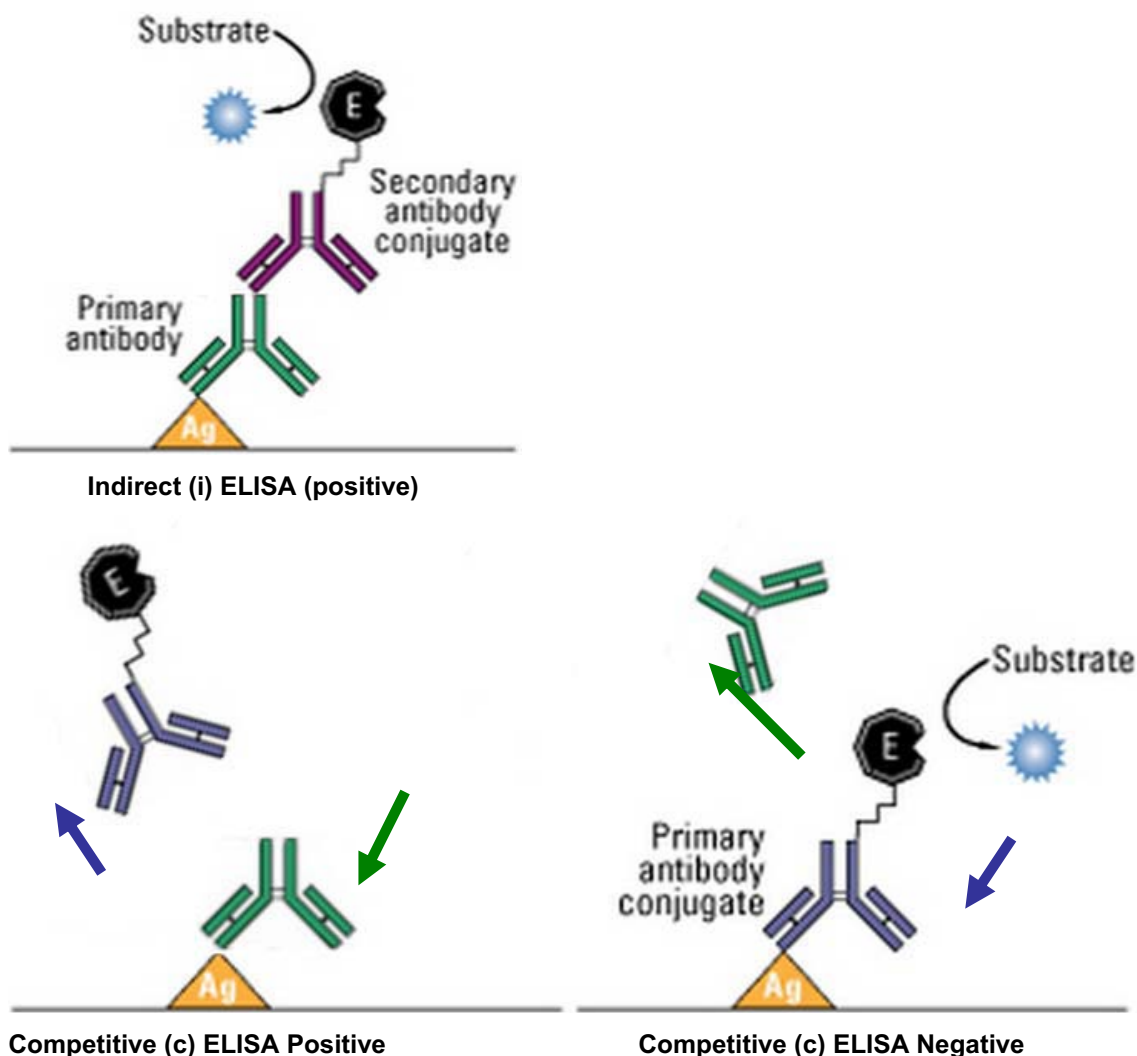


Figure 1.18. Schematic representation of binding in indirect and competitive ELISA

The figure above depicts the indirect (top) and competitive (bottom) ELISA and shows the immunocomplexes formed. The yellow triangle represents the solid phase coated antigen (for example sLPS). Sample antibodies (for example from sera) are shown in green, enzyme (E) conjugated secondary anti-species antibody is shown in purple, and enzyme conjugated anti-antigen mAb is shown in blue. The enzyme is depicted as acting on a substrate to generate colour. In both ELISAs the enzyme complex is built up through the successive addition of sample and conjugates (in the case of the cELISA this is simultaneous) with excess unbound material removed by washing before progression to the next step in the process. In the iELISA, the presence of antigen specific sample antibody enables the subsequent binding and retention of the enzyme conjugated secondary antibody which drives the colour change that indicates the presence of the test analyte. In the cELISA, anti-antigen antibodies within the sample inhibit the binding of the conjugated mAb (left side) which is consequentially washed from the plate prior to the final development stage. When mAb binding is not inhibited, as there may no, few or only low avidity sample antibodies present in the sample, the conjugated mAb is retained for the development stage and consequently colour development occurs (right panel). The addition of fluid phase antigen to the cELISA will create competition for mAb binding which will be manifested as a reduction in final development as mAbs bound to the fluid rather than solid phase antigen are eliminated in the wash. Image has been adapted from the 'Overview of ELISA' available on the Pierce website (<http://www.piercenet.com/method/overview-elisa#elisaformats>).

1.9.7. Contemporary serology: Fluorescent Polarisation Assay

Like the sLPS indirect and competitive ELISAs, the Fluorescent Polarisation Assay (FPA) is also an OIE prescribed serodiagnostic test for use in international trade (Nielsen and Ewalt, 2010). It was developed in the 1990s (Nielsen et al., 1996) and has been applied fairly extensively to bovine sera (Nielsen and Gall, 2001) as well as to sera from swine (Nielsen et al., 1999) and small ruminants (Nielsen et al., 2004a). The FPA is a rapid homogeneous assay that can be done either in single tube or 96 well format. The theory of fluorescence polarisation that underpins the assay is elegantly explained elsewhere (Nasir and Jolley, 1999). The assay employs a purified OPS, or in some instances Native Hapten (Ramirez-Pfeiffer et al., 2008), antigen that has been conjugated to fluorescein and is free in suspension. This conjugated molecule is relatively small and spins rapidly such that upon excitation of the fluorescein with polarised light the subsequent emission of light is depolarised owing to the rate of spin, the time delay between absorption and emission of light. The degree to which this depolarisation occurs can be detected by a bench top (or field portable) reader using polarised filters in the parallel and perpendicular orientation. Any anti-OPS antibodies that are present in serum that is added will rapidly bind to the antigen and cause a decrease in the rate of rotation. This results in a decrease in the depolarisation of emitted light which can be detected by the reader and interpreted, depending upon quantification, as a positive result. A schematic representation of the FPA is presented in figure 1.19.

Although the antibody binding phase can be completed within 2 minutes and the result quantified it is necessary to take a background reading of the serum prior to the addition of antigen to account for any intrinsic fluorescence. Although the method appears to work well with small antigens, the size of the unbound conjugate is limited by the lifetime of the fluorophore and the choice of fluorophore is limited by the intensity of light required. Thus, the antigen must be relatively pure which is why a purified OPS (or Native Hapten) antigen is used. Alternative rapid homogeneous fluorescence based methods for specific antibody detection which do not require a background read and are not size restricted have been developed (McGiven et al., 2009).

There is now a great deal of literature that relates to the validation of the diagnostic properties of the FPA for a variety of host species and circumstances. As well as being a rapid and highly sensitive assay it is also reported to be effective in limiting the detection of antibodies induced through vaccination with smooth strains of *Brucella* (Aguirre et al., 2002) or by infection with Gram-negative

bacteria in possession of OPS epitopes similar to those found in *Brucella* (Nielsen, 2004). However, the abrogation of these responses is far from complete either for serum from vaccinated animals (Nielsen et al., 2007; Samartino et al., 1999b) or from those infected by *Y. enterocolitica* O:9 (McGiven et al., 2008) or field false positive (according to more conventional assays) serum samples (McGiven et al., 2012). In any event, it is difficult to rationalise how an assay that uses purified OPS does not generate positive results when antibodies against this antigen, whatever their origin, are present.

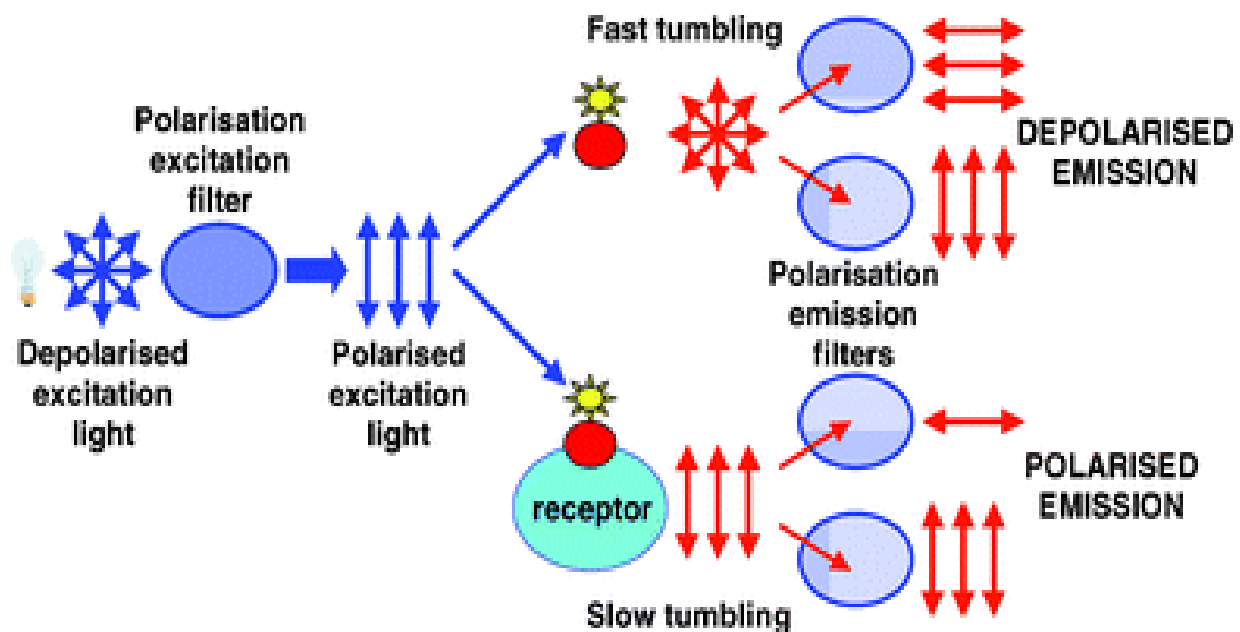


Figure 1.19. Schematic representation of the Fluorescent Polarisation Assay (FPA)

The figure above demonstrates how a small fluorescein conjugated antigen will emit light that remains relatively polarised upon binding a larger molecule (such as an antibody). Excitation of samples with polarised light and detection of emitted light in parallel (polarised) and perpendicular (depolarised) planes may be achieved through a variety of single tube or microtitre plate readers.

1.10. Antibody response to vaccination

Although the use of OPS, or OPS rich, antigens for serodiagnosis provides the assays with good diagnostic sensitivity the reliance upon the OPS creates two main problems. The first issue is the difficulties this creates when trying to differentiate serum samples from animals that have been vaccinated with smooth strains of *Brucella* from those that have been naturally infected. This is a highly important topic and has driven much of the research in antigen discovery. The second issue is

the resolution of false positive serological reactions (FPSRs) that occur due to infection or exposure to Gram-negative bacteria in possession of structures similar to that of the *Brucella* OPS. Attempts to resolve both these issues have focused upon a search for alternative diagnostic antigens that provide assays with workable sensitivity and specificity. Such antigens could be knocked out of vaccine strains to facilitate the production of DIVA assays or may not be shared with those organisms suspected of causing cross reactions.

The control of endemic brucellosis is only achievable via vaccination. At this level of disease other control measures such as husbandry practices may be complementary but are insufficient in isolation. Of the available vaccines two of the three are universally considered to provide useful protective efficacy (Martins et al., 2010). These are the smooth strains *B. melitensis* Rev1 and *B. abortus* S19. However there are considerable drawbacks to their implementation. A critical need for new vaccines was repeatedly identified a recent European Union funded gap analysis review (<http://www.discontools.eu/Diseases/Detail/42>). The following issues were identified. There is no complementary DIVA assay for *B. melitensis* Rev1 and *B. abortus* S19. Both induce antibodies to the OPS that react in the assays that are prescribed by the OIE. In addition the vaccines are live, require extensive quality assurance, may be unstable and require the maintenance of a cold chain. They possess residual virulence in humans and animals (and there is no human or swine vaccine). Vaccination alone does not apply sufficient pressure for eradication. They are insufficiently protective – an extensive and expensive test and slaughter regime is required to eliminate the disease. Without a DIVA test this can only be effectively performed once vaccination has ceased and whereupon animals are once again susceptible to infection.

There has been much research directed towards the generation of improved vaccines (Oliveira et al., 2011), much of it related to finding alternative diagnostic antigens in order to overcome the serological convolutions generated by the currently used smooth strains. A lot of this work also relates directly towards the search for alternative antigens to resolve FPSRs as the antibodies raised in both instances are directed towards the OPS.

1.11. False Positive Serological Reactions (FPSRs)

The *Brucella* OPS is a homopolymer of variably α -(1→2) or α -(1→3) 4-formamido-4, 6-dideoxy- α -D-mannopyranosyl residues. There are a variety of organisms that appear to generate cross reactions

in serology for brucellosis and the common feature appears to be the presence of 4-N-acyl perosamine within their OPS. This particular structural feature has been demonstrated in biochemical studies to be of critical importance to specific antibody binding (Issiki et al., 1995). Some organisms have been demonstrated, by way of experimental infection, to induce anti-*Brucella* antibodies but proving a causal link between such organisms and FPSRs generated in the field is more challenging. The subject of FPSRs and identity of the prime candidates has been previously reviewed (Corbel, 1985) and although this was some time ago the information is still relevant.

This list of organisms includes *Escherichia coli* O:157 (Stewart and Corbel, 1982), *Francisella tularensis* (Francis and Evans, 1926), *Stenotrophomonas maltophilia* (Corbel et al., 1984), *Salmonella* serotypes of Kauffmann-White group N (Corbel, 1975), *Vibrio cholerae* of Inaba and Ogawa serotypes (Feeley, 1969; Wong and Chow, 1937) and *Yersinia enterocolitica* O:9 (Ahvonen et al., 1969). The cross reaction caused by *Y. enterocolitica* O:9 has been demonstrated repeatedly and is the strongest as its polysaccharide O-chain is almost identical to that of *Brucella* in which the A antigen dominates (Caroff et al., 1984a; Caroff et al., 1984b). It is an exclusively α -(1→2) linked 4,6-dideoxy-4-formamido-D-mannose homopolymer (with the exception of the as yet undefined primer and adapter sugars). This cross reaction is the most complete so far described for any antigen sharing antigenic determinants with *Brucella*. Potentially it presents the most serious source of confusion in the serological diagnosis of *Brucella* (Corbel, 1985).

Such is the similarity of the *Y. enterocolitica* O:9 OPS it is incorrect to classify antibodies raised against it as 'non-specific' in the context of conventional and contemporary serology. In such assays, the epitopes against which these antibodies were raised are present in large amounts such is the similarity of the OPS. In areas where *Brucella* is prevalent cross reactions due to epitope similarity may cause only a small percentage of positive serological results and thus be of relatively little significance. However in areas of low incidence or eradication (for example Great Britain) such results may become the significant source of positives (Emmerzaal et al., 2002; Pouillot et al., 1997) and reduce the positive predictive value of the tests to virtually zero.

The influence of cross reactions began to be particularly felt in Northern Europe during the tail end of campaigns to eradicate bovine brucellosis. Although these campaigns were successful in countries such as the Netherlands, Sweden, Finland, Denmark, Belgium, Germany, Austria and Great Britain and France they were prolonged and expensive (Godfroid et al., 2002). It has been estimated that the

cost of eradicating brucellosis in Great Britain, from 1967 to 1981 (although attempts at control predated this time frame), was £200 million.

The increasing influence of false positive serological results identified as a consequence of reducing prevalence of brucellosis created uncertainty and added considerable expense, time and stress to the programmes. In order to maintain officially brucellosis free status these countries had to (and in many cases still do) maintain high levels of surveillance much of which rested upon the serological examination of large swathes of the cattle population. FPSR results required, and still do, considerable investment of time, energy and resources to ensure and demonstrate that such reactions are not due to a re-emergence or reintroduction of disease.

1.11.1. Outbreaks of brucellosis – recent case studies

In 2012 a re-emergence of *B. abortus* biovar 3 in Belgium was confirmed after an abortion within a mixed herd of breeding and fattening cattle was investigated. A second outbreak was confirmed as a result of tracing the animals in the original herd. Subsequently two more cows were confirmed as infected as a result of further tracking of animal movements. A fifth outbreak in cattle was confirmed as a result of enhanced surveillance however this was identified as due to *B. suis* biovar 2. In Calais, France, a further outbreak was confirmed which originated from the first outbreak in Belgium. Belgium was declared officially free of brucellosis in 2003 and it may be that, in the absence of alternative explanatory epidemiological information, that *B. abortus* has re-emerged from some non-livestock niche. Molecular epidemiology relates this outbreak to strains that were present in the original region prior to eradication (Fretin, D. personal communication). During the tracing exercise all the contact farms were serologically tested (98,414 serological tests) with another round of testing to be completed in winter 2012-13. All cattle over 18 months of age that were purchased after the outbreak were also tested (48,583 serological tests). All dairy herds were tested by bulk milk sLPS iELISA. From 23 positive milk ELISA results, follow up serology identified seropositive animals in 11 farms. All seropositive animals were slaughtered and examined by bacteriology. This led to the identification of the animal infected with *B. suis*. None of the other positive animals were bacteriologically positive. As a consequence of the *B. suis* infection an additional 64 contact farms were identified and all animals were serologically tested. All results were negative.

In 2003 a reintroduction of *B. abortus* biovar 1 in Scotland took an estimated £320,000 to resolve (Blissitt et al., 2005). The effective detection and tracing of future incursions of brucellosis would be

significantly enhanced by the ability to eliminate false positive serological reactions from costly and protracted epidemiological enquires and so hasten the transition back to disease freedom.

Since the 1990s the problem of FPSRs seems to have increased, particularly in North Western Europe (MacMillan, 1990; Pouillot et al., 1998), providing fresh impetus into efforts to address this problem which, despite attempts in the 1970s and 1980s, remains unresolved. Although it is difficult to unequivocally prove the aetiological agent of FPSRs, *Y. enterocolitica* O:9 is the primary suspect (Gerbier et al., 1997; Reynaud et al., 1993; Weynants et al., 1996b). Generally, serological titres due to infection with *Y. enterocolitica* O:9 are lower and more transient than those due to infection with smooth *Brucella* strains (Corbel, 1973; Corbel and Cullen, 1970; Garin-Bastuji et al., 1999) but do result in the generation of not only IgM but also IgG₁ and IgG₂ antibody isotypes which result in positive results in agglutination assays, CFT and sLPS iELISAs with conjugates specific to either or both IgG isotypes (Saegerman et al., 2004).

The more transient nature of the antibody titre provides some useful epidemiological evidence if infected individuals are rebleed and the sample retested several weeks after the initial sampling. A reduction in titre is suggestive of a cross reaction, but by no means definitive. Likewise, not all FPSRs will be of such transient nature. The difference in average titre affords some capability to resolve the aetiological agent, for example by a quantitative RBT. This finding relating to the comparative titres of FPSRs and true positives seems to have been perpetuated through the years by the discovery of new techniques with improved specificity but that simultaneously have reduced sensitivity. One of the more promising early methods to differentiate between true and false positive reactions was through the observation of titres against heterologous and homologous antigens and cross absorption experiments (Corbel, 1975).

1.11.2. Use of *Yersinia enterocolitica* antigens in serology

A series of experiments brought comparative serology forward into the 1980s. Serological evaluation of *Yersinia* flagella antigens as well as the OPS from both *Y. enterocolitica* O:9 and *Brucella* demonstrated that the comparative titres against homologous and heterologous antigens gave some indication that FPSRs anti-OPS titres may be due to infection with *Yersinia* (Mittal and Tizard, 1979b). This finding was explored further and supported by the evaluation of cross-absorption agglutination experiments in cattle (Mittal and Tizard, 1979a). However the comparative and cross-

agglutination data from sera obtained from naturally infected cattle was unequivocal (Mittal and Tizard, 1980) although the data from goat sera, and to some extent pig sera, was more supportive. A separate study on sera from pigs experimentally inoculated with vaccine strain *B. abortus* S19 supported the previous observations that in cross agglutination studies the titre to the homologous antigen tended to be higher than the heterologous antigen (Mittal and Tizzard, 1981) but this was not supported by data from field sera.

Similar subsequent work also in cattle demonstrated the application of non-*Brucella* antigens for the differentiation of serological results (Mittal et al., 1985). The use of *Yersinia* specific antigens is a theme that has been explored further elsewhere with some success (Kittelberger et al., 1995b; Schoerner et al., 1990) but the identification of antibodies indicating a *Yersinia enterocolitica* infection doesn't rule out the possibility of dual infection with *Brucella*. For example an ELISA developed with *Yersinia* outer membrane proteins was positive in 10% of bovine samples that were seronegative to brucellosis (Weynants et al., 1996b). Given the prevalence of *Y. enterocolitica* and the lack of specificity of such tests to the O:9 serotype (Godfroid et al., 2002) dual infections are far from being an unrealistic possibility. The cross agglutination assays seem to be too complex and too unreliable for routine diagnosis (Kittelberger et al., 1998; Melzer et al., 2007), but they do provide some interesting observations.

The absorption study using sLPS iELISA for antibody detection is particularly interesting (Kittelberger et al., 1998) due to the focused specificity of the polyclonal antibody population being studied. In a very limited sample set, sera from six cattle experimentally infected with *B. abortus* strain 544 (reference strain for biovar 1, A-dominant) and four cattle experimentally infected with *Y. enterocolitica* O:9, antibodies were absorbed using a concentration range of *Y. enterocolitica* O:9 sLPS or whole cells. The residual antibody titres against *Brucella* A dominant, M dominant or *Y. enterocolitica* O:9 sLPS were measured by iELISA. The results showed that in four of the six samples from *B. abortus* infected cattle, there was a residual antibody titre against the *Brucella* sLPS in absorbed sera from which all reactivity against *Y. enterocolitica* O:9 sLPS had been removed in comparison to the sera from the *Y. enterocolitica* O:9 infected cattle. Sera from two of the six *B. abortus* infected cattle showed the same absorption and reaction profile as the sera from the *Y. enterocolitica* O:9 infected animals. These two sera had moderate titres, approximately equivalent to those from the *Y. enterocolitica* O: infected animals. Of the four samples that showed the different reaction profile, two were of equally moderate titre but two had high titre. This was sufficiently high such that *Y. enterocolitica* O:9 whole cells were required to absorb all of the anti-*Y. enterocolitica* O:9

sLPS titre. The residual titres against the M-type *Brucella* OPS were higher than those against the A-type OPS which suggest that the difference in titres may be due to the structure of the OPS rather than reaction to the rLPS of *Brucella* – although any differences in the lengths of the OPS between the two types may have altered the availability of the rLPS epitopes to the sera. An alternative possibility is that the *Brucella* sLPS iELISAs are detecting anti-M and anti-C (specific) antibodies.

1.11.3. iELISA with *Brucella* sLPS and OPS antigens

Serum obtained from cattle after vaccination with *B. abortus* S19, infection with *Y. enterocolitica* O:9 as well as from those naturally *Brucella* infected have been tested by ELISA with sLPS and OPS as well as competitive ELISA with sLPS (with an anti-OPS mAb). The specific study also showed that, despite some strong responses to the sLPS antigen, sera from the vaccinated and *Y. enterocolitica* O:9 infected animals had a weak or undetectable response to the OPS antigen, either via the OPS iELISA or the cELISA, in contrast to the data from the naturally infected animals (Cherwonogrodzky et al., 1991; Nielsen et al., 1989). The authors speculate that a combination of a shorter period during which the immune system is exposed to the OPS during the more transient carriage of vaccine strains or *Y. enterocolitica* O:9 may result in the generation of antibodies whose specificity is more biased towards the tip of the OPS (as would be presented to the extracellular environment) rather than the length. In sLPS coated plates this tip is always presented towards the liquid milieu. In OPS coated plates the orientation of the tip is more random and access by antibodies to the tip may be hindered if the length of the antigen is bound to the plate.

This is an interesting and plausible theory but is not backed up by observations that show that the FPA, where the OPS is free in solution, is also reputed to be a superior assay for the discrimination of sera from vaccinated or *Y. enterocolitica* O:9 infected cattle (Aguirre et al., 2002; Nielsen et al., 2004c). It may be that the conjugation of fluorescein isothiocyanate (FITC) to the OPS to produce the FPA antigen results in conjugation at the tip (non-reducing end) of the polymer. However, the conjugation process appears to proceed via the introduction of primary amines into the polymer via de-N-formylation with sodium hydroxide (Nielsen et al., 1996). The FITC then spontaneously reacts with these amines which are presumably generated on random residues along the polymer. On the other hand, the passive absorption of OPS to standard ELISA plates is not an efficient or highly reproducible process and the titres described in the report show that the signals generated by sera from naturally infected animals were far lower for OPS than for the sLPS.

1.11.4. Application of sLPS cELISA to FPSRs

The potential of the cELISA (with an sLPS antigen and mouse mAb specific for the *Brucella* OPS) to have improved specificity with regards to FPSR samples was discovered when investigating its properties against sera from cattle vaccinated with *B. abortus* S19 (Nielsen, 1990) as it classified fewer FPSRs samples as positive than iELISA. These properties were affirmed by a later study in which the cELISA demonstrated good diagnostic sensitivity, almost equal to iELISA, and much superior diagnostic specificity (Nielsen, 2004). Similar cELISAs, based on competition for binding to the *Brucella* OPS, have also been applied to sera from *Y. enterocolitica* O:9 infected cattle and field FPSRs (Godfroid et al., 2002; McGiven et al., 2008; Muñoz et al., 2005; Weynants et al., 1996b). Whilst there is some evidence from these studies that the cELISA approach may lead to improved specificity compared to RBT, iELISA and CFT there is also some evidence to show that there is a reduction in sensitivity, especially compared to RBT and sLPS iELISA. Blocking ELISAs using *Brucella* OPS and anti-OPS mAbs (where sera is added and incubated before the addition of the mAb) did not demonstrate any improvement in diagnostic specificity (Kittelberger et al., 1998). One of the studies into the amelioration of FPSRs that included the cELISA had a fairly comprehensive range of alternative assays included (Muñoz et al., 2005) none of which provided a solution to the FPSR problem.

1.11.5. FPSRs in pigs and sheep

The occurrence of FPSR from swine sera is also a significant problem. The frequency of these results has also increased since the 1990s (Wrathall et al., 1993) and, as for cattle, infection with *Y. enterocolitica* O:9 is suspected as being the main cause. This organism is certainly present and circulating within the swine population (Bonardi et al., 2013; Fredriksson-Ahomaa et al., 2007; McNally et al., 2004) and is known to generate FPSRs (Mittal and Tizzard, 1981; Nielsen et al., 2006a) in all conventional assays including cELISA and FPA (Jungersen et al., 2006; McGiven et al.). It has also been isolated from a sheep in Sweden, where brucellosis has never been diagnosed in sheep or goats, that was positive to brucellosis serology by RBT and CFT (Chenais et al., 2012).

1.12. Alternative antigens for immunodiagnosis

The conventional serodiagnostic methods are effective screening tools. However, in areas where the prevalence of disease is low and confirmatory testing is required, a review of the literature demonstrates that serodiagnostic assays that utilise significant quantities of OPS as their diagnostic antigen are not sufficiently reliable to differentiate between false positive and true positive reactions. As many, but by no means all, of the FPSR titres are relatively low compared to the titres of true positives some increase in specificity may be gained by selection of different tests, different test thresholds or by using several tests in series but this leads to a reduction in sensitivity and an increase in risk that becomes unpalatable. In areas of disease management and freedom, where significant resources have already been invested into control, this uncertainty usually leads to the introduction of herd movement restrictions, costly epidemiological investigations involving analysis of animal health and movements, and often repeated blood sampling and testing to gain a longitudinal picture of titre within the herd. Prolonged uncertainty leads to culling of seropositive animals and bacteriological investigation of tissue samples. On the basis of all this evidence, eventually, movement restrictions will be lifted or the herd will be culled.

Given the problems inherent with the OPS based assays a considerable number of studies evaluating the serodiagnostic efficacy of non-OPS antigens have also been undertaken, often with the principle aim of finding alternative 'marker' antigens that could enable the development of novel marker deleted DIVA partner vaccines.

1.12.1. Application of protein antigens in serology

Considerable efforts have been made to discover and apply non-OPS antigens to the serodiagnosis of brucellosis. The approaches taken and the highlights of the research have recently been described in a review (McGiven, 2013). Earlier studies used relatively crude antigens (Baldi et al., 1996; Kittelberger et al., 1995a; Spencer et al., 1994), some were more hypothesis based and targeted (Letesson et al., 1997; Rossetti et al., 1996). Advances in biotechnologies enlarged the target range via more shotgun approaches. Immunoselection procedures applied to proteins derived naturally (Al Dahouk et al., 2006; Connolly et al., 2006; Ko et al., 2012; Yang et al., 2011), through whole genome recombinant gene expression (Lowry et al., 2010) or whole genome protein synthesis (Liang et al., 2010) have all discovered potential diagnostic protein candidates.

The protein candidates identified include ribosomal protein L7/12, Copper/Zinc superoxide dismutase (Al Dahouk et al., 2006), Malate dehydrogenase (Lowry et al., 2010), VirB12 (Rolan et al., 2007) and Lumazine synthase (Goldbaum et al., 1999) among many others. Perhaps the most frequently discovered, investigated and cited is BP26 (Kim et al., 2013), also known as OMP28 (Chaudhuri et al., 2010; Cloeckeaert et al., 2001; Debbarh et al., 1995; Debbarh et al., 1996; Kumar et al., 2008; Liang et al., 2010; Lim et al., 2012; McGiven et al., 2012; Rossetti et al., 1996; Seco-Mediavilla et al., 2003). As well as the complete protein, individual sections of it have also been evaluated (Liu et al., 2011; Tiwari et al., 2011) down to the size of peptides (Qiu et al., 2012). Despite all the effort and the publications, a diagnostic assay based on BP26, or any other protein, is yet to make it beyond the research laboratory. In fact, one of the most recent publications presents a rather negative assessment of the future (Xin et al., 2013), whereby the generation of antibodies against BP26 is not universal but dependent upon infective strain and host. Indeed there seems to be considerable truth to a comment made some time ago 'Our results suggest that the antibody response to OMPs [outer membrane proteins] is different from one animal to another. A combination of several OMPS will be necessary for detection of all infected animals that have anti-LPS antibodies' (Cloeckeaert et al., 1992b). The current situation has been colourfully put thus: "The attempts to find a 'magic' protein antigen for differentiating smooth *Brucella*-infected animals from vaccinated individuals have been finally relegated as experimental relics, mainly because the sensitivity and specificity of protein based assays did not rival those developed with LPS or NH [native hapten]" (Chaves-Olarte et al., 2012). But perhaps the most telling assessment of the lack of success has not been made with words but with actions. The development of a green fluorescent protein marker vaccine for brucellosis (Chacon-Diaz et al., 2011) by a research team with considerable experience and recognition signals a surrender to the domination by OPS of the humoral immune response.

1.12.2. Application of rLPS antigen in serology

Although the search for protein, or peptide, antigens has yet to deliver a routinely applied and widely accepted assay, other non-OPS alternatives may hold more promise. The absence of a phylogenic link between *Brucella* and the organisms that give rise to the most vigorous cross reactive titres, including *Y. enterocolitica* O:9 provides some opportunities. In particular the atypical structure of the core and lipid A of *Brucella*, that appears to be critical to its 'stealthy' strategy and has been described above, may present some unusual epitopes to the host immune system if these become

sufficiently accessible. From what is known of the structure of the core of *Y. enterocolitica* O:9 (Müller-Loennies et al., 1999) it appears to bear no particular structural relationship to that of *Brucella*. For example, unlike *Brucella*, the core of *Y. enterocolitica* O:9 contains heptoses. It is certainly true that *Brucella* possesses the rLPS in abundance and, although hidden by the OPS, it may also be more accessible to the immune system than proteins. There is some evidence that rough forms of smooth strains arise spontaneously during infection (Turse et al., 2011) and this may enhance the antibody response to the rLPS although this should also assist in the generation of anti-OMP antibodies.

It has been demonstrated that antibodies may bind to OMPs and rLPS on the surface of smooth *Brucella* strains, as well as rough ones, but that this binding is inversely related to OPS length (Bowden et al., 1995). The use of rLPS as a diagnostic antigen in iELISA and FPA was shown to be effective in identifying samples from animals infected with rough species and strains of *Brucella* (Nielsen et al., 2004b). The rLPS was then also applied to the serodiagnosis of brucellosis due to infection with smooth strains with fair specificity (90-100%) but highly variable sensitivity (62.9-100%) dependent on host species (Nielsen et al., 2005).

The data from the rLPS iELISA was sufficiently encouraging, especially in view of the lack of advancement elsewhere, to evaluate the antigen against sera from *Y. enterocolitica* O:9 infected cattle and swine, bovine field FPSR samples (those that had reacted with one of more of the SAT, CFT, sLPS iELISA or cELISA) as well as sera from cattle and swine from which *B. abortus* and *B. suis* (the infective biovars were not stated) had been isolated respectively (Nielsen et al., 2006b). The results from the *B. abortus* sLPS iELISA from both bovine and swine sera confirmed previous observations that sera from *Y. enterocolitica* O:9 reacted against the *B. abortus* sLPS but that, on average, the titre was lower than that from sera from the *Brucella* infected animals. When the sera was tested using sLPS from *Y. enterocolitica* O:9 the average titre from sera from *Brucella* and *Y. enterocolitica* O:9 animals was much closer. The sera from the non-infected populations demonstrated no antibody response to either sLPS antigen. The sera from the *Y. enterocolitica* O:9 infected animals, cattle or swine, demonstrated very low reactions against the *B. abortus* rLPS with titres very similar to those from the non-infected populations. In contrast, the samples from the *Brucella* infected animals demonstrated, in most cases, a significant response to this antigen. Based on this data a DS_n of 91.6% could be achieved with the sera from the *B. abortus* infected cattle whilst maintaining 100% DS_p against sera from *Y. enterocolitica* O:9 or non-infected cattle. The equivalent data for the swine sera was: DS_n of 93.5% and DS_p of 100%.

The rLPS iELISA data from the bovine field FPSR demonstrated that 20 of 121 sera (DSp = 83.5%) generated ODs above the assay threshold that generated the DS_n and DSp data described above. Closer inspection of the data reveals that not many of the 121 FPSR samples were positive in more than one conventional assay. The raw data from the bovine rLPS data also demonstrates that many of the samples from the *Brucella* infected animals were only marginally above the positive/negative cut-off selected for the assay. This demonstrates that, on a quantitative basis, there is not a huge difference in titre between large sections of the *Brucella* infected and *Y. enterocolitica* O:9 infected populations. In contrast, the data from the swine sera demonstrates that, although the difference in DS_n between the results for the rLPS iELISA on swine and cattle samples is small, there is a large quantitative difference between the results for the samples from the *B. suis* and *Y. enterocolitica* O:9 infected populations.

Additional data for the rLPS iELISA on swine samples suggests that this has real potential to be a useful confirmatory assay, at least for animals infected with *B. suis* biovar 1 (McGiven et al., 2012). The results from this study gave a DS_n of 91.2 % and DSp of 97.0% for FPSR samples which was significantly superior to any of the other assays evaluated. There were the sLPS iELISA and cELISA, RBT, TR-FRET, FPA, BP26 iELISA and iELISA using a crude protein extract from *B. melitensis* (rough strain) B115. Data generated more recently (McGiven et al, unpublished data) using sera from swine infected with *B. suis* biovar 2 supports these results. Studies conducted at the same time on sera from cattle have shown that the rLPS iELISA is much less effective with DS_n of 79.0 - 94.7% and DSp of 69.4 – 83.3% dependent on the specific population studied (McGiven et al, unpublished data). Whereas it seems as if the rLPS iELISA may provide some relief to the FPSR problem in swine, it doesn't appear to represent a sufficiently complete solution in cattle. This relief is especially welcome considering that the OPS of *B. suis* biovar 2 and *Y. enterocolitica* O: appear to be identical (Zaccheus et al., 2013).

1.13. Diagnostic application of cellular immune responses

1.13.1. Evaluation of cellular immune response: delayed type hypersensitivity reaction (skin test)

Attempts to resolve the problem of FPSRs by non-serological means have lead to the re-investigation of the cellular immune response. This has mainly been evaluated by the measurement of delayed

type hypersensitivity (DTH), or 'skin test'. The first report of the inoculation of *Brucella* antigens in order to facilitate diagnosis of brucellosis is more than 100 years old (McFadyean and Stockman, 1909) and it was applied to cattle. There are also very early reports of such inoculations being applied to humans (Burnet, 1922) and the inoculum being further refined with attempts made to standardise preparations (Huddleson and Johnson, 1933) with the subsequent coinage of the term "Brucellergen" to describe a "protein nucleate fraction" derived from smooth strains of *B. abortus*, *melitensis* or *suis* (Huddleson, 1943). The skin test was used as a regular diagnostic assay in humans (Bradstreet et al., 1970; Howells, 1968) although serological assays became favoured (Bradstreet and Pollock, 1971). Variations of the skin test had also been explored in cattle (Burki and Mosimann, 1956) and swine (Both and Kohl, 1967). It was latter more fully appreciated that preparations enriched in protein and which had been derived from rough strains gave superior diagnostic results from animals infected with smooth strains and resulted in the generation of fewer, potentially confounding, antibody responses (Jones and Berman, 1971; Jones and Berman, 1975; Jones et al., 1973).

Many studies have been performed to evaluate the efficacy of the DTH skin test for the diagnosis of brucellosis in cattle including the following: (Chukwu, 1985, 1986; Fensterbank, 1977; Fensterbank and Pardon, 1977; Nicoletti, 1983; Sutherland, 1983). Selection, recognition, production and batch control of the DTH antigen has been – and remains - an ongoing issue (Bercovich, 2000; Bercovich et al., 1996; Cunningham et al., 1980; de Reviers and Fensterbank, 1984; Pardon et al., 1980; Woodard and Toone, 1980). Alongside issues relating to the content of sLPS, rLPS and OPS, there are at least 20 different proteins which will variably stimulate host immune cells (Denoel et al., 1997b). The value of the skin test for differentiating between animals with brucellosis and those infected with *Y. enterocolitica* O:9 was examined in guinea-pigs with results showing good specificity (Kostov et al., 1980). Unsurprisingly the skin test has also been applied to cattle infected with *Y. enterocolitica* O:9 (Chukwu, 1987; Godfroid et al., 2002). More recent studies on variants of the DTH skin test have demonstrated that the assay has very good specificity, > 99%, in cattle in field trials (Bercovich and Muskens, 1999; Pouillot et al., 1997; Saegerman et al., 1999) even in areas that have increased rates of FPSRs. The DS_n values reported within these studies ranged from 64-95% based on data from experimentally infected animals and also from comparisons with serological results from field infected animals. The reaction induced by Brucellin into *B. abortus* infected animals is more difficult to read than the reaction induced by bovine PPD tuberculin in *M. bovis* infected animals, being only one half to one third the size of the tuberculosis specific reaction (Saegerman et al., 1999).

The production and checking of Brucellin for use in the DTH skin test is carefully described in the OIE Manual of Diagnostic Tests and Vaccines. The skin test is not a prescribed test and although it has good specificity (in non-vaccinated animals) the test is not recommended for the diagnosis of individual animals (Nielsen and Ewalt, 2010). It is my understanding that due to the ongoing strain selection, production and availability issues the revised (combined) OIE chapter on Infection with *Brucella abortus*, *melitensis* and *suis* has revised the advice relating to production of 'Brucellin'. The advice on the selection of strain contradicts that provided in the most recently published review on the topic (Bercovich, 2000) mainly owing to the risk due to induction of specific antibodies (Cloeckert et al., 1992c; Muskens et al., 1996). The risk is presented by the production of internalised OPS by the rough strain *B. melitensis* B115 which had been the strain recommended for use.

After more than 100 years of trial and experimentation, there is no agreed and specified antigen preparation that gives optimal results in the DTH skin test. It may be that the DTH skin test can play a useful role in the resolution of FPSRs, and in some instances this has already been the case (Godfroid et al., 2002). However, the practicalities relating to the difficulties and costs of preparing a quality controlled DTH antigen, coupled with the questionable DS_n, have so far prevented its widespread introduction as even an option within a control or surveillance programme. Implementation of DTH skin testing would also be more expensive than retesting FPSR sera with an effective alternative serological confirmatory assay – were one available.

Most of the large scale studies on the DTH skin test for animal brucellosis have been performed on cattle. Data from small ruminants is sparse but encouraging, one of the most recent reporting DS_n values of 94.0 – 97.1% and DS_p of 100% (Blasco et al., 1994) which were greatly in excess of the comparable results for the RBT and CFT, although the sLPS iELISA had 100% DS_p and DS_n. Despite these excellent results it is interesting that the DTH skin test is not considered as an option in a recent review of control strategies for the control and eradication of *B. melitensis* (Blasco, 2010). Even less data is publically available for the efficacy of the DTH skin test in pigs (Garin-Bastuji and Jungersen, 2010; Riber and Jungersen, 2007; Stuart et al., 1987) but there is some room for optimism about its efficacy although more validation data is required.

1.13.2. In-vitro evaluation of the cellular immune response: Brucellin IFN γ assay

Assays for the *in-vitro* stimulation of T-cells have also been evaluated for their ability to assist in the diagnosis of brucellosis. As with the DTH skin test, such assays are antibody independent and measure the cellular immune response to challenge. In one sense they offer practical advantages over the DTH skin test in as much that no material is injected into animals and assays are done on easily extractable materials. The DTH Brucellin antigen is applied to peripheral blood mononuclear cells. However, the assay depends on the viability of live cells, the material needs to be fresh and preferably stored at 37°C prior to testing. These requirements present high logistical hurdles and, if the IFN γ assay were to be used as a confirmatory test a repeat visit to the farms to bleed FPSR animals would be necessary. The results from one study on cattle experimentally infected with cattle, naturally infected animals and those presenting FPSRs demonstrated that the Brucellin IFN γ assay had superior DS_n and, especially, DS_p than RBT, CFT and sLPS ELISA (Weynants et al., 1995). In another, rather small study, the Brucellin IFN γ assay did not look so encouraging as animals experimentally infected with *Y. enterocolitica* O:9 gave high IFN γ responses (Kittelberger et al., 1997). A later study was almost a composite whereby the Brucellin IFN γ assay offered sensitivity comparable to sensitive serological assays in experimentally infected animals, but this also included sensitivity to animals inoculated with *Y. enterocolitica* O:9 and the serologically unrelated *Y. enterocolitica* O:3 (Godfroid et al., 2002). In a field trial the Brucellin IFN γ assay demonstrated a DS_n of only 42.9% (based on agreement with seropositive animals from herds confirmed brucellosis positive by culture) when the assay cut-off was selected to achieve a DS_p of 90% (McGiven, unpublished data).

There is some data from Brucellin IFN γ assay performed on pigs (Riber and Jungersen, 2007; Thirlwall et al., 2008) and results from these trials were encouraging with reproducible and diagnostically useful differences observed between animals experimentally infected with *Brucella suis* and those infected with *Y. enterocolitica* O:9. Specificity was also examined in field trials on animals presenting FPSRs, and none of these were positive by IFN γ (Riber and Jungersen, 2007). However, the IFN γ assay has not yet been taken forward for further research or application to routine diagnosis. Concerns about the supply and the reproducibility of the DTH skin test antigen may account for this as might the logistical considerations. The Brucellin IFN γ assay has also been applied to whole blood samples from *B. melitensis* infected sheep (Durán-Ferrer et al., 2004) however the context for this was tuned towards developing a better understanding of the protective immune response against

challenge rather than diagnosis and as such no values for DSp or DS_n were computable. Studies on the protective efficacy of vaccine formulations are by far the most common examples of the application of the IFN γ assay – in mice - in which the stimulating antigen may be a specific component of the vaccine be it subunit or DNA based (Commander et al., 2010).

Typically the DTH skin test and the IFN γ test use the same type of antigen, a relatively crude protein extract. This needn't necessarily always be the case and it is possible that some improvements in DS_n and DSp could be made through the evaluation of specific protein or peptide antigens (Denoel et al., 1997a; McGiven et al.). This approach has lead to improvements in the DTH skin test and IFN γ assays for bovine tuberculosis (Vordermeier et al., 2009). However the cost of this research has been considerable and the need has been greater as there is no universally approved serological screening assay for this disease. Given the costs of animal experimentation, especially for large animals, it seems unlikely that sufficiently large studies of this nature will be conducted to address the FPSR issue in brucellosis diagnosis.

1.14. Direct detection of *Brucella* or *Brucella* specific DNA

Clinical diagnosis of animal brucellosis is highly unreliable unless there is very strong supporting epidemiological information however this is frequently absent. Bacterial culture plays an important part in confirming the presence of disease but, other than samples collected from an abortion event, the likelihood of obtaining a positive culture from material from a live infected animal is too low for reliable diagnosis and the absence of a positive result does not confirm absence of infection (Morgan, 1977). Furthermore culture of *Brucella* is not an appropriate technique for routine screening due to the costs, difficulties and dangers that it presents.

Alternative direct detection diagnostic approaches include DNA amplification and detection methods but the effectiveness of such approaches on readily available material such as blood, serum, swabs and milk has yet to be fully evaluated and existing information is conflicting (Whatmore and Gopaul, 2012). PCR directly from tissue has potential as a diagnostic tool but inhibitors and the fact that DNA from the organism itself must be present in the material, negatively affect the sensitivity of the method. During early, latent and sometimes chronic infections the numbers of brucellae may be relatively low making such types of diagnosis difficult (O'Leary et al., 2006). PCR on DNA extracts from lymph nodes from infected flocks of sheep appears to have effective sensitivity and specificity

compared to bacteriology performed on the same tissues (el-Razik et al., 2007; Ilhan et al., 2008) although results from cattle have been less encouraging (O'Leary et al., 2006). Results from PCR from the fetal stomach contents of sheep have been encouraging (Ilhan et al., 2007; Leyla et al., 2003) as have the results from fetal organ homogenates from sheep and cattle (Buyukcangaz et al., 2011). These assays have their place and use but in terms of diagnosis of brucellosis they are somewhat 'after the event'.

Detection of DNA from more routine and readily accessible material would be of much greater value to the diagnostician. Consumption of *Brucella* within milk is the most common means of transmission of brucellosis from animals to humans thus this matrix is a potential source of *Brucella* DNA. The outcome of studies into the diagnostic effectiveness of PCR from milk has been mixed. One study in particular (Marianelli et al., 2008) encapsulates the issues whereby a significant number of culture positive buffalo milk samples were negative by PCR but also, a significant number of culture negative samples were positive by PCR – possibly due to the presence of non-viable cells. PCR from blood samples has provided encouraging data in some cases, for example in sheep (Ilhan et al., 2008) but there is far less encouraging data from cattle (O'Leary et al., 2006) and data from wild boar casts uncertainly over the reliability of bacterial culture and serology as well as PCR (Hinic et al., 2009). To conclude, there is much more validation and development to be done before PCR can be used as a reliable and routine diagnostic assay for animal brucellosis.

However, PCR on serum or whole blood is an important technique in the diagnosis of human brucellosis (Mitka et al., 2007). The occurrence of visible symptoms in humans prompts sampling to be done during periods where bacteraemia is more likely. In livestock, there are no clear symptoms of disease, other than abortion, and therefore sampling is less likely to take place during the bacteremic phase of the infection (although recrudescence of the pathogen is likely to occur during the latter stages of gestation). This adversely affects the diagnostic sensitivity of PCR. Furthermore, screening by PCR is a costly alternative to serodiagnostic techniques.

1.15. MASS SPECTROMETRY

1.15.1 The beginning

In the late 19th century Eugen Goldstein and Joseph John Thomson were independently experimenting on the movement of 'rays' transmitted through gases in electric fields. By the study of 'cathode rays' Thomson discovered the electron and was able to measure its mass at a thousand times less than that of a hydrogen atom. Goldstein was studying fields moving in the opposite direction and concluded that these too contained particles but these were much bigger than an electron and of similar size to a hydrogen atom. Thompson subsequently discovered that these positive rays could be deflected by perpendicular electric fields by detection using photographic plates. Initially with hydrogen, but later with other atoms and molecules, Thomson discovered that each particle was deflected into a parabolic pathway according to both its mass and charge. Further, that by knowing this ratio for one parabolic pathway the mass to charge ratios relating to other parabolic pathways could be deduced. He correctly suggested that the positive rays he was detecting were created by the loss of electrons. J. J. Thomson won the 1906 Nobel Prize for Physics for this work.

These studies gave birth to the field of mass spectrometry and even then encapsulated the capability of obtaining highly sensitive and specific measurements from complex mixtures which are the features that underpin the popularity of mass spectrometry to this day. Essentially, mass spectrometry is the study of measuring the molecular weight of ions in the gaseous state. The critical elements in this process are thus the ionisation process, which is often coupled to conversion to the gaseous state, the analysis (separation) of the ions on the basis of mass and charge and their subsequent detection. Naturally, significant advances have been made since the days of Joseph John Thomson.

1.15.2. Ion sources

1.15.2.1. Ion sources: Electrospray Ionisation (ESI)

The concept of electrospray was conceived by Malcolm Dole in the 1960s who was driven by the desire to measure the mass of macromolecules that were difficult to convert into the gas phase without decomposition. His concept was to use a dilute solution which was nebulised into very small

droplets that perhaps contained only one molecule. If the molecule itself was not charged then the presence of an electrolyte such as Na^+ or Cl^- could lead to the charging of the molecule. However, for such an interaction to occur a suitable functional group must be present on the molecule of interest in order to facilitate such an interaction. The inefficiencies of this process were significantly addressed by the advent of the electrospray which much more efficiently generated charged ions in the gaseous state whilst maintaining the soft ionisation properties of the original approach. Dole came up with this innovation whilst observing the use of an electrospray for painting cars at the paint company where he was working as a consultant.

Dole's paper (Dole et al., 1968) came to the attention of Professor Lipsky at Yale Medical School who understood the potential of this breakthrough. He contacted John Fenn from the Department of Mechanical Engineering at Yale who was a specialist in the nozzle-skimming systems used by Dole in order to create and volatilise his ions. Fenn also saw the potential of this technique and engaged in its development using a quadrupole mass analyser already available in his laboratory. Although based heavily on the work of Dole, Fenn made many significant advancements such as the use of a nitrogen gas counterflow to clean up the spectra by removal of the solvent vapour and the incorporation of a heated capillary at the interface between the ionisation source and the mass analyser. These innovations and their application demonstrated that electrospray ionisation mass spectrometry (ESI-MS) was highly effective at analysing peptides and proteins whose mass, m , was beyond the mass to charge ratio, m/z , of the analyser (Whitehouse et al., 1985). This work earned John Fenn the Nobel Prize for Chemistry in 2002 which was shared with Kurt Wüthrich for his development of NMR applications. It was also shared with Koichi Tanaka for his contribution to mass spectrometry via application of soft laser desorption (SLD) techniques. At a similar time to the development made by Tanaka, and publically disseminated earlier, the technique of MALDI-ToF was developed which was more sensitive and (although not used at the time to ionise proteins) remains widely used in routine mass spectrometry – unlike the SLD technique.

The three major steps in ESI are: the production of charged droplets at the ES capillary tip, shrinkage of the droplets by solvent evaporation and the actual mechanism by which gaseous ions are produced from these droplets. These steps all occur within atmospheric pressure. Some of the resultant ions enter the vacuum region of the mass spectrometer via a small orifice or a capillary. The following description relates to the application of ESI in positive mode. In negative mode, the polarity and charge is reversed.

The electrostatic spraying typically involves passing electrolyte liquids through a needle held at high voltage (typically 4-5 kV) relative to some counter electrode. The electrical charge causes a deformation of the liquid meniscus that is held by surface tension due to polarization and dipole movements of the solvent molecules leading to enrichment of positive ions at the surface of the meniscus. Above a certain voltage threshold the liquid forms a Taylor cone (Fernández de la Mora, 2007; Taylor and McEwan, 1965) pointing downfield and emission from the tip, due to an excess of similarly charged ions, of a fine jet of solvent. This jet breaks up into droplets which remain positively charged, due to the relative abundance of positive electrolyte ions at the surface of the cone and cone jet from which they derived, and are drawn towards the counter charge. This may be a plate with a small orifice or sampling capillary that leads towards the mass analyser (the capillary may be heated to further enhance solvent evaporation) that is held under a (partial) vacuum.

The droplets that derive from the jet become smaller, due to evaporation, leading to an increase in the concentration of positive ions. This evaporation may be assisted, more frequently when flow rates or surface tensions are high, by the addition of an inert nebulising sheath gas such as nitrogen that increases the rate at which the larger droplets shrink. It is also desirable to avoid droplets entering the mass analyser so as to avoid build up of non volatile material within the instrument and bursts of ions that create spikes in the mass spectra. The intrusion of droplets can be minimised by the incorporation of a gas curtain, frequently nitrogen, flowing perpendicular to the ion flight. Or the electrospray capillary may be positioned orthogonal to the sampling orifice so that only objects with lower mass to charge ratios, relative to larger droplets, may divert sufficiently towards the inlet. These modifications are especially useful with high flow applications and instruments.

As the droplets become smaller the repulsion between the charges increases and the droplet becomes unstable. Eventually Coulombic fission occurs almost exactly at the Rayleigh limit, the maximum charge permissible on a liquid surface prior to exceeding the attractive forces of surface tension. This explosion of droplets, which occurs via a cone jet reminiscent of the Taylor cone at the inlet capillary tip, creates smaller progeny droplets and a reiteration of the process until eventually the gaseous phase ions are produced. There are two main theories as to how this process occurs. One, known as the 'charged residue model' predicts that the complete evaporation of solvent leads the generation of the gas phase analyte which carries charges that originate from the surface of the now evaporated droplet (Dole et al., 1968). The second model, the 'ion evaporation model' predicts that as the droplet becomes smaller direct emission of the ions from the droplets becomes more favourable than Coulombic fission (Iribarne and Thomson, 1976). Despite refinements and

extensions to the models neither appears to have gained unequivocal support and the process continues to be the subject of research.

Electrospray ionisation is a highly versatile method in which complex molecules such as polymers, nucleic acids and proteins can be transferred into the gas phase as ions. This is a soft ionisation process meaning that there is minimal fragmentation during this process. The ionic species created during the ionisation process may be inorganic positive metal ions such as Na^+ , M^+ or protonated amines or negative ions such as deprotonated carboxylic acids or sulphates and so on. These may find themselves adducted to compatible neutral molecules to create a charged species. Or the ionic species may themselves have pre-existed as ions in the solution that is sprayed. This versatility may suggest that the approach is suitable for the resolution of any mass analytical problem but electrospray is a selective ionisation technique suitable for some analytes but not others. Molecules that cannot become vapour phase ions cannot be detected. Furthermore, competition between analytes may occur leading to the suppression of the analyte of interest – occasionally completely. In some cases the ions are not formed by the electrospray process itself but the process serves to separate ions from their counter ions to create a net molecular charge.

Some molecules are charged in the electrospray droplets by the gain or loss of one or more protons. Evaluation of the pK values for a particular analyte is therefore a good basis to begin considering the suitability of the electrospray mass spectrometry process. However it should be noted that the conditions suitable for ionisation in a bulk liquid phase are not necessarily those suitable for ionisation within a shrinking electrospray droplet or within the gas phase. This may explain why so many acidic or basic analytes can in fact be ionised at a wide range of pH values. This property provides some flexibility in the range of effective mobile phases that may be used to deliver the analyte to the ESI interface although a balance must be struck between the quality of any online separation and the generation of suitable ionised species. The ESI source is therefore well suited to coupling with HPLC systems.

Molecules that are not inherently charged and resist protonation and deprotonation may still be ionised with electrospray. This occurs through the formation of adducts between polar (although neutral) organic groups and cations such as sodium. For example protonation of non-aminated sugars is predicted to be unfavourable and this has been observed experimentally (Reinhold et al., 1995). Sodiation, on the other hand, does appear to occur with sufficient frequency so as to enable the detection of positive ions (Deery et al., 2001). This may occur through the interaction with

adjacent hydroxyl groups present. To facilitate this, salts may be deliberately added to the solvent although care must be taken not to damage the mass spectrometer due to build up salt within the ESI chamber.

When multiple ionisable molecules exist in the solvent then these may compete with each other to obtain charges from the surface of the electrospray droplet. This becomes more evident when the analytes are capable of carrying multiple charges and when their concentration increases to near or above the concentration of excess charge. Surfactants are particularly effective at competing for charge owing to their increased concentration of the surface of the electrospray droplets in association with the droplet charge. It has been shown that for a constant surfactant concentration, an increase in the concentration of a polar analyte has almost no effect on the response of the surfactant. Whereas, for a given concentration of polar analyte, an increase in surfactant concentration can have a significant suppressive effect (Cech and Enke, 2000).

Studies with molecules, such as acetamide, that model some of the functional groups within proteins demonstrate that these form strong bonds to Na^+ . Comparison with the equivalent Na^+ adducts with acetone demonstrates the importance of the amide group (Hoyau et al., 1999). It is interesting to note, especially within the context of this project, that the Na^+ adduct with N-methylacetamide is more stable than that with acetamide (Klassen et al., 1996). These Na^+ adducts may form with trace amounts of Na and are often undesirable due to the excessively complicated spectra that arise. The more desirable outcome is a net positively charged ion due to protonation of an amide group on the protein which may, in theory, be encouraged by the use a mildly acidic solvent such as millimolar acetic acid. The application of ammonium acetate has also been found to promote protonation over sodiation where the later may be due to trace contamination. In general, gas-phase basicity values would predict that organic molecules containing amines would in general, be easily protonated due to their basic properties.

What does appear to be of significance to the process of ionisation is the nature of the solvent and analyte of interest. When charged analytes, for example proteins and peptides, are present in evaporating droplets ions such as Na^+ will pair with ionised acid residues. This bonding becomes extremely strong once the ion enters the gas phase.

1.15.2.2. Ion sources: MALDI

Matrix Assisted Laser Desorption Ionisation (MALDI) is another soft ionisation technique that has gained considerable prominence in recent years. Prior to the introduction of a matrix, laser ionisation resulted in poor ion yield and high molecular ion fragmentation. Unlike ESI which was developed from a basic understanding of a relatively well established process, MALDI emerged as a 'soft' form of laser ablation. Thus, the early understanding of the MALDI process was less than pre-existed for ESI and lacked much fundamental knowledge. As such, it took empirical testing of hundreds of matrix compounds before those most effective, that are still in mainstream use today, were identified (Fitzgerald et al., 1993). Tanaka won a share of the 2002 Nobel prize for Chemistry for his work demonstrating the ionisation of a large molecular weight protein by laser desorption via the use of a matrix comprised of glycerol and cobalt nanoparticles using a process that became known as Soft Laser Desorption. This form of ionisation is no longer in routine use unlike MALDI which was developed at almost the same time as SLD by Karas and Hillenkamp (Hillenkamp et al., 1986; Karas et al., 1987; Karas et al., 1985).

In MALDI, the analyte of interest is mixed with a large molar excess of matrix that absorbs energy efficiently at the wavelength of the laser used. The most basic function of the matrix is to dissipate the energy from the laser, which is often a nitrogen laser at a wavelength of 337 nm. The subsequent vaporisation is sufficiently vigorous to eject analytes that have been co-crystallised with the matrix. In relation to the speed of intramolecular motions the pulse rates of typical MALDI lasers is slow and energy conversion is also temporally retarded by the storage of energy within the matrix. The period during which the matrix has been energised but not yet expanded is the time during which much of the ionisation is thought to occur. In UV MALDI, the molecules are highly electronically excited at this stage and this may create separated ion pairs. The expansion of the matrix occurs relatively slowly and is the timescale for the secondary reactions. The phase change from solid to gas creates a plume which is largely a consequence of the magnitude of the introduced energy. This is termed desorption when a smooth transition from solid to gas occurs or ablation in when subsurface nucleation occurs leading to the explosive ejection of material. The matrix may take part in secondary reactions upon expansion and these may be proton, electron or cation transfer. As a consequence, matrix ions that take part in all these reactions are themselves charged and observed within the mass spectra.

The mechanisms by which the first ions arise have been the subject of several hypothesis, the 'cluster' and 'photoionisation' models being the ones currently under most investigation. These primary ions may react with neutral molecules during the secondary, expansion phase. In particular biological analytes, such as proteins and peptides are often found as protonated ions. MALDI has also been successfully applied to the analysis of carbohydrates (Mock et al., 1991). When a UV laser is used, common matrices include nicotinic acid, 2,5-dihydroxybenzoic acid (especially popular for glycan analysis), sinapinic acid and α -cyano-4-hydroxy-cinnamic acid. Each of these compounds contains a carboxylic acid group and this may help to achieve proton transfer to create analyte ions.

The nature of the energisation of the matrix and the plume of material that is released causes some variation in the time at which ions are formed and the energy that is imparted to them. As a consequence early MALDI experimental data contained poorly resolved spectra. In order to minimise this unwelcome variation 'delayed extraction' was developed whereby the source electrode is pulsed a short time after laser desorption in order to harmonise ion projection (Brown and Lennon, 1995).

1.15.3. Mass Analysers

Once ions have been formed and directed a means to separate them by a mass analyser is required. The ions are separated according to their mass (m) to charge (z) ratio (m/z) via the use of magnetic and/or electric fields. There are a number of different mass analysers available. Within this project the two that have been used are the quadrupole and Time of Flight (ToF) analysers.

1.15.3.1. Quadrupole analysers

The quadrupole is one of the most common mass analysers and was first described in the 1950s. The unit consists of four parallel rods where those opposite one another are electrically connected. The popularity of this setup stems from its low cost, relatively compact size and fast scanning capability. A voltage of opposite polarity is applied to adjacent rods consisting of a radio frequency (RF) and direct current (DC) component to generate an electromagnetic quadrupole field. Ions are emitted from the source and travel along the axis between the rods. The quadrupole field causes the ions to oscillate between the rods. When this oscillation becomes too large the ions hit the rods and are neutralised. Ions that do not collide with the rods reach the ion detector. The quadrupole field allows ions of specific m/z to reach the detector and when the field is increased (or decreased) this

allows ions of sequentially higher (or lower) m/z to reach the detector. The signal from the detector may be scanned at rates of 35,000 per second and combined with the information relating to the quadrupole field to produce the mass spectrum and they may detect m/z values up to approximately 4000 mass units. They can operate at relatively high pressures and are therefore commonly interfaced with a wide range of inlet systems including separation systems such as gas and liquid chromatography. They couple well to ESI sources and this is a common combination. However they have relatively low resolution and the mass range is limited compared to Time of Flight (ToF) analysers.

1.15.3.2. Time of Flight (ToF) analysers

As implied by their name, the ToF analysers measure the mass to charge ratio by the time taken for a given ion to reach the ion detector whilst travelling through the flight tube. Ions are formed within the source and are then repelled by the application of an electric charge, common voltages would be in the region of 10-30 kV, matching the polarity of the ions of interest. The ions travel down the flight tube, which may be 1 to 2 metres long, at the end of which they are detected. Given that the voltage and the length of the tube remain constant then the time taken between repulsion and detection is a function of ion's m/z . Unlike the quadrupole, the ToF is not a scanning system so theoretically all ions that are produced are detected. This provides the ToF with good sensitivity and minimal artifactual selectivity. In theory even ions with large m/z values greater than 200,000 mass units may be detected if a sufficient amount of time is provided. Thus the ToF has a large dynamic range, good sensitivity and effective quantification capability. It is also amenable to a pulsed torrent of ions and for these reasons virtually all MALDI ion sources are coupled to a ToF analyser and this is a common combination for the analysis of proteins and peptides.

The MALDI-ToF combination does have some drawbacks though, as described above. Owing to the variable distribution of ions in the matrix plume and the uneven distribution of energy imparted, the time at which individual ions of a given species hit the detector within the ToF instrument are also variable. So resolution can be an issue in this system. One technique to overcome this is to employ delayed extraction (as described above) at the source. Another method to improve the resolution can be built into the ToF. This is the 'ion mirror' or 'reflectron' (Cornish and Cotter, 1993; Wiley and McLaren, 1955). This device allows more energetic ions to penetrate deeper into a reflecting electric field effectively increasing the distance travelled to the ion detector in a manner that is proportional to

their energy. This has the effect of neutralising the energy spread between ions of otherwise identical type and improves the resolution of the analyser. One drawback with this approach is that it limits the detection range as higher mass and slower moving ions cannot penetrate the reflection field.

1.15.4. Tandem mass spectrometry (MS/MS)

Whilst soft ionisation mass spectrometry methods may be highly effective at quantifying the mass of the intact molecular analyte further information relating to the structure of the molecule can be derived through subsequent fragmentation. Given the right conditions, predominantly the energy introduced in order to generate ionised fragments, fragmentation follows predictable pathways that are determined by the stability of the bonds within the initial ion. This enables, for example, peptide sequencing. Peptides fragment mainly across the peptide backbone, the –CO-NH- bond being the most susceptible site, and fragment into two major categories, amino-terminal and carboxyl terminal ions (Marino et al., 1988; Morris et al., 1981). These fragments are annotated using a universally recognised nomenclature (Roepstorff and Fohlman, 1984).

This fragmentation and analysis is facilitated by tandem mass spectrometry whereby the first mass analyser can be applied to select the ion of interest from the mix of ions generated at the source. This is then passed into a collision cell that typically contains inert gas such as argon. The subsequent collision-induced dissociation (CID) creates fragment ions from the initially selected molecular ion (other means of fragmentation are also available). These fragments may then be measured by the second mass analyser to provide more detailed information about the original parent molecule. Such tandem mass spectrometers therefore provide substantial structural information from minute quantities of analyte that may be derived from complex and impure sources. Understandably such items of equipment are very popular within analytical molecular laboratories. Ion sources and mass analysers can be put together in a variety of permutations but popular variants are the ESI triple quadrupole (Yost and Enke, 1978), the ESI hybrid quadrupole ToF (Morris et al., 1996), and MALDI-ToF/ToF.

1.15.4.1. MS/MS: Triple Quadrupole (QqQ)

In the triple quadrupole the first and third quadrupoles are operated with both the RF and DC voltages applied and can both select ions. The middle quadrupole is run with an RF only voltage and this serves as the chamber for CID with the application of the collision gas. Owing to the different modes of operation the triple quadrupole is generally annotated as QqQ. In an MS/MS operation, the first quadrupole selects the ion of interest according to its m/z value and this is passed into the RF only chamber for fragmentation. The fragments are then accelerated into the third quadrupole chamber which effectively operates as a standard quadrupole in an MS process whereby its scanning activity allows for the subsequent detection of the m/z for each fragment ion. This is known as the 'parent' mode. The QqQ can be run in four modes. In the 'daughter' mode all ions are scanned and transmitted to the collision chamber and the third quadrupole is set at a single m/z frequency to look for a specific fragmentation ion. In the 'neutral loss' or 'linked scan' mode the first and third quadrupoles are scanned with a specific frequency offset and ions that are detected have lost a common uncharged molecule that can provide information as to the type of molecule detected. In the fourth mode the first and third quadrupoles are set at single m/z frequencies specific for an analyte of interest and one of its daughter fragments. Since the analysers are set for very specific m/z values rather than scanned, the rest of the ions generated are neutralised, the beam of specific ions towards the mass detector allows for very sensitive and specific detection of trace compounds. This type of mode is especially useful for the detection of listed undesirable contaminants and additives. The ability of the QqQ to couple with LC-ESI and operate with high sensitivity and specific, albeit in a very focused fashion, is the reason it remains a vehicle of molecular analysis in many laboratories despite the advantages delivered by alternative MS/MS systems.

1.15.4.2. MS/MS: Quadrupole time of flight (QqToF)

The QqToF rapidly become a popular instrument once it began to become commercially available in the late 1990s. It combined the ability to perform MS/MS with the combined benefits that the ToF method of mass analysis and the ESI interface bring (Chernushevich et al., 2001). These include the ability to simultaneously detect a wide range of ions as the ToF does not use a scanning method of analysis and the ability to readily interface with an LC system. The QqToF contains two quadrupoles and a ToF mass analyser (figure 1.20). The first quadrupole operates in the generic fashion whilst the second operates only in the RF mode and acts as the collision cell – in a fashion similar to that

employed in the QqQ. In most modern instruments the second quadrupole is in fact a hexapole. The method of use is essentially the same as that of the QqQ but with the replacement of the final Q with a ToF.

The QqToF found early use in the analysis of peptides due to the ability to perform some initial separation by online LC, perform effective ionisation at the ESI source, select and fragment the parent ions via the two quadrupoles and deliver all the ions to the ToF for analysis. The QqToF has both high sensitivity and resolution. The ability of the RF only quadrupole to deliver the ions to the ToF as a narrow focused beam in a direction orthogonal to the direction of flight corrects for many of the issues present in the MALDI-ToF system. For example, ions of a specific type each have similar energy and initiate flight from a very similar position (unlike the spatial distribution within the ion plume of the MALDI). As such the ions are delivered to the mass detector in a highly resolved fashion. In addition, the induction of multiple charges on molecules of typically larger molecular weight means that these molecules may have m/z values several multiples lower than their molecular weight. As a consequence the added resolution provided by the ion mirror 'reflecton' is given without much loss of data as most of the larger molecules generate multiply charged ions that give m/z values within the range of detection. However, these multiply charged species do present the need to deconvolute the data. These benefits in terms of precision, especially for larger molecules, are also provided by the QqToF when it is run in singular MS mode. Modern QqToF instruments have been enhanced by features that enable a higher scanning rate and collision cells whereby the ion fragments may be subsequently detected with near 100% efficiency. These features have enhanced the ability to perform high throughput proteomics due to their enhanced peptide sequencing capability.

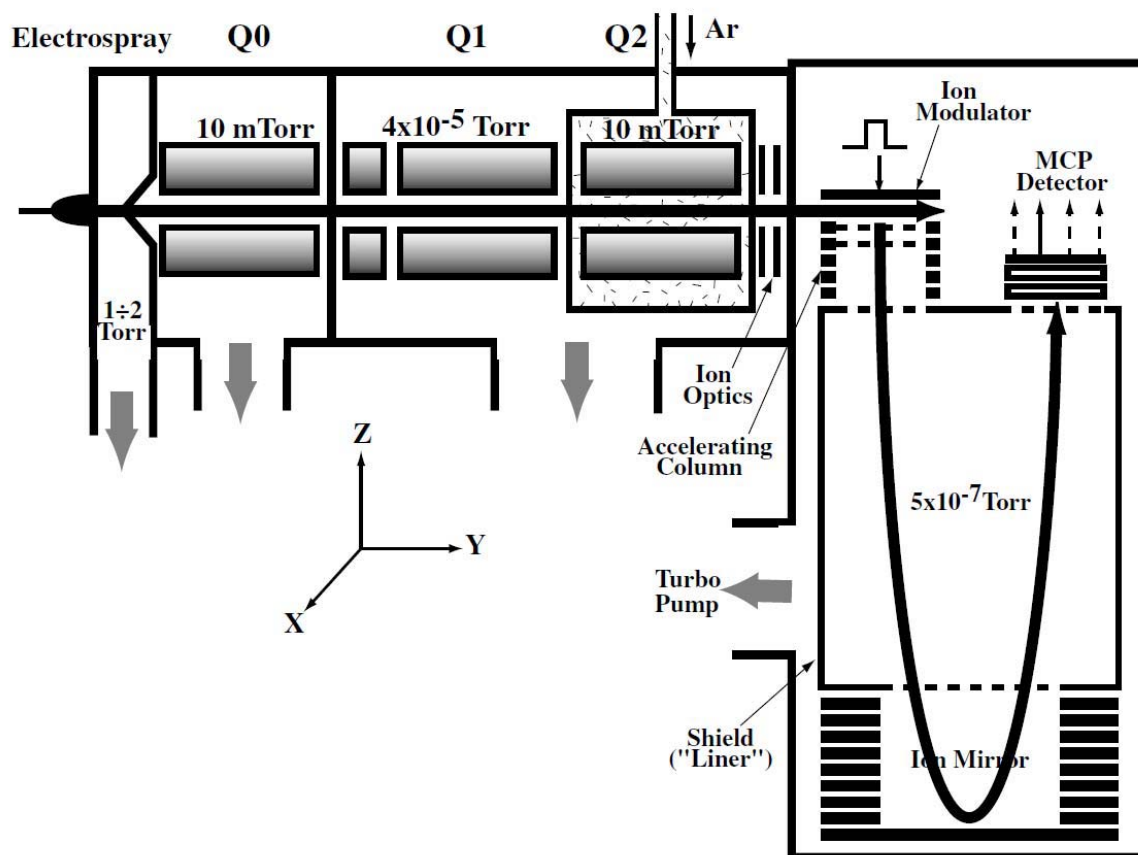


Figure 1.20. Schematic diagram of the ESI-QqToF (Chernushevich et al., 2001)

The sample is ionised by the electrospray and ions are guided towards the mass filters. These typically consists of three quadrupoles with the first (Q0) added to provide collisional damping and ion focusing. In MS only mode the second quadrupole (Q1) acts only as a transition element. In MS/MS mode it acts as a mass filter. In MS/MS mode, fragmentation occurs by CID in the third quadrupole (Q2) and the ions are re-focused by the ion optics. The ions are transferred to the ToF in orthogonal orientation for pulsed acceleration towards the ion detector via a reflection containing an ion mirror.

1.15.4.3. MS/MS: ToF/ToF

Dual ToF/ToF instruments are relatively inexpensive MS/MS instruments and have high transmission and detection sensitivities. In a typical ToF/ToF run, precursor ions are accelerated towards the collision chamber within the flight tube. Plates, based on the Bradbury Nielsen electron filter, are placed in front of this chamber to which a voltage is applied which deflects ions away from the flight path that leads towards the collision chamber. At the time appropriate to the m/z range of interest, the charge on the plates is altered to guide the selected ions into the chamber where collision with an inert gas, typically argon, takes place. Unfragmented parent ions and the daughter fragment ions are subsequently accelerated from the collision chamber into the second ToF analyser which separates

them according to m/z before reaching the ion detector. In many systems it is common to use the reflectron as the second ToF (in which case it would be more accurately referred to as a ToF/RTof). Despite their advantages, the precision of ToF/ToF systems can be limited, especially with regards to their ability to select some precursor parent ions.

1.15.5. Carbohydrate analysis by mass spectrometry

Most carbohydrates are not particularly amenable to mass spectrometry. They are non-volatile due to the abundance of polar interactions between hydrogen and oxygen and are therefore not readily transferable into the gas phase. For this reason they may often be derivatised beforehand, for example by permethylation or the generation of alditol acetates, in order to eliminate the hydrogen bonding and enhance the sensitivity of the mass spectrometry approach. This can also have the added benefit of generating fragmentation processes that are reproducible and provide a means of additional characterisation of the individual saccharides (Fox et al., 1989) and the linkages between them (Carpita and Shea, 1989) by the employment of electron impact ionisation sources. However, as described above, they may form adducts with sodium during electrospray and this can also provide a suitable platform for their analysis. Sugars containing amine groups may also be detected via the addition of proton to the nitrogen to create protonated ions.

In a process analogous to the analysis of peptides, carbohydrates can also be fragmented by tandem mass spectrometry with predictable outcomes to generate additional structural information about the parent polymer. The daughter fragment ions are typically named according to the accepted nomenclature described in the late 1980s (Domon and Costello, 1988). As is the case with peptides so it is that the carbohydrates within a parent polymer will preferentially fragment across the bonds responsible for polymerisation, in this case the glycosidic bond. As shown in the figure 1.21 below, fragmentation may take place either side of the oxygen within the glycosidic bond to create Z and C fragments if fragmentation is on the reducing side (β -cleavage) or Y and B fragments if it is on the terminal side (α -cleavage). Y and Z fragments represent those that contain the reducing end and B and C fragments represent those that contain the terminal end. A fragments are formed by cross-ring fragmentation and represent the fragments that contain the terminal end. The complementary fragment with the reducing end is known as the X fragment. The B, C, Y and Z fragments are numbered according to the number of monosaccharides within. The nomenclature of the cross ring

fragments relates not only to the number of monosaccharides within the fragment but also the position of the carbons across which fragmentation occurred.

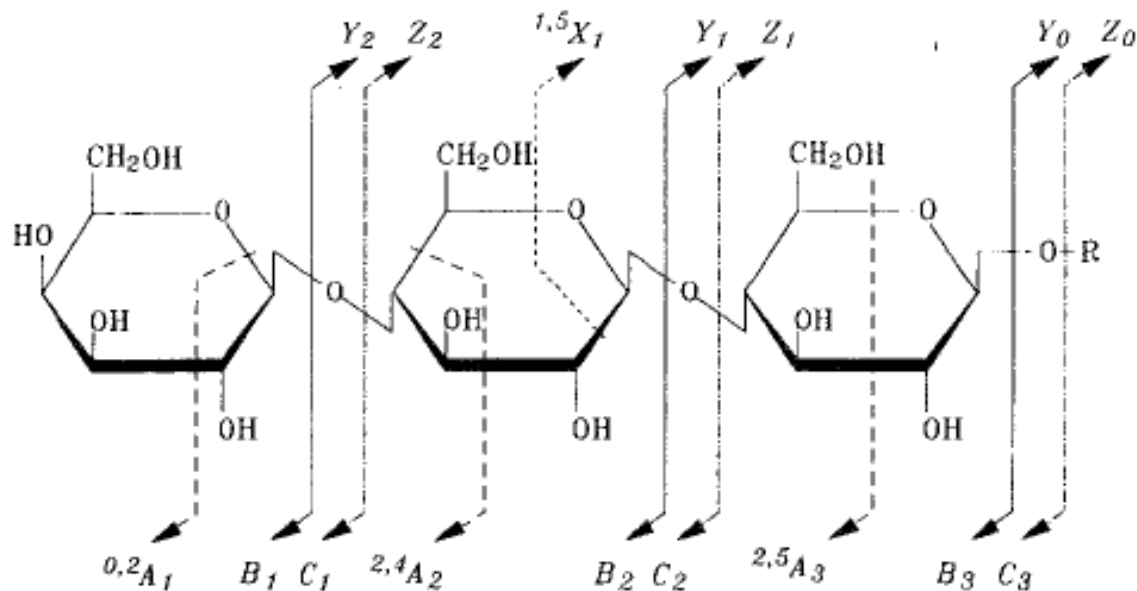


Figure 1.21. The nomenclature of fragmented polysaccharides (Domon and Costello, 1988)

The fragmentation of peptides by MS/MS provides precise sequence information as each of the finite number of amino acids has a specific mass. This, alas, is not the case with carbohydrates owing to their far greater structural repertoire, plethora of stereoisomers, variable linkage types and existence of branched structures.

1.16. NMR

When placed in a powerful electric field, NMR active nuclei such as ^1H or ^{13}C absorb electromagnetic radiation at a frequency characteristic of the isotope. However, the total magnetic field experienced by a nucleus includes local fields created by the surrounding molecular structures. This environment shields the nuclei to a greater or lesser extent from the external magnetic field. Thus as a reproducible consequence of the molecular structure, these nuclei demonstrate variations in frequencies that are referred to as the chemical shift. By applying stronger magnetic fields the signals returned are increased and thus the NMR method may become more analytically sensitive.

There are two main forms of NMR spectroscopy, one is sensitive to the shielding effects that arise via the j-coupling of nuclei that are connected through bonds. The other makes use of the Overhauser effect which is sensitive to interatomic distances rather than through bonds. The quantity of data gathered from an NMR experiment can be increased by the use of 2D NMR. Each 2D experiment consists of 4 steps, the preparation period, the evolution period, the mixing period, and the detection period. The two dimensions of a 2D NMR experiment are two frequency axes representing a chemical shift, each associated with one of the two time variables, the evolution time and the detection time. The end result is a plot showing an intensity value for each pair of frequency (time) variables. The intensity of the peaks may be represented in a third dimension such as through the use of colours or contours. The most popular and powerful 2D technique is homonuclear correlation spectroscopy (COSY) where the data collected relates to the bonds between the NMR active nuclei. Equivalent nuclear Overhauser effect spectroscopy (NOESY) may also be performed to explore the distances between molecular groups.

1.16.1 Application of NMR to carbohydrate analysis

Nuclear Magnetic Resonance is widely used for the structural analysis of carbohydrates. If samples are available in sufficient quantity and purified NMR is a powerful technique for structural resolution. Proton 1D-NMR can be used to determine the anomeric configuration of individual sugars. The connectivity between the sugars can be determined by 2D NMR. Homonuclear COSY experiments are useful for the identification of individual sugars. 2D Heteronuclear methods such as HMBC together with NOESY provide information on linkages and sequences within a saccharide polymer. As the investigations into the structure of *Brucella* OPS have shown (Bundle et al., 1987b; Caroff et al., 1984b; Kubler-Kielb and Vinogradov, 2013b), NMR plays a critical role in the structural resolution of novel carbohydrates.

1.17. PROJECT AIMS

The overarching aim of the project is to develop a new serodiagnostic assay for bovine brucellosis that can be used to effectively discriminate between sera that are, when standard serological techniques are used, true positive or false positive. The OPS from *Brucella* contains antibody epitopes that are, as far as is known, unique to this organism. However these cannot be fully exploited for diagnosis in the native form as the epitopes are conjoined and overlapping with non-specific epitopes in a linear repeating structure. It is hypothesised that the project aim may be achieved by applying the unique OPS epitopes *Brucella* as discrete, separate, individual antigens within serodiagnostic assays.

To develop and test this hypothesis the project aims have been subdivided as follows:

- To demonstrate that the OPS derived from A and from M dominant strains of *Brucella* and from *Y. enterocolitica* O:9 display different levels of reactivity against polyclonal antibodies within bovine sera that are true positives compared to those that are false positives by standard serology.
- To demonstrably generate oligosaccharides of comparable length and structure to those proposed to form discrete antibody epitopes from native *Brucella* and *Y. enterocolitica* O:9 OPS antigens.
- To evaluate the epitopes within the OPS derived oligosaccharides by affinity chromatography using polyclonal and monoclonal antibodies and HPLC-ES-MS.
- To develop an indirect ELISA using the oligosaccharide structures defined by the preceding findings in order to generate a serodiagnostic assay that may be used to assist in resolution between sera that are true or false positives as determined by standard serology.
- To evaluate the diagnostic efficacy of the developed iELISAs using a panel of cattle sera that are either true or false positive by standard serology.

Chapter 2

METHODS and MATERIALS

2.1 Antigen production

2.1.1. Antigen production: Bacteriology

B. abortus biovar 1 strain 99, A dominant (OPS type A, biovar 1), cells were reconstituted from a pure freeze dried source within the culture collection of the OIE (World Organisation for Animal Health) *Brucella* Reference Laboratory at the Animal Health Veterinary Laboratories Agency. The cells were grown on serum dextrose agar plates at 37°C at 10% CO₂ and harvested into phosphate buffered saline (PBS), prepared by adding 1 tablets of PBS (Oxoid, product #BR0014G) to 100 ml of H₂O (all water is at 15 mΩ purity unless otherwise stated). *B. melitensis* strain 16M (OPS type M, biovar 1), was also grown harvested and purified as above. *Y. enterocolitica* serotype O:9 (biotype 2), was reconstituted from a pure freeze dried source within the *Brucella* Reference Laboratory culture collection. The cells were grown on nutrient agar plates at 25°C, atmospheric CO₂ levels and then harvested into PBS. All harvested cells were incubated at 80°C until confirmed non-viable by culture prior to further manipulation.

Culture of *E. coli* O:157 was from strain NCTC 10964 (Furowicz and Ørskov, 1972), received from Public Health England (ACDP level 2). The bacteria were grown on horse blood agar at 37°C at 10% CO₂, harvested into water and heated at 80°C until confirmed by culture as non-viable.

2.1.2. Antigen production: extraction and purification of sLPS from killed bacterial cells

The hot phenol extraction method (Nielsen and Ewalt, 2010; Westphal et al., 1952) was used to produce sLPS antigen from cultured, non-viable, whole cells. Cells harvested in PBS were centrifuged at 10,000 g for 30 mins to form a pellet. This pellet was resuspended in 3.4 mls of water per gram of wet cells and this was warmed to 66°C. This was mixed with 3.8 mls (per gram of cells) of 90% phenol (Sigma, product #P1037) which was pre-warmed to 66°C. This mixture was stirred continuously at 66°C for 15-20 mins and then cooled and centrifuged at 10,000 g for 45 mins at 4°C. The phenol layer was carefully extracted so as to avoid co-extraction of cell debris. The phenol layer was added to 10 mls (per gram of wet cells) of cold methanol containing 1% methanol saturated with sodium acetate (Sigma, product #S2889). This was mixed well and then allowed to settle for 2 hrs at

4°C. The precipitate was resuspended and the mixture centrifuged at 10,000 g for 45 mins at 4°C whereupon the precipitate was retained. This was resuspended in 1.6 mls of water per gram of wet cells and stirred for 4 hours at 4°C. Again the mixture was centrifuged at 10,000 g for 45 mins at 4°C, this time the supernatant was retained. The precipitate was resuspended in the same volume of water for additional extraction of sLPS and this was incubated and centrifuged as above. The supernatants were pooled, 0.05 g per ml of wet cells of trichloroacetic acid (Sigma, product #T6399) added, and then stirred for 10 mins at 4°C then centrifuged at 10,000 g for 45 mins at 4°C. The supernatant was dialysed against water, at least 80 mls per gram of wet cells with two changes of equivalent volume, using dialysis tubing (Medicall 'Visking', product #DTV12000.05.000, size 5 inf diameter 24/32" 19.0 mm, MWCO 12-14 kDa). The dialysed liquid was then freeze dried for quantification by mass and for storage.

2.1.3. Antigen production: extraction and purification of OPS from sLPS

Purified OPS was derived from sLPS by mild acid hydrolysis (Meikle et al., 1989) through incubation of the sLPS in water with 1% acetic acid at 110°C for 2 hrs and 20 mins. The hydrolysed antigen was then centrifuged at 17,000 g for 30 mins to precipitate the lipid A with attached core components. The supernatant was retained and passed through a PD-10 (GE Healthcare, Product #17-0851-01) size exclusion chromatography (SEC) desalting column (5 kDa exclusion limit) equilibrated with water to remove acetic acid as well as small saccharide and oligosaccharide components.

2.1.4. Antigen production: extraction and purification of rLPS from *Brucella*

Rough (r) LPS was produced from *B. abortus* strain RB51, (Schurig et al., 1991). This is a laboratory derived mutated rough (lacking OPS) vaccine strain and was propagated as described for *B. abortus* strain 99 (above). The rLPS antigen was extracted using the petroleum, chloroform, phenol method (Galanos et al., 1972) as described recently (McGiven et al., 2012).

2.2. Antigen Evaluation

2.2.1. Antigen evaluation of sLPS and OPS by UV absorbance

The produced sLPS and OPS antigens were evaluated by UV absorbance from 190 to 290 nm using a Thermo Biomate 3 spectrophotometer.

2.2.2. Antigen evaluation: quantitative protein assay

Quantitative protein assay was performed using the Bradford (Coomassie) protein assay (Bradford, 1976). This was done in 96 well microtitre format according to the manufacturers instructions (Pierce, product #23200). Optical Density (OD) was measured using a POLARstar Omega plate reader (BMG Labtech). Unless otherwise stated, this instrument was used to record all the OD values documented in this study

2.2.3. Antigen evaluation: SDS-PAGE of sLPS and OPS

SDS-PAGE was performed at 200V for 40 mins after the addition of antigen diluted 1/2 in Laemmli buffer (Sigma, product #047K6067) to pre-cast polyacrylamide NuPage® 4-12% Novex Bis-Tris gels (Invitrogen, product #NP0322). Electrophoresis was performed with MOPS SDS running buffer (Invitrogen, product #NP0001-02). The antigen in Laemmli buffer was heated in a water bath at 87°C for 5 mins prior to addition to the gel. For estimation of mass, protein markers ('Rainbow Markers', Amersham, product #RPN756E) were also added to the gels.

2.2.4. Antigen evaluation: protein staining of SDS-PAGE gels

The gels were fixed, washed and then stained with Coomassie dye 'Imperial Protein Stain' (Pierce, product #24615) for visualisation of protein. This was performed in accordance with manufacturers instructions. Proteins were also visualised by silver staining using the 'Colour Silver Stain kit' (Pierce, product #24597) with staining performed in accordance with the manufacturers instructions.

2.2.5. Antigen evaluation: carbohydrate staining of SDS-PAGE gels

The gels were fixed, washed and the contents oxidised with periodate (Fomsgaard et al., 1990), re-washed and stained with Pro-Q® Emerald 300 (Invitrogen [Molecular Probes], product #P20459). This procedure was done in accordance with the manufacturers instructions. The oxidised carbohydrates were visualised using a UV transilluminator and the images captured with a digital camera with a Tiffen Yellow 12 (> 500 nm) filter.

2.2.6. Antigen evaluation: chemical carbohydrate analysis

Chemical carbohydrate analysis was performed using the phenol sulphuric acid method for total carbohydrate analysis (Dubois et al., 1956) following the modifications described for microtitre plate analysis (Masuko et al., 2005). Total carbohydrate analysis was also performed using the BCA assay. This is commonly applied for the determination of protein content (Smith et al., 1985) as originally described but is also sensitive to a wide range of reducing sugars (Doner and Irwin, 1992; Waffenschmidt and Jaenicke, 1987). The BCA was performed according to the manufacturers instructions (Pierce, product # 23227). The OPS samples were hydrolysed by incubation in 2 M HCl at 100°C according to the times shown in the results. Unfortunately no D-Rha4NFO standards were available to assist in the quantification of results derived from these assays.

2.2.7. Antigen evaluation: endotoxin assay

Antigens were also assayed for endotoxin using the Limulus Amebocyte Lysate (LAL) reaction. This was performed using the PyroGene™ rFC assay (Lonza, product #50-658U) as per the manufacturers instructions (Ding and Ho, 2001). The lipid A within the LPS of *Brucella* is atypical and a far less potent endotoxin than that of *E. coli* (Lapaque et al., 2005). It was therefore not possible to quantify in absolute terms the amount of *Brucella* Lipid A present in each of the antigens but it was possible to measure the relative amounts.

2.3 Immunodetection of antigen and antibody

2.3.1. Enzyme Linked Immunosorbent assays (ELISAs)

ELISAs were used in this study for the detection and evaluation of antigens and antibodies. Unless otherwise stated the following parameters were universal. ELISA plates were standard polystyrene (Nunc, product #269620). Water used was deionised to 15 mΩ. Volumes added to the wells of ELISA plates were 100 µl. Antigens were coated to the plate passively by incubation overnight at 4°C in carbonate buffer pH 10, prepared by the addition of one capsule (Sigma, product #C3041) to 100 ml of water. Incubation of ELISA plates was conducted at room temperature (rt) on a rotary shaker at 160 rpm. Washing was with four times 200 µl of PBS-T per well, 1 ltr of PBS plus 0.5 ml Tween 20 (VWR, product #663684B), after which the plate was dried by inversion and tapping on blotting paper. Unless otherwise stated ELISA plates were developed by the addition of 100 µl per well of pH 4.0 buffer (prepared by adding 1 tablet [Flucka, product #82560] to 120 mls of water) containing 0.015% H₂O₂ (Sigma, product #16911) and 0.5 mg/ml 2,2-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS [Sigma, product #A9941]). The plates were left to develop at rt for 10-15 mins without shaking and the reaction stopped by the addition of 100 µl of 1 mM sodium azide (Sigma, product #S2002) per well. For each well Optical Density (OD) at 405 nm was measured.

When ELISAs were used for diagnostic purposes on test serum, positive and negative control sera were included on every plate and used as a reference. A blank well was also included, the OD value of which was subtracted from the OD value of all other wells prior to further analysis.

2.3.1.1. Estimation of sLPS quantity by iELISA

To obtain estimates for the relative content of sLPS in the hot phenol extractions from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 sLPS iELISA was used. ELISA plates were coated with equivalent concentrations of a dilution series of sLPS from each antigen and then washed. A high titre positive serum, derived from a cow experimentally infected with *B. abortus* strain 544 (biovar 1), was added to each well at a 1/200 dilution in PBS-T. The ELISA plates were incubated for 30 mins and then washed as described above. The plates were then incubated with HRP conjugated polyclonal rabbit anti-bovine IgG (Dako, product #P0159), diluted in PBS-T at 1/2000, for 30 mins

and then washed. The plates were then developed with ABTS substrate and OD measured as described above.

2.3.1.2. Purification and quantification of monoclonal antibodies (mAbs)

The mAbs 12G12 and 12B12 were received in ascitic fluid (a generous gift from Professor A. Cloeckaert) and mAb BM40 was prepared from hybridoma supernatant. The mAbs were purified using Protein G HP Spin Trap columns (GE Healthcare, product #28-9031-34) following the manufacturers guide. The elution buffer was 0.1 M Glycine-HCl pH 2.7. Neutralisation buffer was 1 M Trizma base pH 9.1. A total of 250 µl of ascites or hybridoma supernatant was added per Protein G HP Spin Trap column. This was incubated for 5 mins end over end at rt and the column centrifuged at 100 g for 45 secs. Elution buffer was added, 400 µl, and mixed. The column was then centrifuged for another 45 secs at 100 g and the eluate neutralised with 30 µl of neutralisation buffer. All eluates for the same mAb were pooled and then buffer exchanged into PBS using PD-10 (GE Healthcare, Product #17-0851-01) disposable size exclusion chromatography (SEC) columns (contain sephadex G-25 matrix) following the manufacturers instructions. The preparations were then concentrated using an Amicon® Ultra Centrifugal Filter (10 kDa molecular weight cut off), (Millipore, product #UFC801008). The final concentration of mAbs was determined by UV 280 nm absorbance measured using a Thermo Biomate 3 spectrophotometer.

2.3.1.3. sLPS iELISA for determination of mAb binding (BM40, 12G12, 12B12, 4B5A)

ELISA plates were coated with equivalent concentrations of *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 sLPS. The plates were then washed. The mAbs were prepared at a 100 nM concentration in PBS-T and double diluted, in duplicate, across the plate in successive 100 µl volumes each half the concentration of previous dilution. The ELISA plates were incubated for 1 hr and then washed. The plates were then incubated for 30 mins with HRP conjugated goat anti-mouse polyvalent immunoglobulins (Sigma, product #A0412) diluted 1/2,500 in PBS-T and then washed. They were then developed with ABTS substrate and OD measured as described above. The mAb 4B5A was received pre-conjugated with HRP and was evaluated without the need for an anti-antibody conjugate.

2.3.1.4. The Standard *Brucella* cELISA

The *Brucella* ('standard') cELISA is a contemporary assay which is applied to routine serology at the Animal Health Veterinary Laboratories Agency (AHVLA) in a manner that conforms to the requirements of the OIE 'Manual for Diagnostic Test and Vaccines for Terrestrial Animals' (Nielsen and Ewalt, 2010). The details of the assay have been described elsewhere (Perrett et al., 2010; Thompson et al., 2009) but are as follows.

ELISA plates are coated with *B. melitensis* 16 M sLPS and then washed (optimal antigen concentration is determined for each antigen batch by checkerboard titration against serum standards but is approximately 0.25 µg/m). Samples are tested by adding 20 µl of serum and 100 µl of HRP-labelled BM40 mAb (Greiser-Wilke and Moennig, 1987; Greiser-Wilke et al., 1985) diluted in PBS-T, to each well. Optimal BM40-HRP concentration for each conjugated batch is determined previously by checkerboard titration against serum standards. Each plate has a negative control, high and low-titre positive serum controls, and conjugate-only controls (no serum added) added (100 µl per well). ELISA plates are incubated for 30 min before being washed. Plates are developed by the addition of 0.012% H₂O₂ substrate and 0.4 mg/ml o-Phenylenediamine dihydrochloride (OPD) chromogen (Sigma, product #P8412) in water and are incubated at room temperature (rt) for 15 mins. After this time the reaction is stopped by the addition of 25 mM citric acid (Sigma, product #C3041) to each well. The OD for each well at 450 nm is measured. Results are considered positive if the percentage of the conjugate control was < 60% (which equals > 40% inhibition of the conjugate control sample). This is the cELISA method that was used to test the pig and cattle sera, the results for which are shown in chapter 3.

2.3.1.5. *Brucella* cELISA (BM40 mAb) for the detection of free, fluid phase, antigen

This 'standard' cELISA method can be adapted to suit several different tasks. The competitive method of antibody detection also enables the detection of free, fluid phase, antigen via competition of the free and solid phase bound antigen for binding to BM40. The analytical sensitivity of this process may be increased by increasing the volume of sample added and to compensate, increasing the concentration of BM40-HRP added in the remaining well volume (total volume of 120 µl). This

approach was used for the detection of antigen eluted from the CarboLink™ affinity chromatography columns post antigen conjugation where the protocol for the standard cELISA was followed with one modification which was to increase the sample volume to 40 µl, decrease the BM40-HRP volume from 100 to 80 µl but also to increase the concentration by 25%. Analytical sensitivity may also be increased by decreasing the concentration of competing BM40 mAb as a lower concentration of analyte has a proportionally higher inhibitory impact. To compensate for loss of OD signal as a consequence of this, a different conjugate can be used. This was done for the quantification of the binding of free antigen (OPS and synthetic D-Rha4NFo tetrasaccharide [TSM] antigen) to the BM40 mAb. The method is as follows.

Corning CarboBind™ plates were coated with 0.1 µg/ml of *B. melitensis* 16M OPS (as described below). The test, fluid phase, antigens (*B. abortus* S99, *B. melitensis* 16M, *Y. enterocolitica* O:9 OPS and the tetrasaccharide 'M' antigen [TSM]) were prepared at 64 µg/ml concentration in water and double diluted, in duplicate, across the plate in successive 50 µl volumes each half the concentration of the previous dilution. A biotinylated BM40 conjugate was added in 50 µl volumes, in 2x working strength casein buffer (Sigma, product #B6429), to each well for a final concentration of 0.1 µg/ml. Biotinylation of BM40 was achieved using EZ-Link Sulfo-NHS-Biotin (Pierce, product #21326) and following the manufacturers instructions. Conjugate controls were prepared with 0.1 µg/ml of conjugate without free phase antigen. The plates were incubated for 1 hr then washed. A streptavidin poly-HRP (Pierce, product #21140) conjugate was added to the wells at 0.2 µg/ml in casein buffer. The plates were incubated for 1 hr then washed. The plates were developed using ABTS substrate and the OD recorded as described above. This method was also used to detect the presence of *B. melitensis* 16M OPS antigen being eluted from BM40 mAb conjugated AminoLink™ affinity purification columns.

2.3.1.6. *Brucella* cELISA (monospecific A and M sera) for the detection of free, fluid phase, antigen

Fluid phase antigen was also measured by cELISA using rabbit anti-A and anti-M monospecific sera, as used for the serotyping of *Brucella* strains (Alton et al., 1994). The test antigens (of varied type and dilution as shown in results) and anti-OPS monospecific sera, at a 1/10 dilution, were pre-incubated for 30 mins in casein buffer at rt on a rotary shaker at 160 rpm. This antigen and antibody mix was then added to the wells of Corning CarboBind™ plates coated with 0.1 µg/ml of either *B.*

abortus S99 (for evaluation with anti-A monospecific sera) or *B. melitensis* 16M (for evaluation with anti-M monospecific sera) OPS as described below. The monospecific sera and antigen were incubated on the plate for 30 mins then washed. A biotinylated goat anti-rabbit IgG conjugate (Sigma, product #B6648) was diluted 1/125 in casein buffer and added to each well of the plate and incubated for 1 hr and then washed. A second conjugate, streptavidin poly-HRP (Pierce, product #21140) at 0.4 µg/ml in working strength casein buffer, was added to the wells and the plates were incubated for 1 hr then washed. The plates were developed with ABTS substrate and OD was measured at 405 nm.

2.3.1.7. Anti-Brucella antibody absorption assay

Antibodies were absorbed from a high titre positive serum, derived from a cow experimentally infected with *B. abortus* strain 544 (biovar 1), using killed whole cells from *B. abortus* S99 and *Y. enterocolitica* O:9. The serum was diluted 1/5 in PBS-T such that a number of aliquots were prepared that contained between 0 – 200 µg/ml of cells (as shown in the results). The sera were incubated with the cells for 18 hrs at rt on a rotary shaker at 160 rpm. The aliquots were centrifuged at 10,000 g for 10 mins to sediment the cells and associated antibodies.

The supernatant was tested by iELISA using plates coated with 0.2 µg/ml of *B. abortus* S99 or *Y. enterocolitica* O:9 sLPS and then washed. They were then blocked with 100 µl/well of 1% bovine serum albumin (BSA) (Sigma, product #A6003) in PBS-T for 2 hrs at rt on a rotary shaker at 160 rpm after which they were washed. The serum supernatants were further diluted 1/10 in PBS-T with 1% BSA and added to each well of the sLPS coated and BSA blocked plates. The ELISA plates were incubated for 30 mins and then washed. The plates were then incubated with HRP conjugated polyclonal rabbit anti-bovine IgG (Dako, product #P0159) diluted in PBS-T at 1/2,000 for 30 mins then washed. The plates were then developed with ABTS substrate and OD was measured at 405 nm. Sera from cattle experimentally infected with *Y. enterocolitica* O:9 (n = 3) were also tested in this manner as were an additional 3 sera from cattle infected with *B. abortus* biovar 1. In samples with lower titre, pre-ELISA incubation with sLPS, up to 50 µg/ml, was sufficient to eliminate detectable antibody binding to solid phase antigen.

2.4 Affinity Chromatography

2.4.1. OPS conjugation to Carbo-BIND™ ELISA plates and CarboLink™ columns

The purified OPS antigens were covalently conjugated to Carbo-BIND™ ELISA plates (Corning, product #2507) and CarboLink™ affinity chromatography columns (Pierce, product #20355). For conjugation to the Carbo-BIND™ plates the OPS was oxidised, at 100 µg/ml, by incubation with 10 mM sodium metaperiodate in 50 mM sodium acetate buffer pH 5.5 for 30 mins in the dark. After oxidation, the OPS was diluted to working strength (0.1 to 0.5 µg/ml, dependent on exact assay) in sodium acetate buffer pH 5.5 and 100 µl of this was added per well of a Carbo-BIND™ plate. The plate was incubated at 37°C for 1 hr in the dark. After this time the plates were washed. The plates were then ready for use and could be stored dry in the dark for at least 2 months. Prior to conjugation to the CarboLink™ affinity chromatography columns the OPS was oxidised at 1 mg/ml by incubation in 10 mM sodium metaperiodate in 50 mM sodium acetate buffer pH 5.5 for 60 mins in the dark. After this period the oxidised OPS was separated from excess sodium metaperiodate using a PD-10 column pre-equilibrated with sodium acetate buffer pH 5.5, and 2 mg was added to the CarboLink™ affinity chromatography column (also pre-equilibrated in sodium acetate buffer pH 5.5). The column was incubated at 37°C for 20 hrs and then washed with PBS. Reagents are added and eliminated from the column by centrifugation as described in the manufacturers instructions.

2.4.2. sLPS, OPS and rLPS iELISAs for detection of OPS purified polyclonal antibodies

Initial exploration of antibody elution from OPS conjugated CarboLink™ affinity chromatography columns by iELISA was performed with *B. abortus* S99 and *B. melitensis* 16M sLPS antigen coated ELISA plates at 0.2 µg/ml. Column wash and eluates were diluted 1/50 or 1/200 in casein buffer respectively, and a dilution series of the source positive sample was prepared ranging from a 1/50 to a 1/819,200 dilution in casein buffer. These dilutions were added to the ELISA plates and the assay was performed as described above (2.3.1.1. 'Estimation of sLPS quantity by iELISA') with the exception that the anti-bovine conjugate was used at 1/4,000.

Further exploration of eluted antibody titre was performed using Carbo-BIND™ ELISA plates coated with 0.25 µg/ml of OPS antigen from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 (as described above). A range of dilutions, in casein buffer, for the wash, eluates and source sera were tested by the method described below for the OPS iELISA for cattle sera (section 2.5.3.) except that sample and conjugate incubations were both only 30 mins and conjugate was used at a 1/2,000 dilution. The rLPS iELISA for the eluted fractions was performed as described below for cattle sera (section 2.5.4).

2.4.3. OPS Affinity chromatography for purification of anti-*Brucella* OPS antibodies

Antigen capture affinity chromatography was performed using CarboLink™ columns (Pierce) conjugated with OPS as described previously. Columns were prepared with 2 mg of OPS antigen from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9. Each step and iteration in the conjugation, washing and elution process involved the addition and mixing (and incubation if necessary) of the desired reagent and buffer followed by centrifugation at 1000 g for 1 minute to evacuate the fluid (which may be retained for analysis) from the column prior to the next step in the process.

The development of the method for the production of purified anti-*Brucella* OPS antibodies is described in the results, chapter 5. The final protocol used for the production of the purified anti-*Brucella* OPS polyclonal antibodies to be used in the affinity selection of homo-D-Rha4NFO-oligosaccharides is as follows. A 400 µl volume of positive, high titre, source serum derived from a cow experimentally infected with *B. abortus* strain 544 (biovar 1, A dominant OPS) was diluted in 2.6 mls of casein buffer and added to the *Y. enterocolitica* O:9 OPS conjugated column. The column was incubated for 3 hrs at 37°C with end over end mixing. The serum flow through after centrifugation was collected and the column was then washed with 6x 2 mls of PBS followed by addition of 6x 2 mls of 0.1 M glycine-HCl pH 2.5 elution buffer. This process was reiterated four further times such that 2 mls of source serum was used in total.

The eluted fractions were neutralised to approximately pH 7.0 with 1 M Trizma base (Sigma, product #T1503) pH 9.0 and concentrated using Amicon® Ultra centrifugal filter with a molecular weight cut-off of 30,000 kDa (Millipore, product #UFC903008). They were then buffer exchanged into PBS

using PD-10 SEC columns. These buffer exchanged eluates from all five rounds of chromatography were pooled and concentrated by centrifugal filtration as just described.

The antibody fraction contained in each 3 ml serum flow through from the *Y. enterocolitica* O:9 OPS column was applied sequentially to the *B. abortus* S99 OPS column. Each was incubated in the column for 1 hr at 37°C with end over end mixing after which time the column was centrifuged to remove the unbound contents and the next 3 mls of serum flow through from the *Y. enterocolitica* O:9 column was added. After all 15 ml of the serum flow through from the *Y. enterocolitica* O:9 OPS column had been incubated within the *B. abortus* OPS column it was washed with 6x 2 mls of PBS. This was followed by the addition of 6x 2mls of 0.1 M glycine-HCl pH 4.0 elution buffer, then 6x 2mls of 0.1 M glycine-HCl pH 2.7 elution buffer. These fractions were neutralised, buffer exchanged and concentrated as described above.

The antibodies in the fraction eluted from the *Y. enterocolitica* O:9 OPS column constituted one population ('Y') to be used for the affinity chromatography of homo-Rha4NFO-oligosaccharides and the combined eluted fractions from the *B. abortus* S99 OPS column constituted the second ('YxA') population. The final concentration of antibodies was determined prior to conjugation to the AminoLink® columns by UV 280 nm absorbance measured using a Thermo Biomate 3 spectrophotometer.

2.4.4. Conjugation of antibodies to AminoLink® Columns

As with the CarboLink™ columns, each step and iteration in the conjugation, washing and elution process for the AminoLink® Columns involved the addition and mixing (and incubation if necessary) of the desired reagent and buffer followed by centrifugation at 1000 g for 1 minute to evacuate the fluid (which may be retained for analysis) from the column prior to the next step in the process.

The three antibody sets (BM40 mAb, 'Y' and 'YxA') were coupled to AminoLink® Columns (Pierce, product #44894) via the binding of primary amines on the antibodies to aldehyde groups on the surface of the agarose beads within the column. The conjugation was performed based upon the manufacturers instructions. The antibodies were added to the column in pH 10 kit coupling buffer (to form Schiff base bonds). After incubation at 37°C for 4 hrs with end over end mixing, excess material was removed from the column by centrifugation. The covalent links were then stabilised by the

addition of 2 mls of 100 mM sodium cyanoborohydride in kit pH 7.2 coupling buffer and the column was incubated for 1 hr at rt with end over end mixing. Unconjugated aldehyde groups on the agarose beads were then blocked by the addition of 3 times 2 mls of kit 'quenching buffer'. The columns were then washed 5x with 2mls of kit wash and a further 3x with 2ml of PBS. The columns were then left in sterile PBS at 4°C until use. To evaluate the success of this conjugation process, the samples and all eluted fractions were evaluated by Bradford (Coomassie) protein assay. A total of 1000 µg of BM40 mab, 525 µg of *Y. enterocolitica* O:9 OPS affinity purified polyclonal antibodies ('Y') and 750 µg of *Y. enterocolitica* O:9 OPS deselected and *B. abortus* S99 OPS affinity purified polyclonal antibodies ('YxA') were added to individual AminoLink® columns

2.4.5. Antibody affinity chromatography of homo-D-Rha4NFO-oligosaccharides

The development of the affinity chromatography process is described in the results section chapter 5 and the separation was based upon the methods described in the AminoLink® Column manufacturers instructions. The developed and selected process applied for purification was the addition of the homo-D-Rha4NFO-oligosaccharides to the antibody conjugated AminoLink® Columns in 2 mls of 25% PBS and incubation for 3 hrs at 37°C with end over end mixing. PBS was then removed from the column by centrifugation. The columns were then washed 5x with 2 mls of water. The elution process then began with the addition of 5x of 2 mls of 0.00625 M HCl (in water only) to each column. Followed by 5x elution with 2 mls of 0.0125 M HCl and 5x elution with 2 mls of 0.025 M HCl. The wash and eluates were all retained for analysis by ESI-MS as described below.

2.5. Serology

2.5.1. Serum Samples

2.5.1.1. Cattle sera

There were five populations of cattle sera evaluated within this study. These were as follows. A total of 46 samples from individual animals confirmed by culture to be field infected with *B. abortus* biovar 1 (A dominant). Also 68 samples from individual animals whose sera was collected from within Great Britain from 1996 to 1999, more than 10 years since the declaration of officially brucellosis free

status for Great Britain, that were positive in conventional serology for brucellosis such as *B. abortus* S99 sLPS iELISA, SAT and CFT (Nielsen and Ewalt, 2010) but for which there was no cultural or epidemiological evidence of the disease. For the evaluation of BSA conjugated synthetic oligosaccharide antigens 125 randomly selected sera from non-*Brucella* infected cattle sample from Great Britain collected since 2007 were also included. Also evaluated by OPS iELISA were sera from four cattle experimentally infected with *Brucella abortus* strain 544 (an A dominant strain) and four experimentally infected with *Y. enterocolitica* O:9 as described previously (McGiven et al., 2008). One of the samples from the *B. abortus* experimentally infected animals was used as the source material for generation of OPS affinity purified polyclonal anti-*Brucella* OPS antibodies as described above.

2.5.1.2. Pig sera

There were also five populations of pig sera evaluated within this study. These were as follows. There were 41 samples from individual swine that were positive to the Rose Bengal Test (RBT) and iELISA (Olsen, 2010) and from herds confirmed by culture to be infected with *B. suis* biovar 1, an A dominant OPS biovar (Meikle et al., 1989; Olsen, 2010). A further 21 serum samples from serologically positive pigs from herds confirmed by culture to be infected with *B. suis* biovar 2 were also evaluated. An additional 52 samples were tested which were collected from individual swine in Great Britain, officially free of *B. suis*, within herds from which one or more samples were positive in conventional serology such as RBT, cELISA, iELISA (Olsen, 2010) and where there was no epidemiological evidence of brucellosis. Four sera from pigs experimentally infected with *Y. enterocolitica* O:9, described previously (McGiven et al., 2012) were also examined. Conventional assays, RBT and cELISA were also applied to 161 samples from randomly selected individual pigs from within Great Britain.

2.5.2. Rose Bengal test

The Rose Bengal test (RBT), a conventional assay, was performed as described in the OIE 'Manual for Diagnostic Test and Vaccines for Terrestrial Animals' (Nielsen and Ewalt, 2010).

2.5.3. OPS iELISA for detection of antibodies in pig and cattle sera

B. abortus S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS was oxidised and conjugated to Carbo-BIND™ ELISA plates as described above at a final concentration of 0.25 µg/ml. Test sera and positive and negative controls were diluted 1/50 in casein buffer and added to the plates. The plates were incubated for 30 mins for pig sera and 60 mins for cattle sera then washed. For pig sera HRP conjugated goat anti-pig IgG Fc (Serotech, product #AAI41P) was diluted 1/2,500 in PBS-T and added to each well of the plate. For cattle sera HRP conjugated polyclonal rabbit anti-bovine IgG (Dako, product #P0159) was diluted in PBS-T at 1/4,000 and added to the plate. Incubation of conjugate was 30 mins for pig sera and 45 mins for cattle sera after which the plates were washed. The plates were developed with ABTS substrate and OD measured at 405 nm. Results for individual samples were determined relative to the positive control.

2.5.4. rLPS iELISA for detection of antibodies in pig and cattle sera

The rLPS iELISA for pig sera was performed as described recently (McGiven et al., 2012). The anti-rLPS antibody response from cattle sera was of comparatively low titre and was therefore amplified by use of streptavidin poly-HRP. The cattle sera were diluted 1/200 in casein buffer added to the wells of an ELISA plate precoated with rLPS. The plates were incubated for 30 mins and then washed. A biotinylated goat anti-bovine IgG conjugate (Jackson ImmunoResearch, product #101-065-003) was diluted 1/20,000 in casein buffer and added to each well. The plates were incubated and washed as described for the first incubation. The second conjugate, streptavidin poly-HRP was diluted to 0.05 µg/ml in casein buffer and added to each well. The plate was incubated and washed as for the first incubations and then developed with ABTS substrate and OD measured at 405 nm. The results were expressed as a percentage of a positive control sample.

2.5.5. Oxidised tetrasaccharide M antigen iELISA for detection of antibodies in cattle sera

The synthetic TSM antigen was oxidised at a final concentration of 500 µg/ml of TMS antigen and 2 mM sodium metaperiodate in 50 mM sodium acetate buffer pH 5.5 for 2 hrs 30 mins in the dark at 4°C. The resin from a Sephadex G-10 MiniTrap column (GE Healthcare, product #21-9180-10) was removed from its column and mixed with 3 mls of sodium acetate buffer pH 5.5 and 600 µl, containing 200 µl of Sephadex G-10 resin, was returned to the original column. The column was filled with sodium acetate buffer which passed through the resin to ensure all storage buffer had been eliminated. Then 400 µl of the oxidised antigen in sodium acetate buffer and residual sodium metaperiodate was added to the column. All the flow through was collected. An additional 600 µl of sodium acetate buffer at pH 5.5 was added to the column and the flow through was collected. The total volume of 1000 µl contained approximately 200 µg of oxidised TSM antigen.

The oxidised TSM antigen was diluted in sodium acetate buffer to 10 µg/ml and was added to Carbo-BIND™ ELISA plates. The plates were incubated in the dark for 3 hrs at 37°C then washed. Serum was tested as for the OPS iELISA (described above) except that the second incubation, with the anti-bovine HRP conjugate, was for 1 hr and conjugate was used at a 1/2,000 dilution.

2.5.6. Conjugate penta- and nonasaccharide iELISA for detection of antibodies in cattle sera

The two BSA conjugated synthetic D-Rha4NFo oligosaccharide antigens supplied by Professor D. Bundle (University of Alberta) were used to develop iELISAs for the detection of anti-*Brucella* antibodies in cattle sera. Each antigen was diluted to 2.5 µg/ml and coated to ELISA plates. The assay was then performed as described above for the oxidised TSM antigen iELISA except that both the serum and the conjugate incubation periods were 30 mins.

2.5.7 Statistical analysis of serological data

The results of the developed putative diagnostic OPS iELISAs were evaluated by Student's t test for unpaired samples. When the results for two assays were being compared, the results for each sample were expressed as a ratio of one result relative to the other. The populations compared were the ratiometric results for all samples derived from either the *Brucella* infected or non-*Brucella* infected animals. The hypothesis being tested was that there was a significant difference between the mean ratiometric results of the two populations under evaluation.

The OPS iELISAs were also compared against the iELISAs developed with the synthetic antigens. For each assay the maximum (optimal) Youden Index ($YI = \text{diagnostic sensitivity [DSn]} + \text{diagnostic specificity [DSp]} - 1$) was calculated. This was done with the assistance of GraphPad Prism 6 software. DSn (diagnostic sensitivity) and DSp (diagnostic specificity) were calculated according to standard convention (Jacobson, 1998). Receiver Operator Characteristics (ROC) analysis was also applied with the assistance of GraphPad Prism 6 software in order to plot the ROC curves and calculate the Area Under the Curve (AUC) and its 95% confidence interval. The testing for significant differences between AUC values was performed according to published methods (Hanley and McNeil, 1982, 1983).

2.6. Acid Hydrolysis of OPS

A variety of acid hydrolysis conditions were evaluated for their suitability to the production of fully formylated homo-D-Rha4NFO-oligosaccharides, this included testing on some non-OPS substrates such as mannan and maltotriose. These conditions were centered around incubation in 2 M trifluoroacetic acid (TFA) at 121°C or 10 M HCl at 40 or 60°C. TFA was removed from the sample by evaporation under a continuous flow of nitrogen. HCl hydrolysis was terminated by the addition of 10x volume of ice cold water followed swiftly by freezing at -80°C. The samples were then freeze dried overnight at -50°C at less than 100 µbar (using a Thermo ModulyoD Freeze Dryer) with an acid trap fitted to the exhaust. The final protocol used to generate the homo-D-Rha4NFO-oligosaccharides used for analysis by antibody affinity chromatography was as follows. Incubation in 200 µl of 10 M HCl per mg of OPS for 3 mins 45 secs at 60°C followed swiftly by addition of 10x vol of ice cold water and freezing at -80°C. This was followed by three rounds of freeze drying and reconstitution in water. This was required to eliminate all hydrolytic activity within the reconstituted sample. This activity was evaluated by incorporating a maltotriose standard and monitoring the production of maltose. A visible white fluffy product, attributable to the hydrolysed OPS, was not apparent in the sample until after the second round of freeze drying. Hydrolysis remained evident until the third round.

2.7. Modified Fluorophore Assisted Carbohydrate Electrophoresis (FACE)

Modified FACE was adapted from published methods (Buzzega et al., 2010; Oonuki et al., 2005) so that a wider mass range carbohydrates could be visualised. Carbohydrates were derivatised with 8-

aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS), a negatively charged fluorophore (Invitrogen, product #A-350). This was prepared to a 30 mM concentration in 15% glacial acetic acid and 25 µl of this added per mg of sample. The samples with ANTS were left to stand for 10 minutes and a fresh 1 M solution of sodium cyanoborohydride (Sigma, product #15,615-9) was prepared in DMSO (Sigma, product #D4540) which was added, after the ten minute period, to the sample at 25 µl per mg of carbohydrate. The samples were mixed and incubated in the dark at 37°C for 16 hrs.

The samples were diluted with 1.5 times volume of 40% aqueous glycerol prior to addition to the electrophoresis gel. The volume of sample plus aqueous glycerol added to the gel was 10 or 20 µl dependent on the likely abundance of the constituents within the sample. Agarose gels of between 1.5 and 3% (25 cm length) were used for electrophoresis dependent upon the nature of the separation desired. Electrophoresis was performed at 150 volts for between 2 to 4 hrs at 4°C (in order to prevent overheating). The fluorescence of the ANTS conjugates within the gels was captured using a UV transilluminator (peak excitation at 350-360 nm) and digital camera with a Tiffen Yellow 12 (> 500 nm) filter to capture maximal emission (540-550 nm).

2.8. Mass Spectrometry

2.8.1. Gas Chromatography Electron Impact Mass Spectrometry analysis of OPS

The OPS antigen from *B. melitensis* 16M OPS and selected carbohydrate standards, including an O-linked oligosaccharide control (Lacto-N-hexaose [3x Gal, 2x GlcNAc, 1x Glc]) were subjected to acid hydrolysis using 0.5 and 2 M trifluoroacetic acid (TFA) (Hitchen et al., 2002) after which the samples were reduced and derivatised to alditol acetates (Hitchen et al., 2002). These derivatives were cleaned using chloroform and water and the chloroform phase dried under nitrogen then diluted in hexanes.

Incubation of the OPS and carbohydrate standards with 100 µl of 98% trifluoromethanesulfonic acid (Sigma, product #347817) at room temperature and 4°C for 18 hrs was also performed to attempt depolymerisation through solvolysis (Knirel and Perepelov, 2002). The reaction was neutralised with ammonia and the samples were dried under nitrogen. The samples were then reduced and derivatised into alditol acetates as referenced above.

GC-MS analysis was carried out on a Perkin Elmer Clarus 500 GC-MS Instrument. The samples were injected on a RTX-5MS fused silica capillary column (30mx0.25mm internal diameter). The oven was held at 60°C for 1min and increased to 190°C at 20°C min⁻¹, from where the temperature was increased to 230°C at 1°C min⁻¹. The final temperature increment was to 300°C raised at 25°C min⁻¹ and held for a total of 5mins.

2.8.2. MALDI-ToF

2.8.2.1 Permethylation of OPS

Permethylation of OPS was carried out in a microcolumn format (Mechref et al., 2009). Briefly, sodium hydroxide beads were packed into 1 ml solid phase extraction columns with frits to a depth of 3 cm and 300 µl of acetonitrile was added. The columns were centrifuged at 100 g for 1 min and washed 3 times with DMSO to replace the acetonitrile and condition the column. The OPS samples were dissolved in DMSO at 1 mg/ml with 0.4% H₂O and 100 µl of this was mixed with 44 µl of iodomethane. This mix was applied to the columns and the gravity fed flow throughs were collected. A further 150 µl of DMSO was applied to the each of the columns and allowed to flow through under gravity. These were collected and passed through the columns five more times and then added to the first samples. The columns were then washed twice with 100 µl of DMSO which was also collected and added to the samples. The samples were then mixed with 400 µl of chloroform and 400 µl of water and the aqueous layer discarded. The chloroform layer was washed a further three times with water. The retained samples were dried under nitrogen and re-dissolved in 100 µl of 50% methanol containing 1 mM sodium acetate.

2.8.2.2. MALDI-ToF of native and permethylated hydrolysed and non-hydrolysed OPS

MALDI-ToF was conducted on OPS (permethylated and non-permethylated) using 2 µl of 20 mg/ml DHB matrix (2,5-Dihydroxybenzoic acid) in 50% methanol and 1 mM sodium acetate per spot on the source plate. This was allowed to dry before adding 2 µl of sample to the spot. The OPS samples were added at a concentration of 100 mg/ml. Where the OPS was processed prior to MALDI-ToF, for example by permethylation, acid hydrolysis or both, the concentration of OPS is based on the assumption that there was no loss (or gain) of material during these processes. MALDI-ToF was

performed once using a Voyager-DE STR but subsequently using a Bruker Autoflex II in linear and reflection modes with and without matrix suppression (as described in the results section). Data was analysed using FlexControl software (Bruker, version 3.3).

2.8.3. HPLC and HPLC-ESI-MS

2.8.3.1 Size Exclusion HPLC-ESI-QqQ of *B. abortus* S99 and *E. hermannii* OPS

The OPS from *B. abortus* S99 and *E. hermannii* was evaluated by SEC HPLC using a Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column (7.8 mm internal diameter x 30 cm length) plus matching guard column (6 mm internal diameter x 4 cm) (Tosoh, product #21873). HPLC separation was performed using an Agilent Infinity Bioinert 1200 System. UV absorbance was measured at 210, 230 and 280 nm. The volume of OPS from *B. abortus* S99 and *E. hermannii* applied was 50 µl at 1 mg/ml. The mobile phase was 60% 20 µM NaCl (aqueous) and 40% methanol with a 0.8 ml/min flow rate. The SEC was interfaced with an Agilent 6410 Triple Quadrupole (QqQ) mass spectrometer via a splitter which delivered the mobile phase to the QqQ at a flow rate of 17 µl/min. The *B. abortus* S99 fraction eluting from the SEC column between 8.5 and 10.5 minutes was retained for further analysis. This collection process was reiterated 4 times to increase the quantity of material collected in this fraction. Analysis of data from the HPLC system was performed using ChemStation software version B.04.03 (Agilent). Analysis of data from the ESI-QqQ was performed using Mass Hunter WorkStation version B.02.01 (Agilent).

2.8.3.2. Reverse Phase HPLC-ESI-QqToF of *B. abortus* S99

The *B. abortus* S99 SEC purified OPS from the 8.5 to 10.5 minute fraction (see section 2.8.3.1. above) was evaluated by reverse phase HPLC SEC using a wide bore C₁₈ column (Vydac, product #218TP) with a 4.6 mm internal diameter and 25 cm length plus matching guard column (6 mm internal diameter and 4 cm length). Reverse phase HPLC separation was performed using an Agilent Infinity Bioinert 1200 System. UV absorbance was measured at 210, 230 and 280 nm. The volume of OPS from *B. abortus* S99 applied was 10 µl at 1 mg/ml. The mobile phase was: 20 µM NaI (aqueous) and 40% methanol with a 0.6 ml/min flow rate. The gradient was increased from 5% to 80% methanol linearly from 0 to 20 mins and at 80% until 24 mins. The C₁₈ column was interfaced with an Agilent 6520 Quadrupole Time of Flight (Q-ToF) mass spectrometer via a splitter which

delivered the mobile phase at 13 µl/min. Analysis of data from the ESI-QqToF was performed using Mass Hunter WorkStation version B.04.00 (Agilent).

2.8.3.3. SEC-HPLC of HCl acid hydrolysed *Brucella* and *Y. enterocolitica* O:9 OPS

The acid hydrolysed (10 M HCl, 3 mins, 45 secs, 60°C) OPS from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS was fractionated using the Tosoh SEC columns and HPLC system described above (see section 2.8.3.1.) The volume of hydrolysed OPS applied was 50 µl at 4 mg/ml. The mobile phase was 100% water with a flow rate of 1 ml/min. UV absorbance was measured at 210, 230 and 280 nm and 0.5 ml fractions were collected from 1 to 12 minutes. This process was reiterated to fractionate all of the hydrolysed OPS (from each source) and the fractions from each run with common source material and elution time were pooled together.

2.8.3.4. Graphitised carbon column HPLC-ESI-MS of hydrolysed and intact OPS and tetrasaccharide M antigen

The pooled SEC fractions of hydrolysed OPS were freeze dried and reconstituted in 50 µl of water for subsequent use and for analysis by ESI-MS (QqQ and QqToF) using the internal HPCL Chip Cube with a graphitised carbon (GC) column (Agilent, product #G4240-62003). The volume of samples applied was 0.5 µl and these were run in a mobile phase of 5 mM ammonium formate increasing in linear fashion from 0% to 54% acetonitrile from 0 to 20 mins with flow rate of 3 µl/min. This was also the method used to evaluate the oligosaccharides eluted from the antibody conjugated AminoLink® affinity chromatography columns. Native OPS from *B. abortus* S99 was also evaluated by this method with injection of 3 µl of 10 mg/ml OPS into the GC column. The synthetic D-Rha4NFO tetrasaccharide antigen was analysed by using the GC column chip interfaced with the ESI-QToF and a mobile phase of 0.1% formic acid increasing to 0.1% formic acid in 90% acetonitrile over an 18 minute period. Collision induced dissociation (CID) for both QqQ and QqToF was performed on selected ions related to the D-Rha4NFO tetrasaccharide by collision with nitrogen in the second quadrupole chamber.

Chapter 3

NATIVE ANTIGEN PRODUCTION, EVALUATION AND IMMUNOASSAY

3.1. INTRODUCTION

The concept of the project is that epitopes that exist on the *Brucella* OPS, that are, as far as currently known, unique to this organism can be used in serology to improve the specificity of diagnosis and aid in the resolution of FPSRs. To investigate this hypothesis would require the identification and generation of the discrete forms of the specific epitopes and their evaluation in immunoassay. This study would be time consuming and relatively high risk. The objective of the work described in this chapter is to find further evidence that supports this concept and thus gain a better understanding of the likelihood of success that subsequent investigation may bring and so determine whether such a pursuit is a realistic undertaking.

The specific aim of the work described in this chapter is to investigate the antibody response to the D-Rha4Nfo homopolymer that comprises the majority (as non-repeating elements at the reducing end are of different composition) of the OPS from *Brucella* (A and M type) and *Yersinia enterocolitica* O:9. In particular to evaluate whether the magnitude of the response of antibodies within the sera of cattle and pig, experimentally and naturally infected, differs between OPS antigens and the aetiological agents responsible for antibody induction. The hypothesis is that the magnitude of the antibody response to the *Brucella* OPS antigens is greater for true positives than for false positives as compared to their response against the OPS from *Y. enterocolitica* O:9. This may be more readily encapsulated by appreciating the antibody response against the different antigens as a ratio of the response of the *Brucella* and *Y. enterocolitica* OPS antigens. It is hypothesised that this 'B/Y' ratio will be greater for true positives than for false positives. If true, this will confirm that anti-OPS antibodies raised do not bind uniformly to the different OPS types. This would imply that the different structural features within these antigens are recognised by antibodies within a polyclonal population within natural hosts. Both A dominant (*B. abortus* S99) and M dominant (*B. melitensis* 16M) will be evaluated alongside the *Y. enterocolitica* O:9 OPS.

3.2. Antigen Production

Bacteria were grown and harvested under the conditions described in the materials and methods section. Antigen production was as described in the methods following the hot-phenol extraction method for production of sLPS antigen (Westphal et al., 1952) and the phenol, chloroform, petroleum ether method for production of rLPS antigen (Galanos et al., 1972; Nielsen et al., 2006b). The OPS

was separated from the sLPS by mild acid hydrolysis. This is made possible due to the particularly acid sensitive linkage of α -3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues to adjoining sugars within the LPS core (Caroff and Karibian, 2003; Meikle et al., 1989). The OPS was purified by centrifugation and de-salting.

3.3. Chemical analysis of extracted antigens

3.3.1 Quantification of amino sugars, Morgan-Elson and Elson-Morgan

The sLPS and OPS antigens extracted were primarily quantified by dry mass and antibody titre. Evaluation of carbohydrate content by chemical means did not allow for an accurate determination of OPS content. Mainstream assays for the determination of amino sugars such as the Morgan-Elson (Morgan and Elson, 1934) and the Elson-Morgan assays (Elson and Morgan, 1933) are only applicable for the determination of sugars aminated on the 2nd carbon – whereas D-Rha4Nfo is aminated on the 4th. In any event, these assays apply to reducing sugars – excluding all but one of the sugars within a polysaccharide.

3.3.2. Phenol Sulfuric acid assay for quantification of total carbohydrate

A commonly used and simple assay for measuring total carbohydrate content is the phenol-sulfuric acid method (Dubois et al., 1956) and adaptations for use in microtitre format have been published (Masuko et al., 2005). The sulfuric acid hydrolyses existent carbohydrates and then generates furfuranol via reaction between the 2nd and 5th carbon on the sugar ring. This reacts with the phenol to create a coloured reagent that can be measured by UV absorbance. The nature of this reaction is the reason why it may be unsatisfactory for the determination of some deoxy and aminated sugars, many of which are aminated on the 2nd carbon. Theoretically the phenol-sulfuric acid assay should work with the OPS from *Brucella* and *Y. enterocolitica* O:9. The results (not shown) of phenol-sulfuric assay on the antigens extracted within this project were insufficiently reproducible to be reliable for quantification. A major reason for this could be the poorly characterised nature of the acid hydrolysis reaction for this polymer. However it is noted that this assay has been successfully applied, with the use and help of a synthetic standard, by others for the quantification of D-Rha4Nfo polymers (Iwashiki et al., 2012).

3.3.3. Bicinchoninic acid assay for reducing sugars

Bicinchoninic acid (BCA) is better known as a reagent used for the quantification of proteins however it is also very sensitive to the presence of reducing sugars (Waffenschmidt and Jaenicke, 1987) including aminated sugars derived from chitin (Doner and Irwin, 1992). This feature was investigated with the extracted *B. abortus* OPS antigen and monosaccharide standards. Hydrolysis with 2 M HCl at 100°C did result in an increased response to this assay suggesting that some reducing sugars are being released. This data is shown in figure 3.1. The results suggest that the OPS is being hydrolysed and that liberated reducing sugars are being detected. However, there are some anomalies in the data, chief among them the increasing values over time for mannose and glucose (although the values for GlcN and GlcNAc remained relatively constant). This discrepancy and the requirement that quantification of OPS required prolonged incubation in hot HCl counted against the method as a means of OPS quantification. Furthermore, D-Rha4Nfo will not be the only carbohydrate component in the antigen. Other sugars will be present including, at the very least those relating to the non-repeating section at the reducing end of the OPS and probably also sugars from the core. The degree to which these other carbohydrates make up the antigen content will sway the results of any chemical analysis and this would create uncertainty when comparisons are made between quantitative data from *Brucella* and *Y. enterocolitica* O:9 antigens.

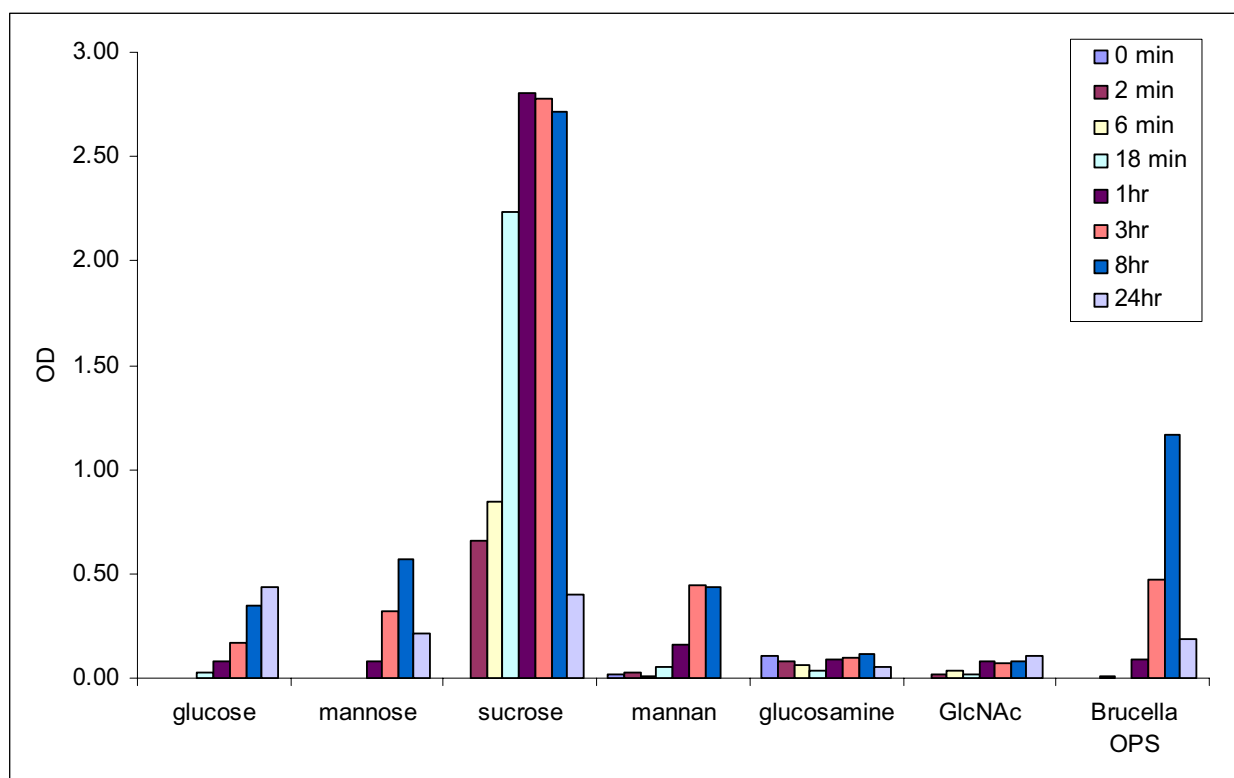


Figure 3.1. Quantification of carbohydrates by the BCA assay

Results for the BCA assay (OD, y-axis) for different mono and polysaccharides (x-axis) after different time periods of incubation in 2 M HCl at 100°C (shown as different bars for each saccharide).

3.3.4. Bradford (Coomassie) assay for quantification of total protein

The purity of the sLPS antigen preparations was examined using the Bradford (Coomassie) protein assay. The level of protein detected in by this assay ranged from 2.8 to 6.8 percent of the total dry mass of the hot-phenol extracted sLPS antigen. This was not considered to be significant at the antibody immunoassay level. Given that the effective concentration of the sLPS as an antigen in serodiagnostic ELISA is approximately 0.2 µg/ml, then, if approximately 5% of this may be protein this would have a concentration of approximately 0.01 µg/ml of protein. Further, given the relative ineffectualness of proteins for the detection of antibody responses to infection with brucellosis (McGiven, 2013) the level of protein contamination was considered to be serologically insignificant. The level of protein in the OPS preparations (after PD-10 desalting), as determined by the Bradford assay was from 5.4 to 8.6% of the total dry mass. It would thus appear that the OPS separation assay applied was not effective at reducing the level of protein contamination in the sample. In fact,

the removal of other components from the sLPS preparation had the effect of increasing the overall concentration of protein.

3.4. Analysis of sLPS and OPS antigens by SDS-PAGE

3.4.1 SDS-PAGE: Protein (Coomassie) stain

The antigens were also evaluated by SDS PAGE with proteins stained using Imperial blue, a modified formulation of the Coomassie reagent. The results, shown in figure 3.2, demonstrate that much of the protein from the sLPS antigens is presented as a broad smear within the gel that ranges from approximately 50 to 30 kDa against the protein ladder. This is typical of the range over which the sLPS migrates (Garin-Bastuji et al., 1990a) and corresponds to the results from Western blot with polyclonal anti-*Brucella* sera (results not shown). In some cases specific bands can be seen that would relate to proteins of specific molecular weight although these are not universal between antigens

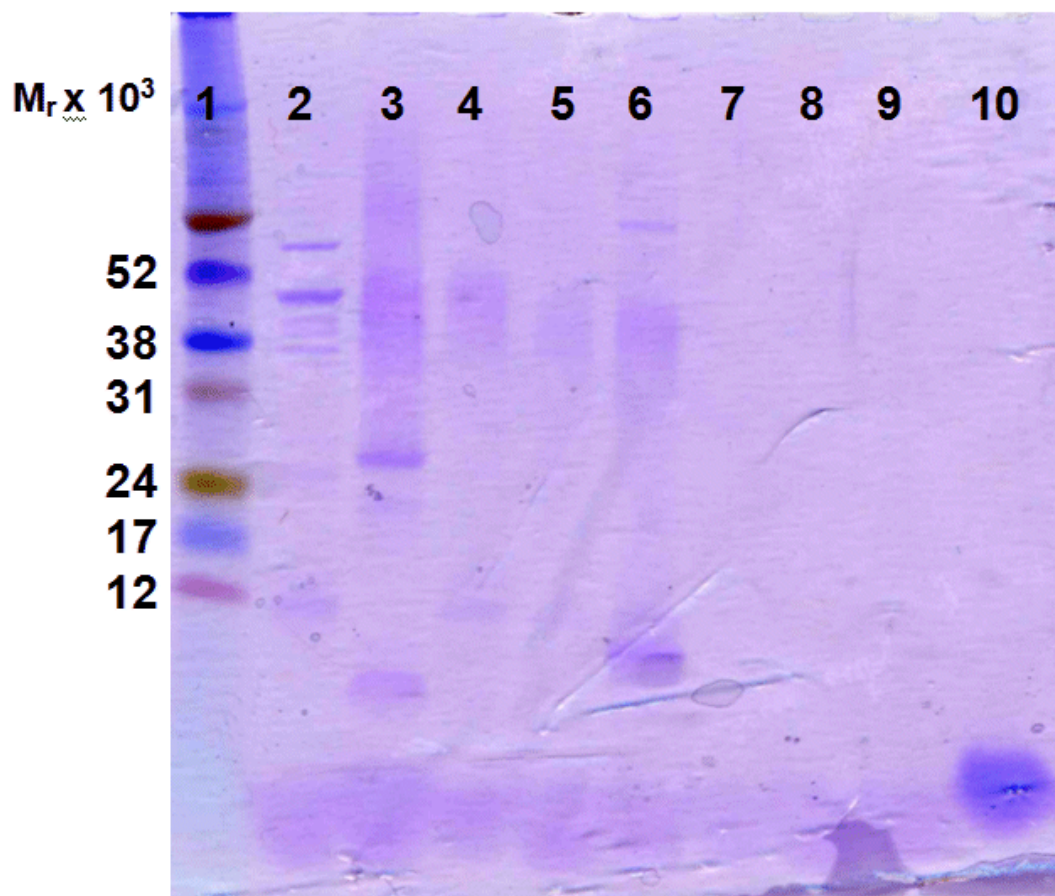


Figure 3.2. Antigen evaluation by SDS-PAGE and Coomassie stain

Imperial blue (Coomassie) stained SDS PAGE containing: lane 1 – rainbow protein marker, lane 2 – *B. melitensis* cytoplasmic protein extract (Brucellergene™) 80 µg, lane 3 – *B. abortus* S99 sLPS 50 µg, lane 4 – *B. melitensis* 16M sLPS 10 µg, lane 5 – *B. abortus* S99 sLPS 3 µg, lane 6 – *Y. enterocolitica* O:9 sLPS 12 µg, lanes 7, 8, 9 – *B. abortus* S99 OPS 1.5 µg, lane 10 – *B. abortus* RB51 (rough strain) 10 µg.

3.4.2. SDS-PAGE: Protein (Silver) stain

The results from the Silver stained SDS PAGE where the antigen concentrations have been equalized on the basis of mass are shown in figure 3.3. The image shows a dark smear between 50 and 30 kDa for the sLPS but not the OPS antigens. The protein contaminants are visible as relatively sharp bands within the lanes. The proteins visible in the *Brucella* and *Y. enterocolitica* O:9 antigens above 70 kDa appear very similar. Below that, differences are visible including faint bands at approximately 35 kDa in the *Brucella* sLPS antigens. There are also different banding patterns between the *Brucella* and *Y. enterocolitica* O:9 sLPS antigens below 24 kDa. The OPS antigens do

not have any visible stains below 55 kDa. As carbohydrates are not stained by this method, pure OPS would not be visible and proteins that were attached to the rough LPS would have been removed. Centrifugation and desalting of the sLPS on its own (lane 10) is insufficient to remove such proteins in the absence of hydrolysis. The images from lanes 7-8 showing the protein content from the variably acidified, heated, centrifuged and desalted sLPS antigens show that the combination of acetic acid and heat is required to obtain the distinct profile for the OPS antigens.

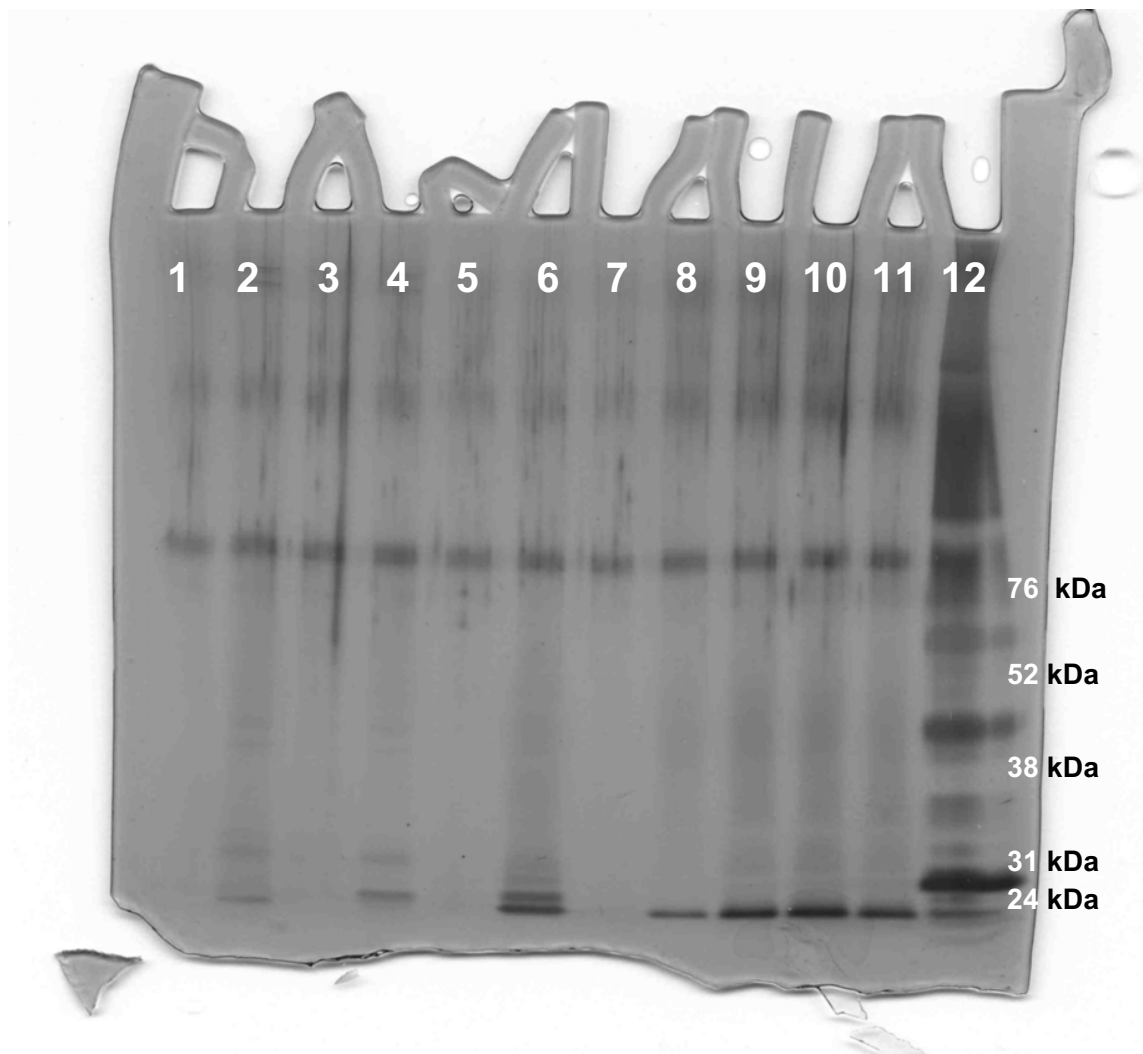


Figure 3.3. Antigen evaluation by SDS-PAGE and Silver stain

A Silver stained SDS PAGE gel showing, 2.75 µg per lane per antigen, of: *B. melitensis* 16M OPS (lane 1) and sLPS (lane 2), *B. abortus* S99 OPS (lane 2) and sLPS (lane 4), *Y. enterocolitica* O:9 OPS (lane 5) and sLPS (lane 6). Lanes 7 – 11 show antigens from various regimes of OPS extraction. Lane 7 contains *B. abortus* sLPS/OPS antigen prepared in 1% acetic acid and heated at 110°C for 140 mins then centrifuged and desalted. Lane 8 as lane 7 but without acetic acid. Lane 9 as lane 7 but at 4°C for 140 mins. Lane 10 contains *B. abortus* sLPS after centrifugation and desalting. Lane 11 contains *B. abortus* S99 sLPS without additional processing. Lane 12 contains molecular weight markers.

3.4.3. SDS-PAGE: Carbohydrate conjugation and fluorescence

The sLPS was also evaluated by SDS PAGE using an LPS gel stain kit ('Pro-Q® Emerald 300': Molecular probes) which is specific for carbohydrates. Once *in situ* within the gel susceptible carbohydrates are oxidised by periodate allowing the subsequent conjugation of a fluorescent marker – the Pro-Q® Emerald 300 dye which is visible using a UV transilluminator. The carbohydrate within the sLPS antigens appear as smears within the gel highly reminiscent of the major feature of the sLPS protein stain. The OPS antigens have a very different pattern whereby the majority of the carbohydrate remains close to the sample addition well. SDS is a negatively charged surfactant that binds to the hydrophobic portions of proteins and unravels them causing them to migrate to the positively charged anode. SDS will also bind LPS by virtue of the hydrophobic lipid A causing the molecule to migrate through the acrylamide gel. Binding to the OPS portion will be substantially reduced and thus for its mass it is probable that the sLPS will have a lower charge than the equivalently sized protein. A direct comparison of size between sLPS and proteins on a gel is therefore not justified. Purified OPS has completely lost the lipid A and consequently binding to SDS will be substantially reduced, if not eliminated, as will the migration towards the anode. Therefore, the SDS PAGE approach does not provide a means to evaluate the size distribution of the purified OPS. Nonetheless, the results from the SDS PAGE do, somewhat serendipitously, demonstrate that separation of the OPS from the lipid A during the extraction process has been successful.

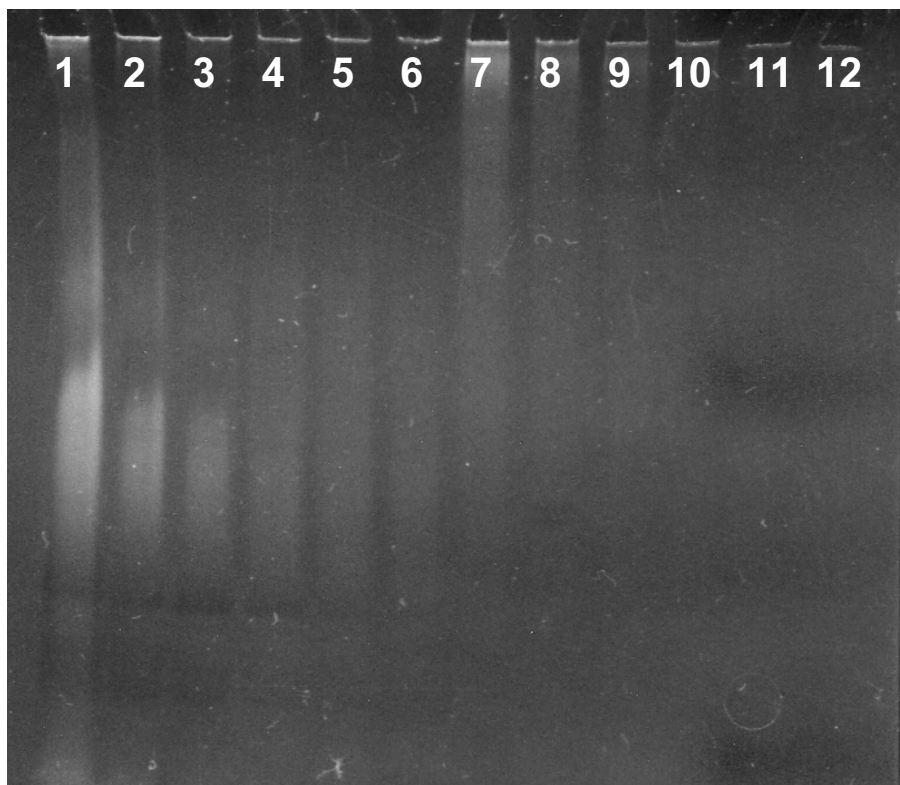


Figure 3.4. Antigen evaluation by SDS-PAGE and carbohydrate stain

A periodate oxidised and Pro-Q® Emerald 300 (Invitrogen) stained SDS-PAGE gel containing: Lanes 1-6: 32, 16, 8, 4, 2 & 1 µg *B. abortus*, lanes 7-12: 32, 16, 8, 4, 2 & 1 µg *B. abortus* S99 OPS.

3.5. Analysis of sLPS and OPS antigens by endotoxin (Limulus Amebocyte Lysate) assay

The antigens were also evaluated for the presence of endotoxin by Limulus amebocyte lysate (LAL) assay using recombinant factor C (Ding and Ho, 2001). This detects the presence of the lipid A moiety and even though this antigen has unusually low toxicity in *Brucella* (Moreno et al., 1981b) it is readily detectable by LAL assay. The results, shown in figure 3.5, demonstrate that concentrations of sLPS antigens that were less than 0.1 µg/ml had higher RFU values than OPS concentrations in excess of 100 µg/ml. Therefore there has been at least a 1000 fold reduction the endotoxin content, as determined by LAL, as a consequence of the OPS separation and extraction process verifying that this has been successful.

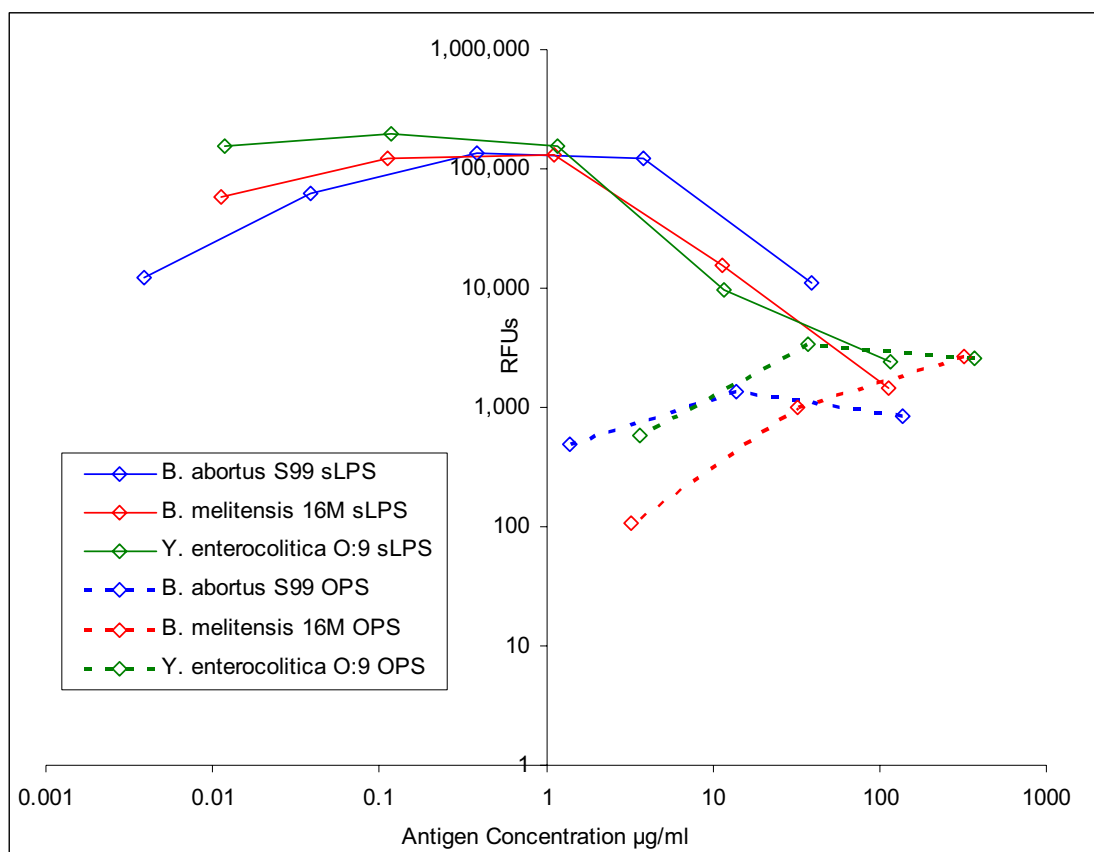


Figure 3.5. Limulus Amebocyte Lysate (LAL) recombinant factor C Assay for sLPS and OPS

LAL assay showing antigen concentration on the x-axis and RFUs on the y-axis. The results, in RFUs are shown for: OPS (dotted lines) and sLPS (solid lines) from *B. abortus* (blue lines), *B. melitensis* 16M (red lines) and *Y. enterocolitica* O:9 (green lines)

3.6. Analysis of sLPS and OPS antigens by UV absorbance

The antigens were also evaluated by UV absorbance as shown in figure 3.6. This shows that purified OPS absorbs very little light above 220 nm. It was also found (data not shown) that absorbance at 210 nm was linearly dependent on the concentration (µg/ml) of antigen. This may be due to the presence of the aldehyde like group within the N-formamido on carbon 4 of the D-Rha4NFo OPS unit. Therefore, despite the lower overall absorbance values for the OPS compared to the sLPS it is possible to conclude that the decrease in absorbance to background levels above 240 nm is a feature of antigen type rather than it's concentration. The absorbance curves for the sLPS antigens are similar but display a 'hump' at 230 nm that may be due to the presence of proteins. This is not shared by the OPS antigens and the absorbance for sLPS antigens does not decrease to the

baseline. This absorbance profile proved to be a useful tool to monitor the progression of the mild acid hydrolysis reaction.

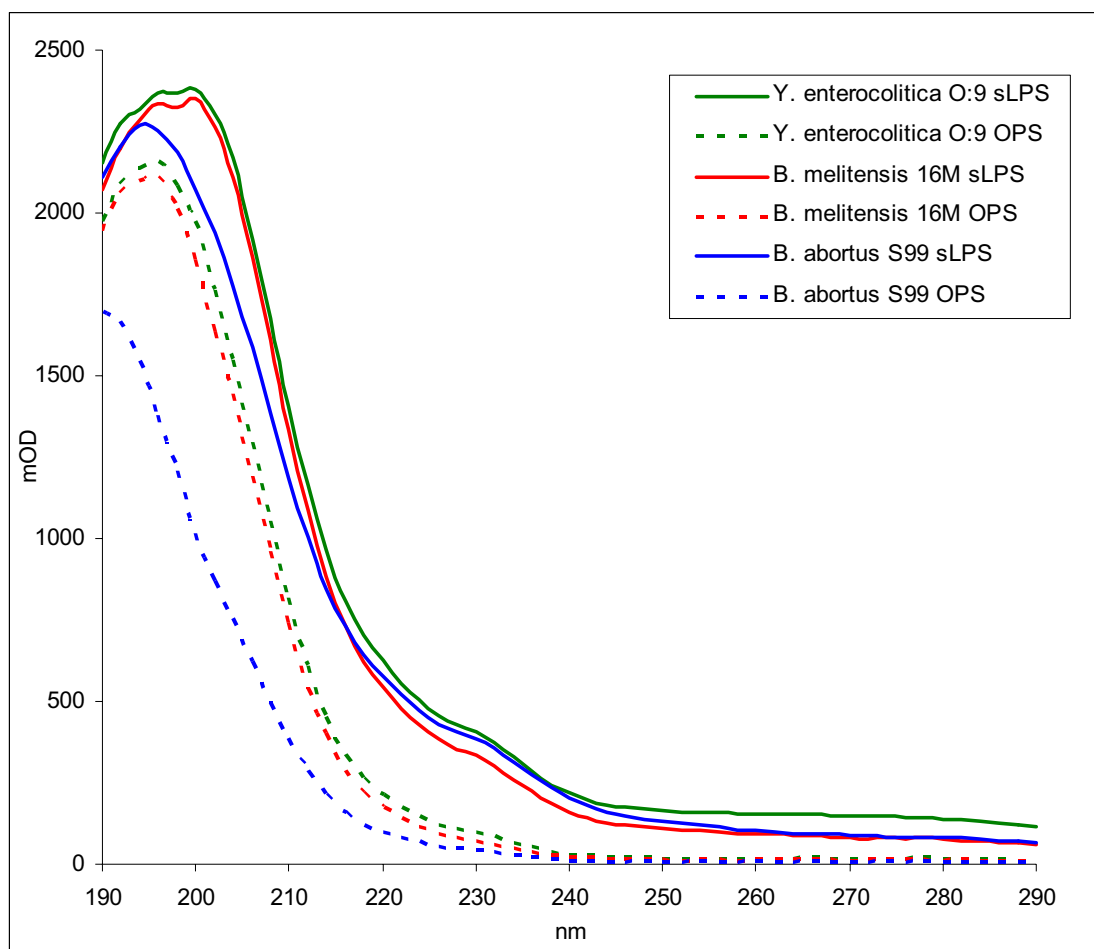


Figure 3.6. UV absorbance of OPS and sLPS antigens

A line graph showing the absorbance (mAU, on the y-axis) at different wavelengths (in nm) as shown on the x-axis. The solid lines show the absorbance profiles for sLPS and the broken lines the profiles for OPS. The results for the different bacteria are shown as: green line *Y. enterocolitica* O:9, red line *B. melitensis* 16M and blue line *B. abortus* S99.

3.7. Analysis of sLPS and OPS antigens by polyclonal and mAbs

3.7.1. Analysis of polyclonal anti-*Brucella* antibody and sLPS antigen binding by iELISA

Given the difficulties in defining a chemical assay to quantify sLPS and OPS content within antigens and antigen batches the pragmatic way forward was to evaluate antigens according to their antibody

titre, dry mass and protein content. Given that the crux of the project relates to the reactivity of the antibodies against the extracted antigens and that comparisons of titre could be relative there was no absolute requirement for the accurate quantification of pure OPS.

The hot-phenol extracted sLPS antigens from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 gave similar titres in iELISA immunoassay when probed with bovine polyclonal antisera raised against infection with *B. abortus* strain 544 (A type OPS). The iELISA used for determination of antigen titre was similar in terms of reagents and reagent concentrations used to that applied for the routine serodiagnosis of cattle sera (Thompson et al., 2009). A concentration of approximately 0.2 µg/ml of sLPS gave a good signal in iELISA with the positive sera (and a low signal with negative sera) with the variation between the antigen concentrations, of the different types, that give equivalent antibody signals of less than 10% of the dry mass. As a means of quantification, the antibody titres are informative but not absolute. Without a pure standard the results may produce relative data that is useful but remains somewhat arbitrary and the (hypothesised) possibility that anti-OPS antibodies may not react against all D-Rha4Nfo OPS types in a uniform manner poses a question about the precise nature of the relativity.

3.7.2. Analysis of anti-*Brucella* mAb and sLPS antigen binding by iELISA

The sLPS antigens were evaluated against mAbs by iELISA. The OPS did not bind to standard polystyrene ELISA plates owing to its hydrophilic and uncharged nature so these were evaluated against BM40 mAb by cELISA. Binding by four mAbs was investigated: BM40 (previously reported as an M specific (Greiser-Wilke and Moennig, 1987; Greiser-Wilke et al., 1985)), 12G12 (previously reported as C specific (Weynants et al., 1997)), 12B12 (previously reported as C,M >A (Cloeckaert et al., 1993)), and HRP conjugated 4B5A (previously reported as C specific (Portanti et al., 2006)). The mAbs 12B12 and 12G12 were generous gifts from Professor A. Cloeckaert. The mAb BM40 is held at AHVLA under licence with the Hannover University of Veterinary Medicine. These mAbs were purified from ascites (12B12 and 12G12) and hybridoma supernatant (BM40) by protein-G affinity chromatography and quantified by UV 280 nm spectroscopy. The mAb 4B5a was a generous gift from Dr M. Tittarelli and arrived pre-conjugated with HRP but unquantified.

The unlabelled mAbs BM40, 12B12 and 12G12 were tested by sLPS ELISA at varied concentrations against three different sLPS antigens at equal concentrations. Figure 3.7 shows the binding efficacy

of mAb BM40 for sLPS is *B. melitensis* 16M > *B. abortus* S99 > *Y. enterocolitica* O:9 and confirms the reported specificity for this mAb as M specific with minimal attraction to *Y. enterocolitica* O:9 sLPS unless at high concentrations – higher than used here. The results for mAb 12B12, shown in figure 3.8, demonstrate a similar ranking of avidity but of a lower magnitude and a lower degree of stringency as reaction against the *Y. enterocolitica* O:9 sLPS is evident. The results for the binding of mAb 12G12 are shown in figure 3.9. The ranking of binding efficacy is the same as the BM40 and 12B12 mAbs: *B. melitensis* 16M > *B. abortus* S99 > *Y. enterocolitica* O:9. However 12G12 does show less selectivity for the *Brucella* sLPS and less avidity. The binding of mAb 4B5A was equal against all antigens (data not shown).

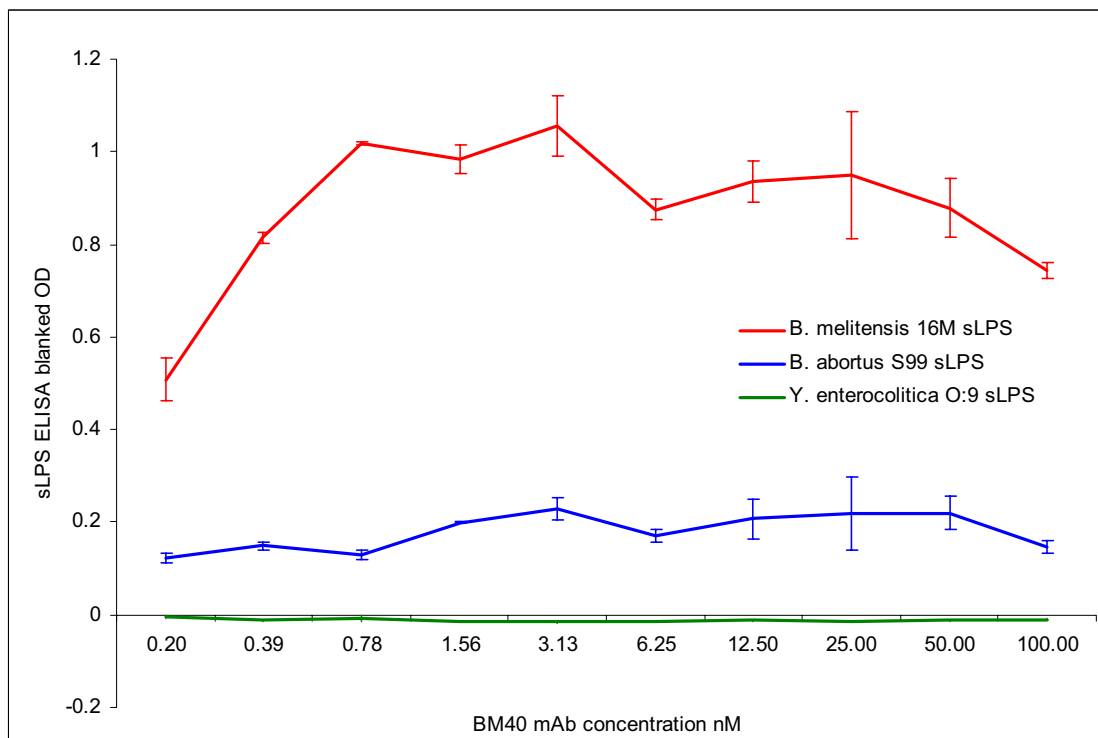


Figure 3.7. The binding profile of BM40 mAb against *Brucella* and *Y. enterocolitica* O:9 sLPS

The binding of BM40 mAb (anti-M epitope) at different concentrations (nM, x-axis) against *B. melitensis* 16M (red line), *B. abortus* S99 (blue line) and *Y. enterocolitica* O:9 (green line) sLPS antigens as measured by iELISA (blanked OD, y-axis). The lines are drawn through the average of two replicates, the maximum and minimum of which is shown by the error bars.

The mAb binding data re-affirms that differences within the structures of the OPS (although binding against sLPS was evaluated here, the mAbs have previously been reported to be specific to OPS) from *B. melitensis* 16M, *B. abortus* S99 and *Y. enterocolitica* O:9 have a readily detectable impact on antibody binding. Each mAb shows a different binding profile which is consistent with the variable

proportion of M epitopes on each of the antigens and variable stringency of the mAbs. BM40 mAb in particular being very specific to *Brucella* antigens. The data presented here does not support the existence of a C specific epitope. Both mAbs with previously reported specificity to this hypothetical structure bound *Y. enterocolitica* O:9. In the case of mAb 12G12 the binding profile would more readily be explained by preferential binding to the M epitope. In the case of the 4B5A antigen the binding profile would appear to match that of an antibody with preference for a C/Y (common, non-specific epitope).

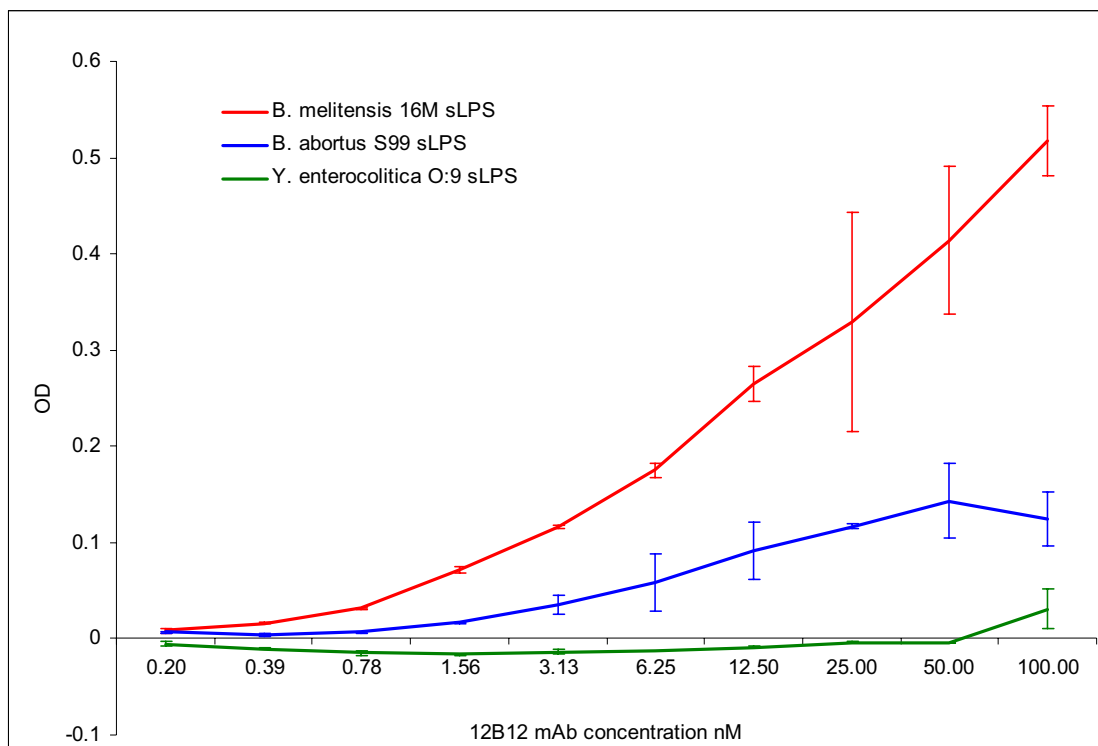


Figure 3.8. The binding profile of 12B12 mAb against *Brucella* and *Y. enterocolitica* O:9 sLPS

The binding of 12B12 mAb (anti-M epitope) at different concentrations (nM, x-axis) against *B. melitensis* 16M (red line), *B. abortus* S99 (blue line) and *Y. enterocolitica* O:9 (green line) sLPS antigens as measured by iELISA (blanked OD, x-axis). The lines are drawn through the average of two replicates, the maximum and minimum of which is shown by the error bars.

These results are not in complete agreement with published material and this may be due to differences in the exact circumstances within which the antibodies were evaluated (Cloeckert et al., 1998). However, the data presented here is more readily explained by the existing knowledge of the OPS structure. However it is noted that the existence of non-D-Rha4NFO sugars on the OPS (Kubler-Kielb and Vinogradov, 2013b) may give rise to a raft of alternative epitopes, some of which may be specific to *Brucella* and present in equal measure in all significant smooth strains. Epitopes that

overlap the junction between the OPS and the core may also have such properties. However both these epitopes are likely to be sequestered under the non-reducing portion of the OPS and less exposed to the immune system.

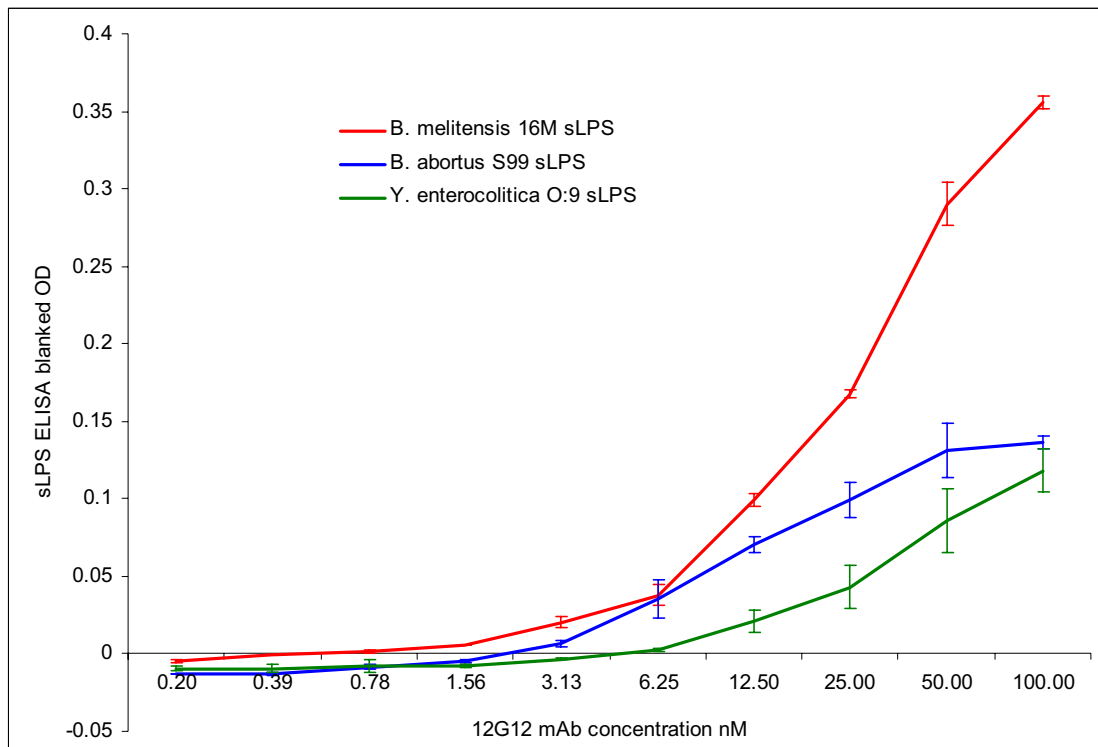


Figure 3.9. The binding profile of 12G12 mAb against *Brucella* and *Y. enterocolitica* O:9 sLPS
The binding of 12G12 mAb (anti-C epitope) at different concentrations (nM, x-axis) against *B. melitensis* 16M (red line), *B. abortus* S99 (blue line) and *Y. enterocolitica* O:9 (green line) sLPS antigens as measured by iELISA (blanked OD, y-axis). The lines are drawn through the average of two replicates, the maximum and minimum of which is shown by the error bars.

3.7.3 Analysis of anti-*Brucella* mAb and OPS antigen binding by cELISA

A cELISA that is typically used for the detection of antibodies against *Brucella* OPS antigen (Perrett et al., 2010) was used to detect OPS antigen in the liquid phase, rather than antibody. This was relatively straightforward as either antigen or antibody will compete with the liquid phase HRP-conjugated mAb (BM40) for binding to the *B. melitensis* 16M sLPS adsorbed to the surface of the ELISA plate. The OPS antigens were evaluated at a range of concentrations for their ability to inhibit the binding between the labelled mAb and the solid phase bound antigen and the results are shown in figure 3.10. The data shows that the order of inhibition is *B. melitensis* 16M > *B. abortus* S99 > *Y.*

enterocolitica O:9 OPS. This confirms the observations made using iELISA and thus further emphasises the specificity of this mAb, BM40, to elements within the OPS that are not shared with *Y. enterocolitica* O:9 and more prevalent in *B. melitensis* 16M than in *B. abortus* S99.

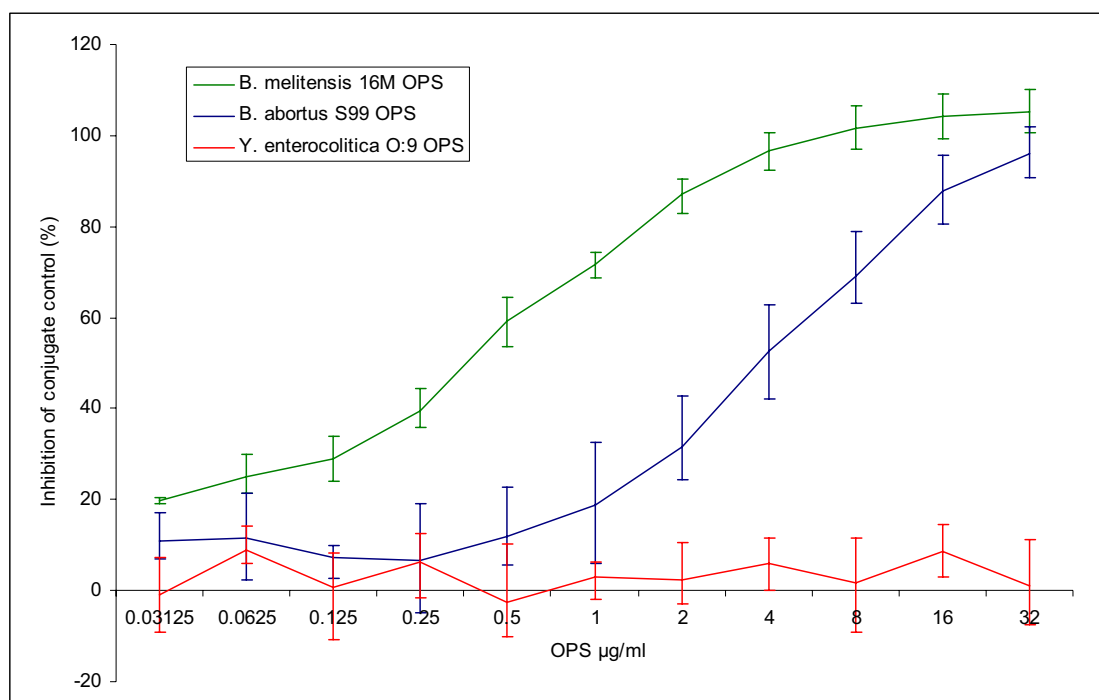


Figure 3.10. BM40 mAb cELISA with *Brucella* and *Y. enterocolitica* O:9 OPS

The results of a competitive ELISA using *B. melitensis* 16M OPS coated plates and an anti-M epitope mAb (BM40) showing the inhibitory effects on optical density (OD, y-axis) of different quantities of native M dominant (*B. melitensis* 16M), A dominant (*B. abortus* S99) and A exclusive (*Y. enterocolitica* O:9) OPS. Error bars represent the maximum and minimum data points from triplicate assays with the lines plotted along the mean result.

3.8. Absorption of anti-*B. abortus* and *Y. enterocolitica* O:9 antibodies and sLPS ELISA iELISA

B. abortus S99 and *Y. enterocolitica* whole cell antigens were used in antibody absorption experiments using adaptations of methods described previously (Kittelberger et al., 1998). Serum from a cow experimentally infected with *B. abortus* stain 544 (A dominant) was incubated with the cells and centrifuged to pellet the cells and any antibodies bound to them. The supernatant containing the unbound antibodies was evaluated by iELISA with *Y. enterocolitica* O:9 and *B. abortus* S99 sLPS solid phase antigens. The results, shown in figure 3.11, demonstrate that at a 100 µg/ml concentration of absorbing *B. abortus* S99 cells there is effectively no remaining antibody titre on either the *B. abortus* S99 or *Y. enterocolitica* O:9 sLPS coated plates. This is in contrast to the results

when sera is absorbed by *Y. enterocolitica* O:9 cells where residual antibody is present on the *B. abortus* S99 but not the *Y. enterocolitica* O:9 sLPS coated plates. These results suggest that cattle may produce a subtly different anti-sLPS antibody response dependent upon whether the infective organism is *B. abortus* or *Y. enterocolitica* O:9. The caveat of this work is that the sLPS used includes the core and lipid A and although the OPS is serologically immunodominant, these structures may have a role in the generation of the observed differences. These results originate from just one serum sample from one cow experimentally infected with *B. abortus* but supports previously published results (Kittelberger et al., 1998).

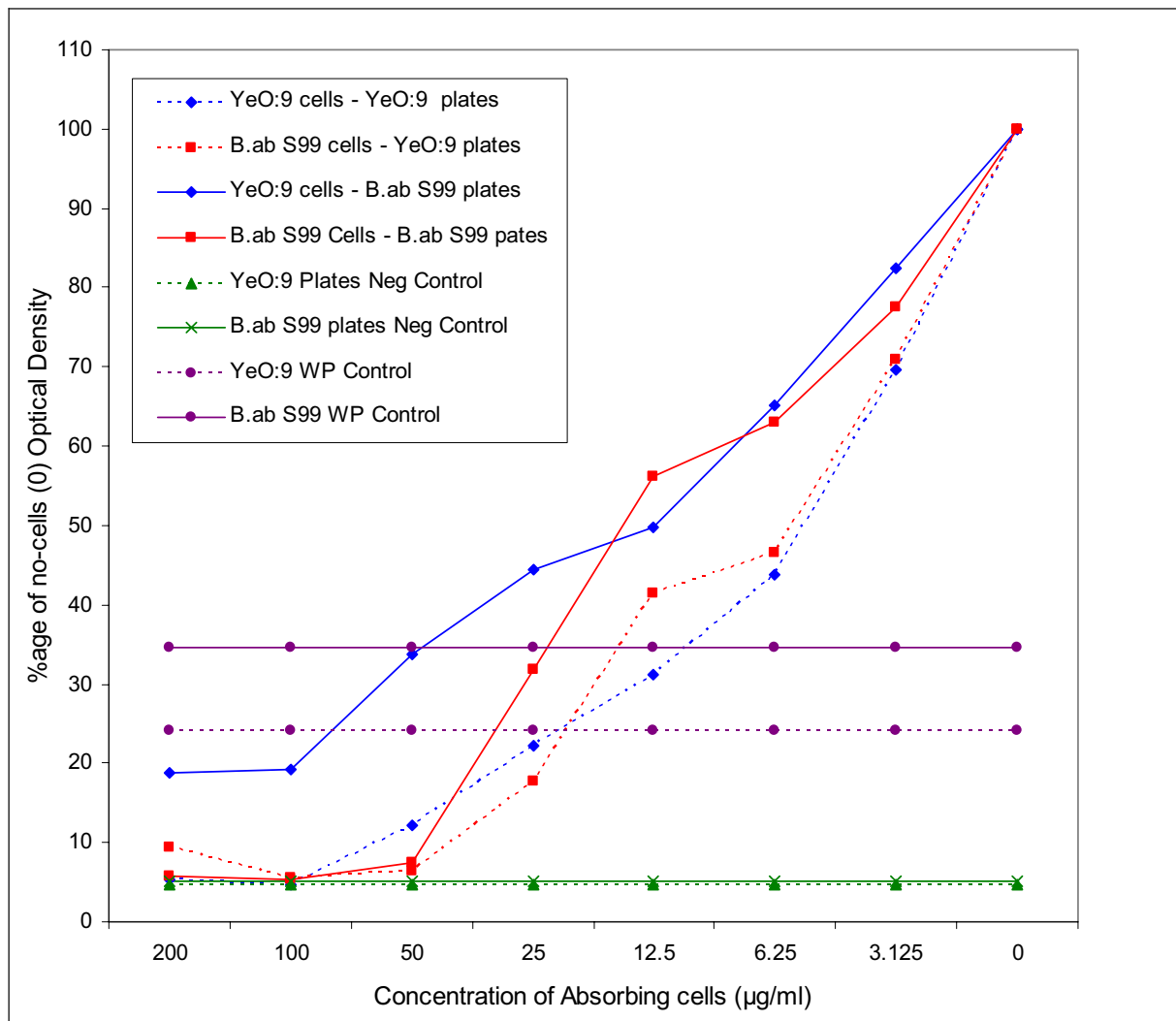


Figure 3.11. *Brucella* and *Y. enterocolitica* O:9 whole cell O:9 Antibody Absorption and sLPS iELISA
The results of iELISAs (expressed as a percentage of optical density of the unabsorbed serum) using *Y. enterocolitica* O:9 (YeO:9) and *B. abortus* S99 (B.ab S99) sLPS on serum from a *B. abortus* infected cow. The

sera was pre-absorbed with *Y. enterocolitica* O:9 or *B. abortus* S99 cells prior to the iELISA (as shown on the x-axis). The blue lines represent the results from sera absorbed with *Y. enterocolitica* O:9 cells, and the red lines the results from sera absorbed with *B. abortus* S99 cells. The purple lines represent the results from a weak positive (WP) control sample as tested on the different plate types. The green lines represent the results from negative serum. The dashed lines represent the results from the ELISA plates coated with *Y. enterocolitica* O:9 sLPS and the solid lines represent the results from the ELISA plates coated with *B. abortus* S99 OPS.

The antibody absorption method was subsequently applied to other sera from additional animals that were experimentally infected with *B. abortus* 544 or *Y. enterocolitica* O:9. Dependent on the titre of the serum the antibody reactivity could be reduced by pre-incubation with sLPS prior to ELISA or, with high titre sera, incubation with whole cells and centrifugation (as described above). The results from this evaluation (not shown) were mixed and did not show any significant difference in the residual antibody response between infection types. However, during the course of this work differences between the unabsorbed titres, the ELISA antigen type and the infection type were observed. This line of enquiry was pursued further with some refinements to the iELISA method, foremost amongst these was the use of a purified OPS antigen so that questions related to the role of the rough antigen in the differential antibody response could be resolved.

3.9. Immunoassay for antibody detection

3.9.1. Immunoassay: pig sera

Antibody detection immunoassays were applied to the serum populations described in the methods chapter (section 2.5.1.2.). Briefly there were 41 sera from pigs considered to be infected with *B. suis* biovar 1. A further 21 considered to be infected with *B. suis* biovar 2. There were 52 FPSR samples and four samples from pigs experimentally infected with *Y. enterocolitica* O:9. There were also 161 randomly acquired samples from non-*Brucella* infected pigs. The populations of sera evaluated by each assay is described below.

3.9.1.1. Immunoassay: pig sera, RBT and cELISA

The results of the conventional cELISA (McGiven et al., 2012; Perrett et al., 2010) and RBT assays for the samples from the *B. suis* biovar 1 infected animals, the *Y. enterocolitica* O:9 experimentally infected animals, the FPSR samples and those from the 161 non-*Brucella* infected animals are

shown in figure 3.12. The quantitative output for the cELISA is the OD of the sample as a percentage of the conjugate only (uninhibited binding of the HRP conjugated BM40 mAb) control. The lower the percentages the greater the inhibition and thus positive results have lower percentages. The qualitative interpretation is made using a cut-off of 60%, as shown in the figure 3.12, with results lower than the cut-off determined as positive and those greater as negative.

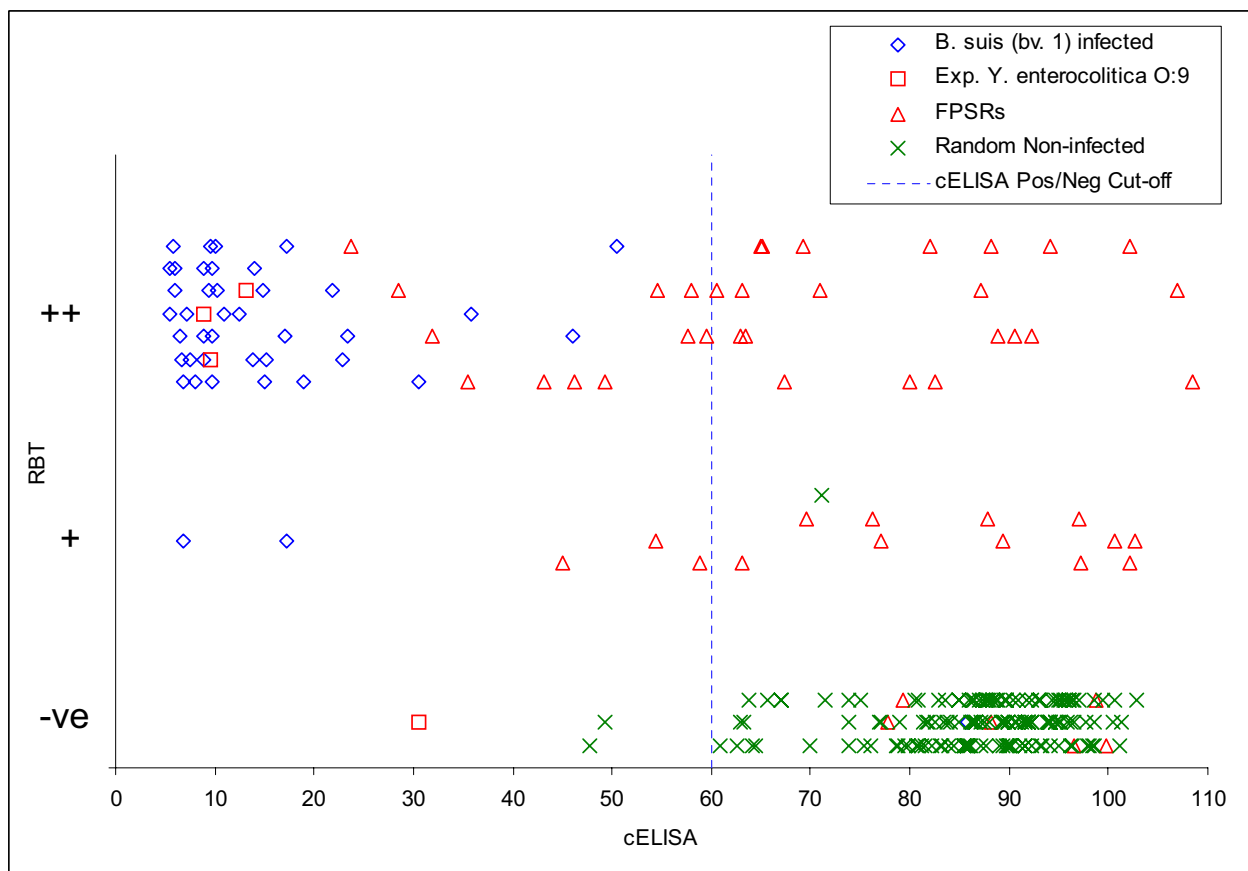


Figure 3.12. RBT and cELISA results for pig sera

Results for RBT and cELISA assays used in standard practise to test pig sera for the presence of anti-*Brucella* antibodies. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9, the green crosses are results from non-*Brucella* infected pigs (serum randomly collected from within Great Britain). cELISA results less than 60 are considered positive, those greater than 60 are considered negative. The RBT results are expressed as no reaction (-ve), weak (but positive) reaction (+), and strong positive reaction (++)

The cELISA and RBT are valuable assays as can be seen by the negative results from the randomly selected samples from non-infected animals and the positive results for the samples from *Brucella*

infected animals. However, in common with all assays that utilise the native OPS antigen as a diagnostic tool, the cELISA and RBT are both susceptible to FPSRs. The results shown in figure 3.12 demonstrate that the FPSRs occur in both assays. However the results are not always in agreement and neither is an effective confirmatory assay. The titre of the cross reactions may vary from moderate to high whereas the titres, especially in cELISA, from the true positive sera are, on average, higher. The existence of FPSRs in the cELISA occurs despite the anti-OPS mAb used (BM40) having high specificity for *Brucella* OPS (as shown above) and the antigen used being one rich in M epitopes (*B. melitensis* 16M sLPS).

3.9.1.2. Immunoassay: conjugation of OPS to ELISA plates

The sLPS antigen possesses many fine attributes that make it highly suited as an antigen for ELISA. The amphiphilic nature of this macromolecule presents a lipid anchor to the surface of a standard polystyrene plate and allows the hydrophilic sugar moieties to extend into the liquid milieu and readily interact with antibodies. In the context of the current study, where the reaction to the OPS alone is of interest, this desirable amphiphilic nature is offset by the presence on the antigen of the additional antigenic structures. The purification of the OPS at least partly eliminated confounding epitopes but created a practical challenge in that the elimination of the lipid anchor prevented the OPS from passively binding to the plate surface. In order to resolve this issue an OPS iELISA was developed whereby the OPS antigen was covalently attached to the plate surface. This was achieved using ELISA plates with surface hydrazide groups (Carbo-BIND™, Corning) on the end of a spacer arm. These groups react with aldehyde groups formed within oxidised carbohydrates to form stable covalent hydrazone bonds. The aldehyde groups in the OPS were created by mild oxidation with sodium metaperiodate. Under mildly oxidising conditions only vicinal diols will react to create aldehyde groups (Sussich and Cesaro, 2000). These are present in the *Brucella* and *Y. enterocolitica* O:9 OPS on the terminal D-Rha4NFO on the second and third carbons. The oxidised OPS was then diluted in sodium acetate buffer and added to the functionalised ELISA plates for covalent attachment. The use of the Carbo-BIND™ plates did increase the level of non-specific signal although this was capably reduced by the use of a casein based serum diluent in the developed ELISAs.

3.9.1.3. Immunoassay: pig sera, OPS iELISAs

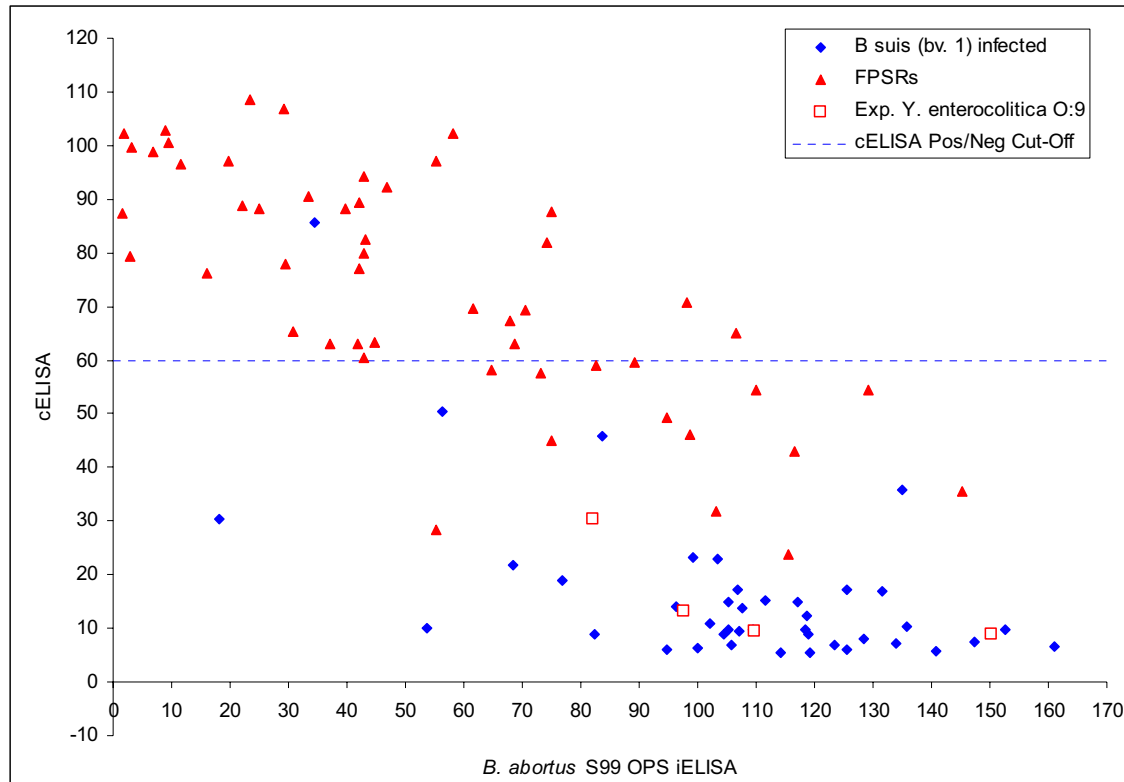


Figure 3.13. *B. abortus* S99 OPS iELISA and cELISA results for pig sera

The results of iELISA with *B. abortus* S99 OPS (x-axis) and cELISA are shown above. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

The samples from the *B. suis* biovar 1 infected pigs and the FPSR samples were then tested by the *B. abortus* S99 OPS iELISA. The results, shown in figure 3.13 as a scatter plot against the cELISA results, demonstrate the considerable reaction of the FPSR sera to this *Brucella* antigen in this format. The data also shows that the iELISA presents more cross reactions than the cELISA as there is more overlap between the results of the two populations. The results from the OPS iELISA using the antigen prepared from *B. melitensis* 16M are shown in figure 3.14. As in the previous figure, many of the FPSR samples react extensively against this antigen although these appear to be fewer in number than was the case with the *B. abortus* S99 OPS antigen.

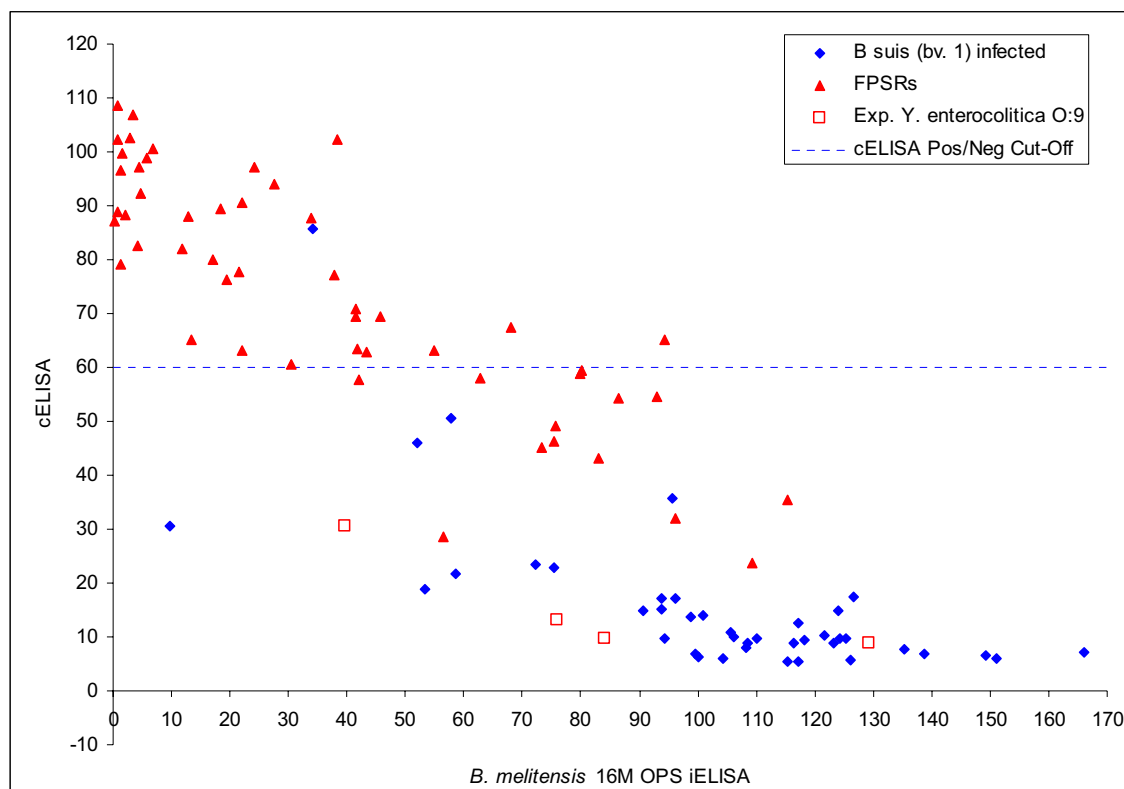


Figure 3.14. *B. melitensis* 16M OPS iELISA and cELISA results for pig sera

The results of iELISA with *B. melitensis* 16M OPS (x-axis) and cELISA are shown above. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

These samples were also tested in iELISA with the *Y. enterocolitica* O:9 OPS antigen preparation and the results are shown in a scatter plot against the results from the equivalent *B. abortus* S99 OPS assay in figure 3.15. The results show a considerable amount of cross reaction but when the results for each sample are expressed as a ratio of the *B. abortus* S99 to *Y. enterocolitica* O:9 OPS iELISA results there is a significant difference between the FPSR sera and the sera from the *B. suis* biovar 1 infected animals ($P = 0.023$). This difference presents itself graphically as a tendency for the samples from the *Brucella* infected animals to be on the right hand side of the graph compared to the FPSR sera with equal titre against the *Y. enterocolitica* O:9 OPS.

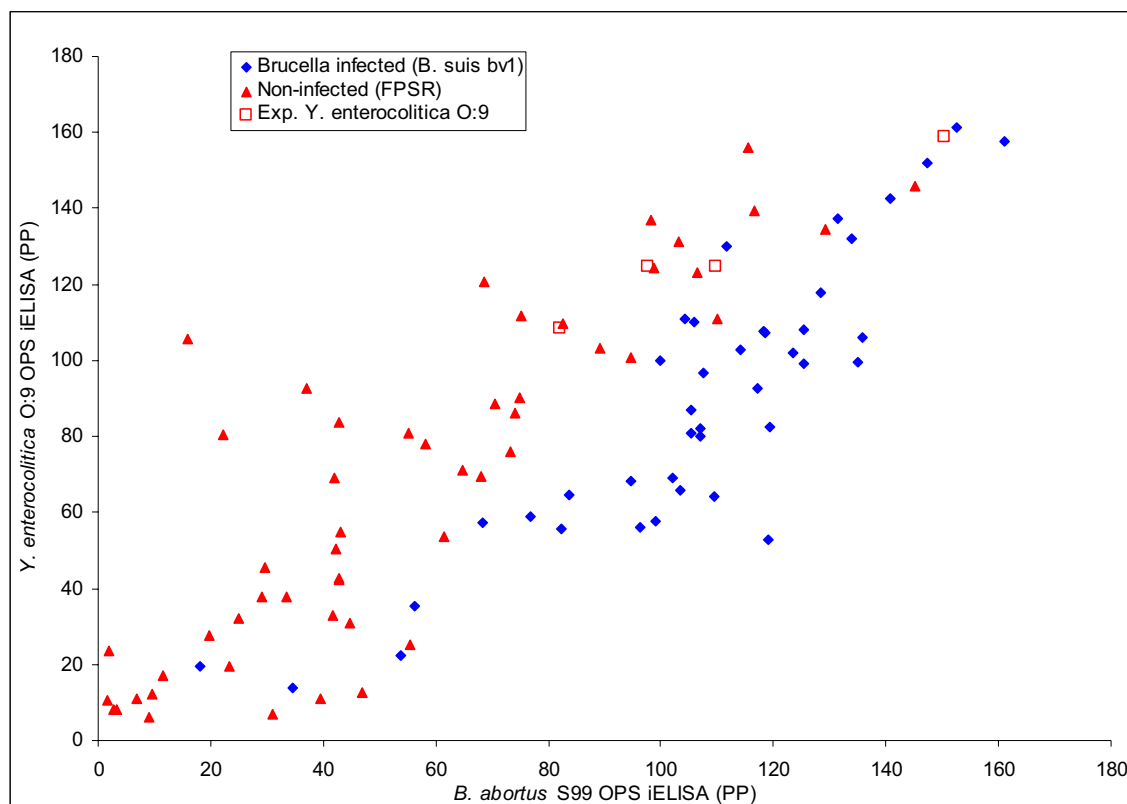


Figure 3.15. *B. abortus* S99 against *Y. enterocolitica* O:9 OPS iELISA results for pig sera

The results of iELISA with *B. abortus* S99 and *Y. enterocolitica* O:9 OPS (x-axis) are shown above. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

The results of the *B. melitensis* 16M OPS assay are shown in figure 3.16 by scatter plot against the results from the *Y. enterocolitica* O:9 OPS. Once again there is a large degree of cross reaction between the sera and antigens. However there is an uneven relationship between serum titre and antigen. In this case statistical analysis of the ratio of the *B. melitensis* S99 to *Y. enterocolitica* O:9 OPS iELISA results reveals that there is a very highly significant difference between the FPSR sera and the sera from the *B. suis* biovar 1 infected animals ($P = 0.000000006$). As can be seen on the graph the sera from the *Brucella* infected animals have a higher titre, on average, against the *B. melitensis* 16M antigen than the FPSR samples of equivalent titre against the *Y. enterocolitica* O:9 OPS antigen.

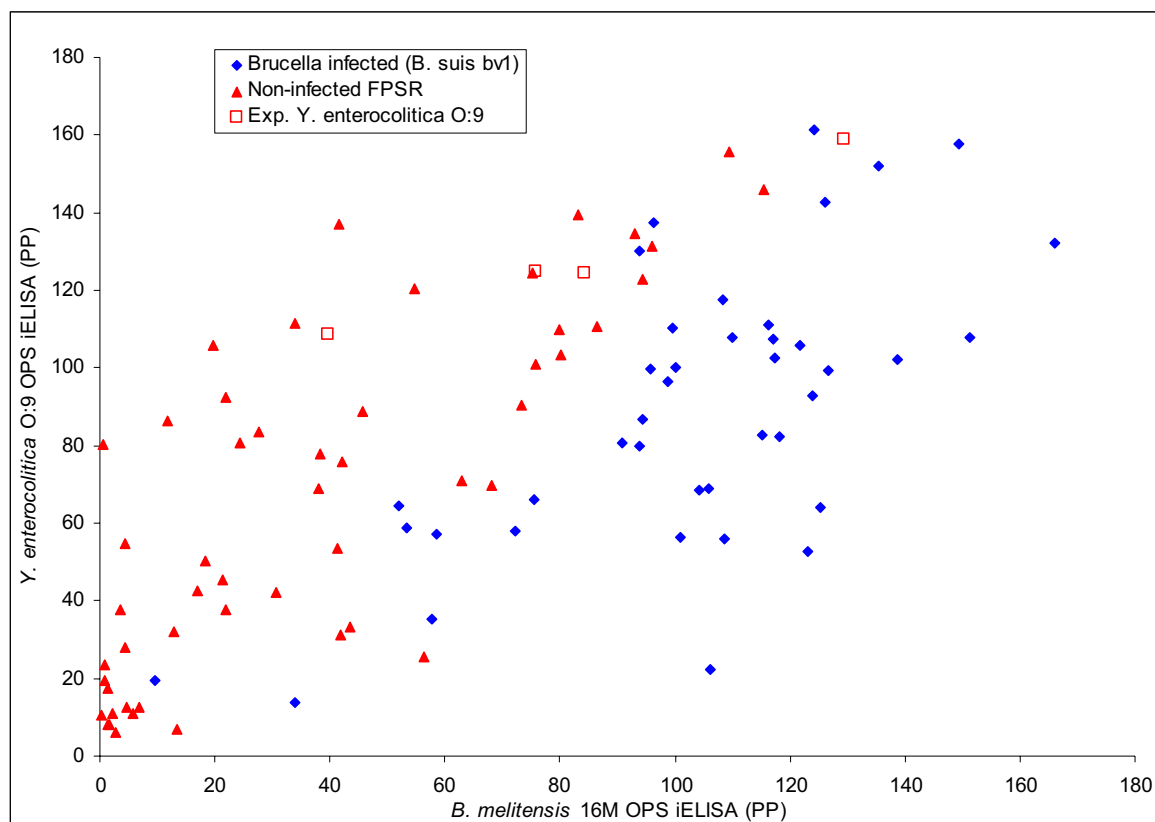


Figure 3.16. *B. melitensis* 16M against *Y. enterocolitica* O:9 OPS iELISA results for pig sera

The results of iELISA with *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS (x-axis) are shown above. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

When the iELISA results from the iELISAs performed with the two *Brucella* OPS antigens was compared, as shown in figure 3.17, there was also a highly significant, serum population dependent, differential response to these two antigens ($P = 0.000000016$). This difference was more significant than the difference between the *B. abortus* and *Y. enterocolitica* O:9 OPS antigens. The difference between the response to the two *Brucella* OPS antigens suggests that the structural component responsible is not part of the core (as this may also be within the OPS antigen preparations) and not due to the non-repeating elements of the OPS at the reducing end. The differences between the antigens does reflect the known structure of the 3 antigens with the A dominant OPS of *B. abortus* S99 having only approximately 2% α -1,3 linkages compared to the M dominant *B. melitensis* 16M OPS with 20% and *Y. enterocolitica* with 0%.

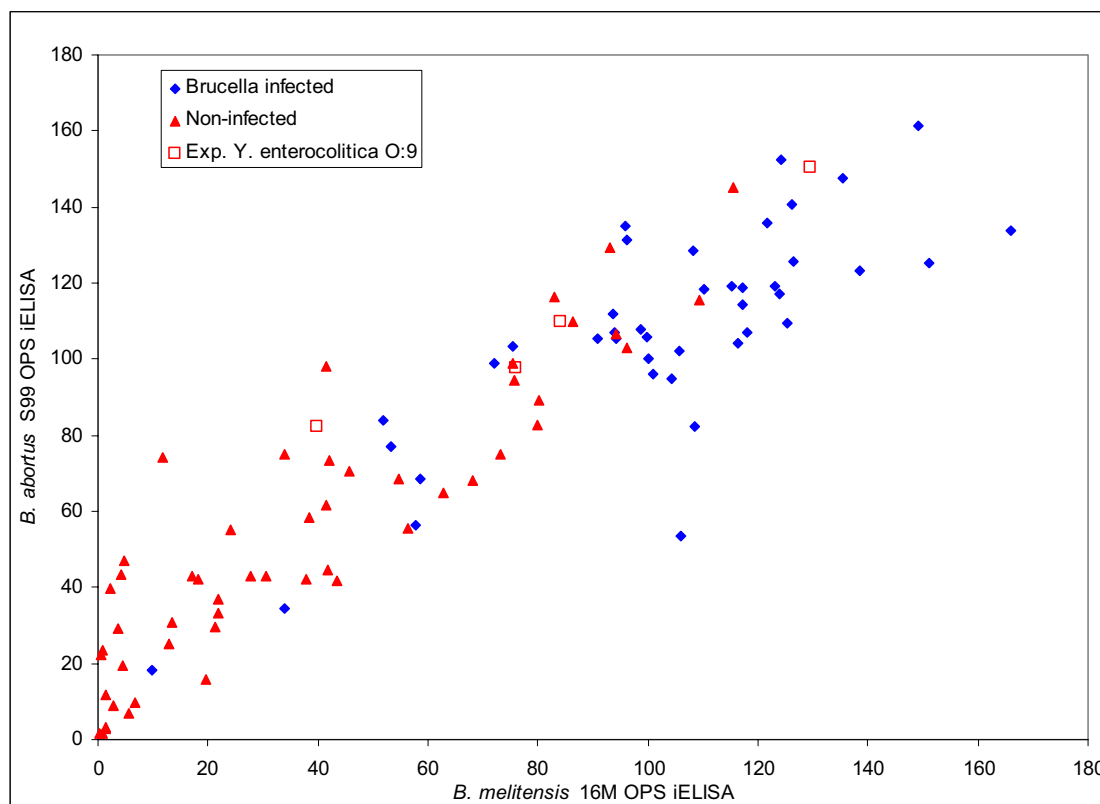


Figure 3.17. *B. melitensis* 16M against *B. abortus* S99 OPS iELISA results for pig sera

The results of iELISA with *B. abortus* S99 OPS (x-axis) and cELISA are shown above. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

3.9.1.4. Immunoassay: pig sera, statistical analysis of OPS iELISA's diagnostic capability

The results from all three of the OPS iELISA assays were evaluated for their diagnostic capability to minimise the FPSR problems by considering the FPSR samples as the only group within the non-infected population and the samples from the *B. suis* biovar 1 infected animals as the group comprising the infected populations. A 4th metric was contrived by dividing the result from the *B. melitensis* OPS iELISA by that from the *Y. enterocolitica* O:9 OPS iELISA to derive a simple ratiometric value (henceforth described as the 'Bm16M/YeO:9' ratio). Thus higher Bm16M/YeO:9 ratio values signify that the intensity of the reaction to the *B. melitensis* 16M OPS antigen was greater than the intensity of the reaction to the *Y. enterocolitica* O:9 antigen relative to lower Bm16M/YeO:9 ratio values.

The four sets of data were analysed for their maximum Youden Index (YI = diagnostic sensitivity [DSn] + diagnostic specificity [DSp] - 1). Receiver Operator Characteristics (ROC) analysis was also applied, the ROC curves have been plotted in figure 3.18. This shows a clear division between the curves generated from the *Brucella* and *Y. enterocolitica* O:9 OPS antigens, those from *Brucella* demonstrating superior diagnostic performance. The Area Under the ROC Curve has been tabulated together with the Youden Index in table 3.1. The optimised Youden Index, for each of the ELISA outputs are of the order: Bm16M/YeO:9 > *B. melitensis* 16M > *B. abortus* S99 > *Y. enterocolitica* O:9. The AUC is of the order *B. melitensis* 16M > Bm16M/YeO:9 > *B. abortus* S99 > *Y. enterocolitica* O:9. In this context, the AUC represents the ability of the assay to correctly classify samples from animals that are *Brucella* infected and those that are not.

Assay	Optimal Youden Index (YI=DSn+DSp-1)			ROC - Area Under Curve	
	YI Estimate	DSn %	DSp %	AUC	95% Confidence Interval
<i>Y. enterocolitica</i> O:9 OPS iELISA	0.3588	87.80	48.08	0.6445	0.5328 - 0.7562
<i>B. abortus</i> S99 OPS iELISA	0.6472	87.80	76.92	0.8607	0.7832 - 0.9382
<i>B. melitensis</i> 16M OPS iELISA	0.7087	80.49	90.38	0.9135	0.8559 - 0.9710
<i>B. melitensis</i> 16M / <i>Y. enterocolitica</i> O:9	0.7678	90.24	86.54	0.9085	0.8437 - 0.9733

Table 3.1. - Performance statistics for OPS iELISAs as tested on pig sera

The AUC for the *Y. enterocolitica* O:9 OPS iELISA was significantly lower ($P < 0.0001$) than for the other three outputs, data shown in table 3.2. There was a difference of weak significance between the AUC for the *B. abortus* and *B. melitensis* OPS iELISAs ($P = 0.066$).

Assay	<i>B. abortus</i> S99	<i>B. melitensis</i> 16M	Bm16M/YeO:9
<i>Y. enterocolitica</i> O:9 OPS iELISA	< 0.0001	< 0.0001	< 0.0001
<i>B. abortus</i> OPS iELISA (B.ab S99)		0.066	0.174
<i>B. melitensis</i> 16M OPS iELISA (B.mel 16M)			0.452

Table 3.2 - Comparison of ROC AUC statistics for the OPS iELISAs as tested on pig sera

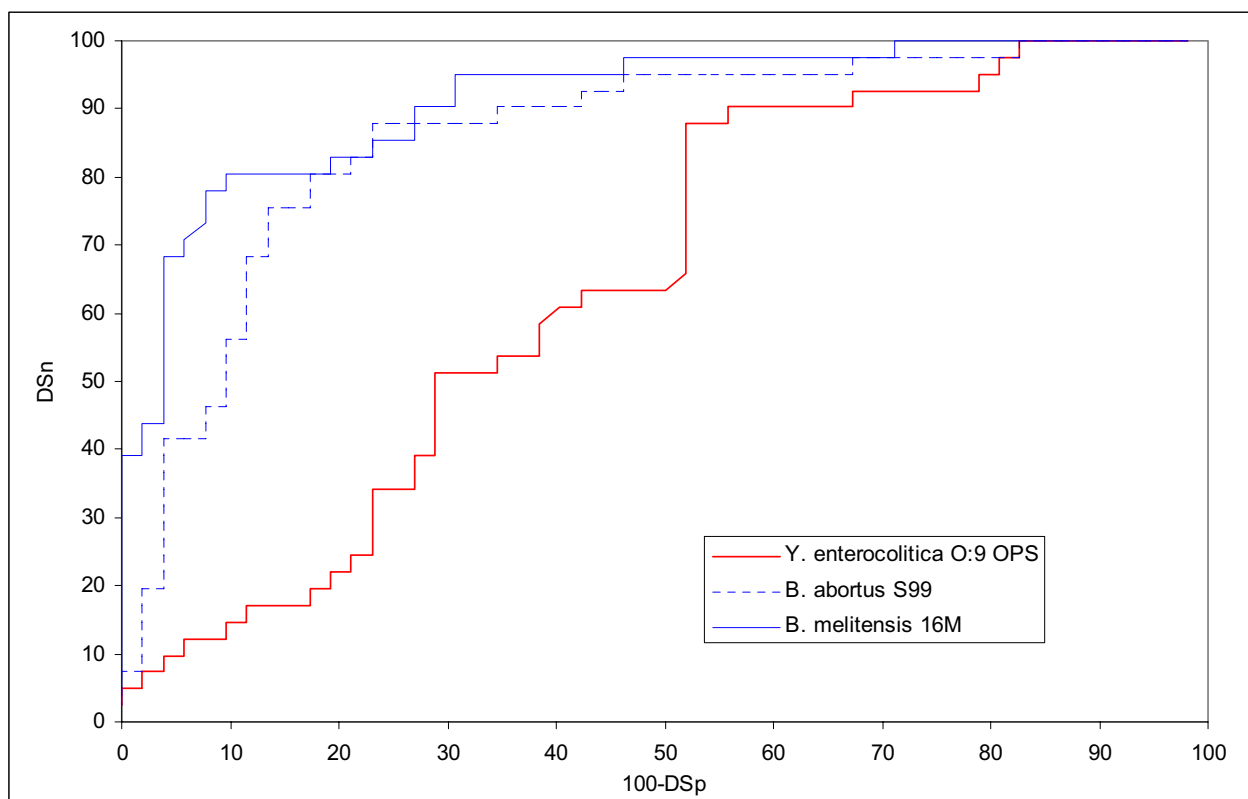


Figure 3.18. ROC curves for the *Brucella* and *Y. enterocolitica* O:9 OPS iELISAs applied to pig sera
ROC Curves generated from the results of the *Y. enterocolitica* O:9 OPS, *B. abortus* S99 OPS, and *B. melitensis* OPS iELISAs as applied to sera from pigs confirmed as infected with *B. suis* biovar 1 (n=41) and sera from non-*Brucella* infected pigs from herds from which there was one or more samples positive in conventional serodiagnostic assays (n=52).

3.9.1.5. Immunoassay: OPS iELISA on pig sera from animals infected with *B. suis* biovar 2

The same OPS iELISAs were applied to 21 serum samples from serologically positive pigs from herds confirmed by culture to be infected with *B. suis* biovar 2. These results were compared to the

existing data from the 52 FPSR samples, as well as to the 41 samples from *B. suis* biovar 1 infected animals. There was no significant difference in the Bm16M/YeO:9 ratio results between the sera from the *B. suis* biovar 2 infected animals and from sera from the non-infected animals ($P = 0.926$).

These results were initially considered disappointing as they did not agree with the data from the samples from the *B. suis* biovar 1 infected pigs and did not support the project hypothesis. However, it has recently been discovered that, uniquely for *Brucella*, the OPS from *B. suis* biovar 2 contains no α -1,3 linkages (Zaccheus et al., 2013), and no C specific epitopes, and therefore the OPS is highly similar, if not almost identical, to that of *Y. enterocolitica* O:9. As such, samples from animals infected with this biovar would, according to the hypothesis, react in identical fashion to those from *Y. enterocolitica* O:9 infected animals. This data suggests that they do. This is therefore powerful evidence that the differences observed above are due specifically to the M epitope, the presence of the $\alpha(1\rightarrow3)$ linkage.

3.9.1.6. Immunoassay: rLPS iELISA on pig sera

The pig sera was also tested by iELISA against rLPS extracted from *B. abortus* RB51. Some of this data has already been published (McGiven et al., 2012) but the relevant details are worth including here. The results of the rLPS assay for the pig sera from *B. suis* biovar 1 infected animals, the FPSR sera and the sera from animals experimentally infected with *Y. enterocolitica* O:9 are shown in figure 3.19 in a scatter plot against the results from the *Y. enterocolitica* O:9 OPS iELISA. The results show that the rLPS antigen is very effective at discriminating between the sample populations. The rLPS iELISA also has good diagnostic specificity when applied to randomly selected samples from non-infected pigs (McGiven et al., 2012). The rLPS iELISA appears to be equally effective at detecting anti-rLPS antibodies in the sera of pig infected with *B. suis* biovar 2 (unpublished data) and may be the solution to the FPSR issue in pigs.

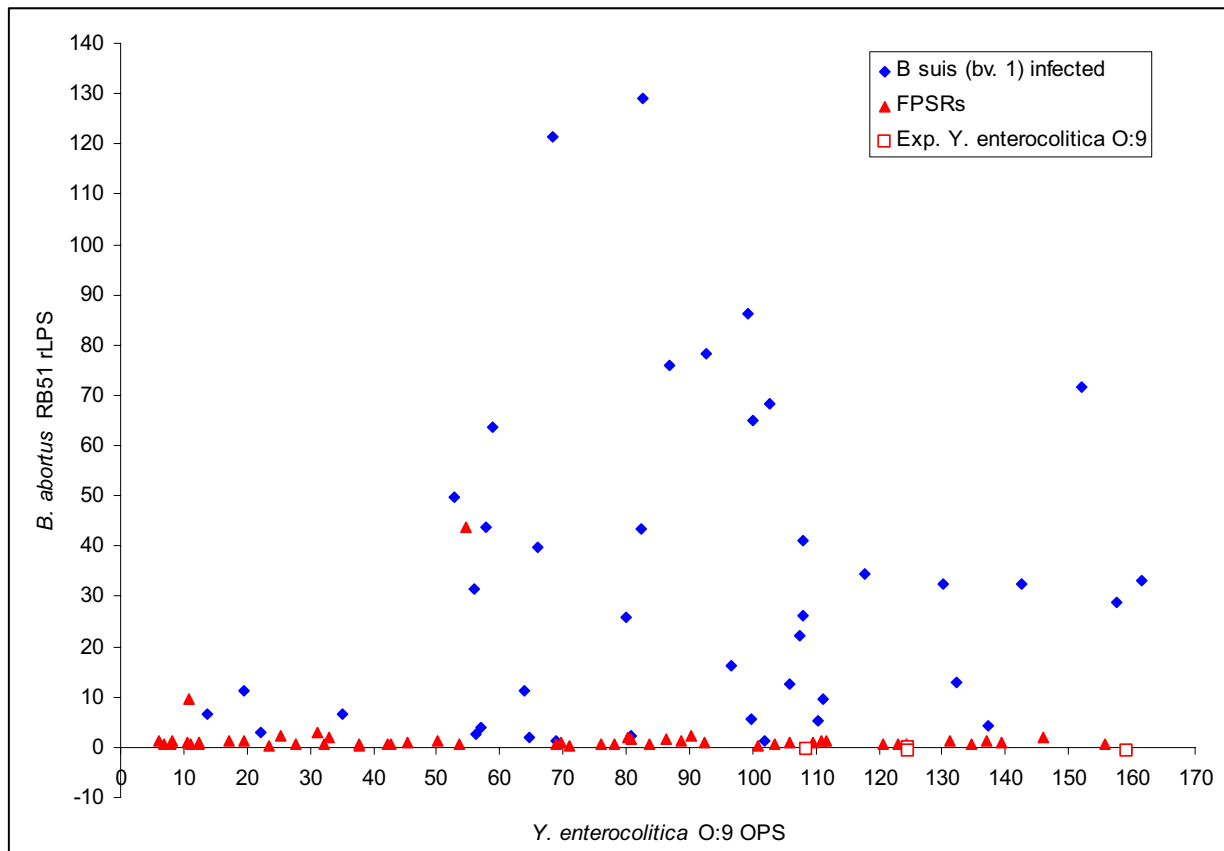


Figure 3.19. *B. abortus* RB51 rLPS against *Y. enterocolitica* O:9 OPS iELISAs for pig sera

Results for iELISA with *Y. enterocolitica* O:9 OPS (x-axis) and *B. abortus* RB51 rLPS (y-axis) on pig sera. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9.

Given the absence of specific epitopes in the OPS of *B. suis* biovar 2 it is most unlikely that any assay based on this structure will be capable of confidently addressing the issue of FPSRs in pigs. The evidence from the rLPS iELISA suggests that there is no additional need to develop an alternative confirmatory assay and this lessens the disappointment that the OPS based approach described herein will almost certainly be unsuccessful for confirmatory testing of pig infected with *B. suis* biovar 2. In fact, the situation presents valuable, and somewhat unforeseen, opportunities that will be discussed subsequently. The rLPS data does have value in providing further proof that the nature of the linkage in the OPS is the determinant behind the differences in relative titre against the different OPS antigens.

The evaluation of the rLPS response set against the Bm16M/YeO:9 ratio, shown in figure 3.20, demonstrates that there is no relationship between this ratio and the response to the rLPS antigens.

When all samples are included in the analysis the relationship is, in fact, negative. If a potential outlier is removed from the analysis (the point with ratio value > 4.5) the relationship does become positive but only to a very minor degree.

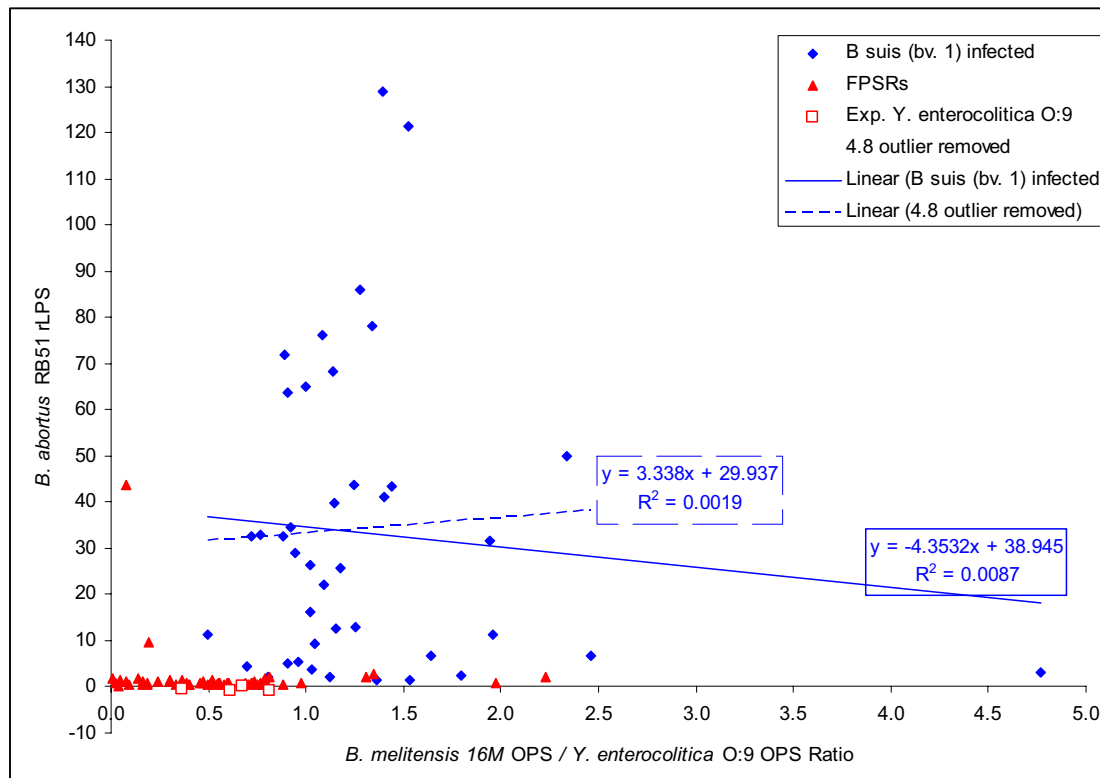


Figure 3.20. *B. abortus* RB51 rLPS against Bm16M/YeO:9 ratio for pig sera

Results for iELISA Bm16M/YeO:9 ratio (x-axis) against *B. abortus* RB51 rLPS (y-axis) on pig sera. The blue diamonds are results from *B. suis* biovar 1 infected pigs, the red triangles are results from non-*Brucella* infected pigs from herds with FPSRs, the red squares are results from four samples collected from four pigs experimentally infected with *Y. enterocolitica* O:9. The data in the coloured boxes show the equation of the best fit lines (in blue) and the coefficient of determination (R^2). The dashed blue line and box represents the data when the potential outlier ($x > 4.5$) is removed.

3.9.1.7. Summary of serological data from pig sera

To summarise, the serological data from the pig samples demonstrates the problem of false positive serological reactions. It demonstrates that the field FPSRs behave in a manner highly similar to that of the sera from the animals experimentally infected with *Y. enterocolitica* O:9 (epidemiological evidence suggests that infection with *Y. enterocolitica* O:9 is a probable explanation for the induction of these field FPSRS). The antibodies from true and false positive samples do not react with equal

vigour to the different types of OPS antigen extracts evaluated, even though their structures are highly similar. Antibodies induced by infection with *Brucella* in possession of M epitopes – even in relatively low abundance - react preferentially against antibodies rich in these epitopes, compared to FPSR sera. Comparison of the results between the *B. abortus* S99 and *B. melitensis* 16M OPS antigen preparations also show highly significant differences in the manner in which the serum populations react. This supports the theory the M epitopes, created by the presence of $\alpha(1\rightarrow3)$ inter glycosidic linkages, are responsible for these differences. The role of the C specific epitope, if indeed it even exists, seems to be inconsequential. Furthermore, and significantly, this potential role for the M epitope has been demonstrated using sera from animals infected with *Brucella* in possession of A dominant OPS and thus low in relative $\alpha(1\rightarrow3)$ abundance. This theory is supported by the results on the sera from pigs infected with *B. suis* biovar 2 and by the results from rLPS iELISA.

These differences are highly significant at the population level but not at the level of the individual and thus the approach, as it stands, is not sufficient to aid in confirmatory serodiagnosis. The additional convolutions created by the nature of the overlapping epitopes may be masking the true diagnostic potential of the OPS antigen, potential that may yet be realised from the evaluation of the individual epitopes on their own. The results from the rLPS iELISA applied to pig sera suggest that such a confirmatory assay may not be needed. The results from cattle sera, as shall be shown, suggests otherwise.

3.9.2. Immunoassay: cattle sera

Antibody detection immunoassays were applied to the serum populations described in the methods chapter (section 2.5.1.1.). Briefly there were 46 sera from cattle infected with *B. abortus* biovar 1. There were 68 FPSR samples. There were also samples from four cattle experimentally infected with *B. abortus* biovar 1 and from four cattle experimentally infected with *Y. enterocolitica* O:9. The populations of sera evaluated by each assay are described below.

3.9.2.1. Immunoassay: sera from experimentally infected cattle, OPS iELISAs

Following on from the evaluation of the OPS specific serum antibody response in pigs a similar study was performed on cattle sera. This began with an evaluation of sera from four cattle experimentally

infected with *Brucella abortus* strain 544 (an A dominant strain) and four experimentally infected with *Y. enterocolitica* O:9. Only the OPS from *Y. enterocolitica* O:9 and *B. melitensis* 16M was used in ELISA as these showed the greatest difference with pig sera and represent the two extremes in terms of the proportion of M epitopes within. As anticipated, there was much cross reaction between the *Brucella* and *Y. enterocolitica* O:9 OPS antigens with both sets of sera. The results for the different OPS iELISAs are shown in figures 3.21 and 3.22.

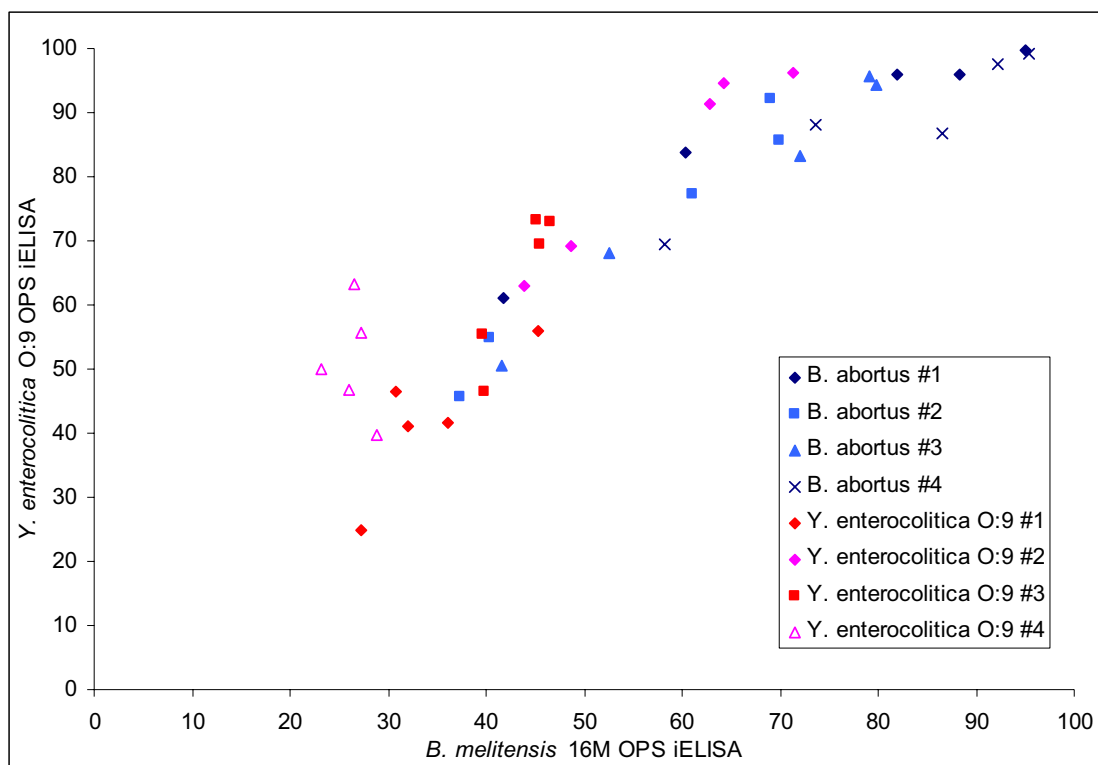


Figure 3.21. Results of *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS iELISA on sera from experimentally infected cattle

The results of iELISA with *B. melitensis* 16M (x-axis) and *Y. enterocolitica* O:9 (y-axis) OPS antigens are shown above. The blue data points are results from four *B. abortus* strain 544 infected cattle. The red data points are results from four *Y. enterocolitica* O:9 experimentally infected cattle. There were five timed samples taken from each animal, each character type denotes results for a specific animal.

Figure 3.21 shows the quantitative data for each OPS iELISA. Sera collected at five different time points for each animal (#1 to #4), 3, 7, 16, 24 and 53 weeks post infection was analysed. The data points show a similar distribution, relative to the serum populations and antigen types, to that observed previously in the pig sera. The same data is presented in figure 3.22 with time, in weeks as a categorical variable on the x-axis and the Bm16M/YeO:9 ratio on the y-axis. This data shows that the sera from the *Brucella* infected cattle has a higher ratio, on average, than the sera from the *Y.*

enterocolitica O:9 infected cattle. The distinction, based on this ratio, of the infection types was not perfect, at the first time point at three weeks, the ratios for the two infection types were at their closest. The difference in the ratio between the two infection types was highly significant ($P = 0.008$).

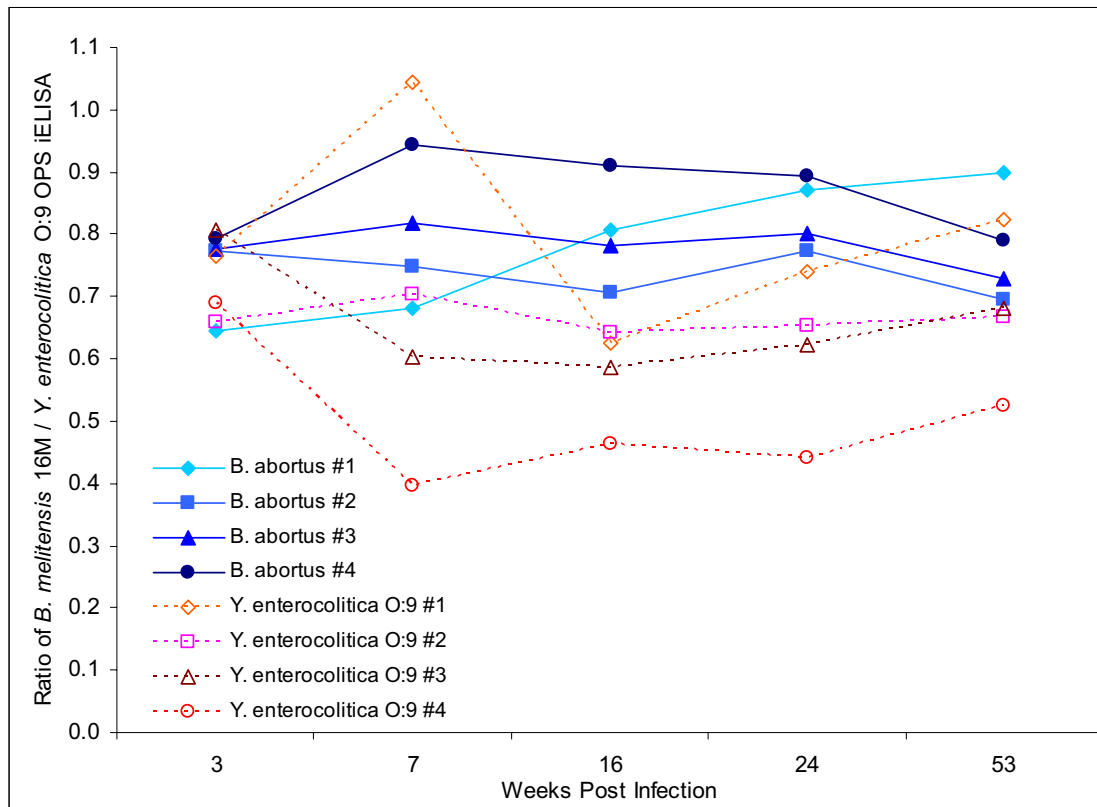


Figure 3.22. The Bm16M/YeO:9 ratiometric results against time post infection for sera from experimentally infected cattle

The Bm16M/YeO:9 ratiometric results of iELISA with *B. melitensis* 16M by *Y. enterocolitica* O:9 OPS antigens (y-axis) against weeks post infection (x-axis) are shown above. The blue data points are results from four *B. abortus* S544 infected cattle. The red data points are results from four *Y. enterocolitica* O:9 experimentally infected cattle. There were five timed samples taken from each animal, each character type denotes results for a specific animal which are linked in time series.

3.9.2.2. Immunoassay: cattle sera from the field, OPS iELISAs

Following on from this finding with the sera from experimentally infected animals the same methods were applied to archived cattle field sera from Great Britain. A total of 46 samples from individual animals confirmed by culture to be infected with *B. abortus* biovar 1 (A dominant) were tested. Also 68 samples were tested from individual animals whose sera was collected from 1996 to 1999, more than 10 years since the declaration of officially brucellosis free status for Great Britain, that were

positive in conventional serology for brucellosis such as *B. abortus* S99 sLPS iELISA, SAT and CFT (Nielsen and Ewalt, 2010) but for which there was no cultural or epidemiological evidence of the disease.

The cross reactivity of the 68 field FPSR samples is evident in figures 3.23 and 3.24 where the results for the cELISA have been set against the results for the two OPS iELISAs, *B. melitensis* 16M in figure 3.23 and *Y. enterocolitica* O:9 in figure 3.24, in scatter plot. These graphs show that most of the FPSR sera are positive by cELISA and most also have a significant titre in both the OPS iELISAs. They also show that the titres of the true positive samples are, on average, higher in cELISA than the titres of the FPSRs. However, adjustments to the assay cut-off to take advantage of this would result in an increase of false negative results. Comparing the data from the two OPS iELISAs reveals that the titre of the FPSR sera in the *B. melitensis* 16M OPS iELISA, relative to the true positives, is lower than their relative titre in the *Y. enterocolitica* O:9 OPS iELISA. This relationship is more clearly visible when the data is plotted together as shown in figure 3.25. As with the data from the experimentally infected animals there was a highly significant difference ($P = 0.000012$) between the Bm16/YeO:9 Ratio values for the two different serological groups.

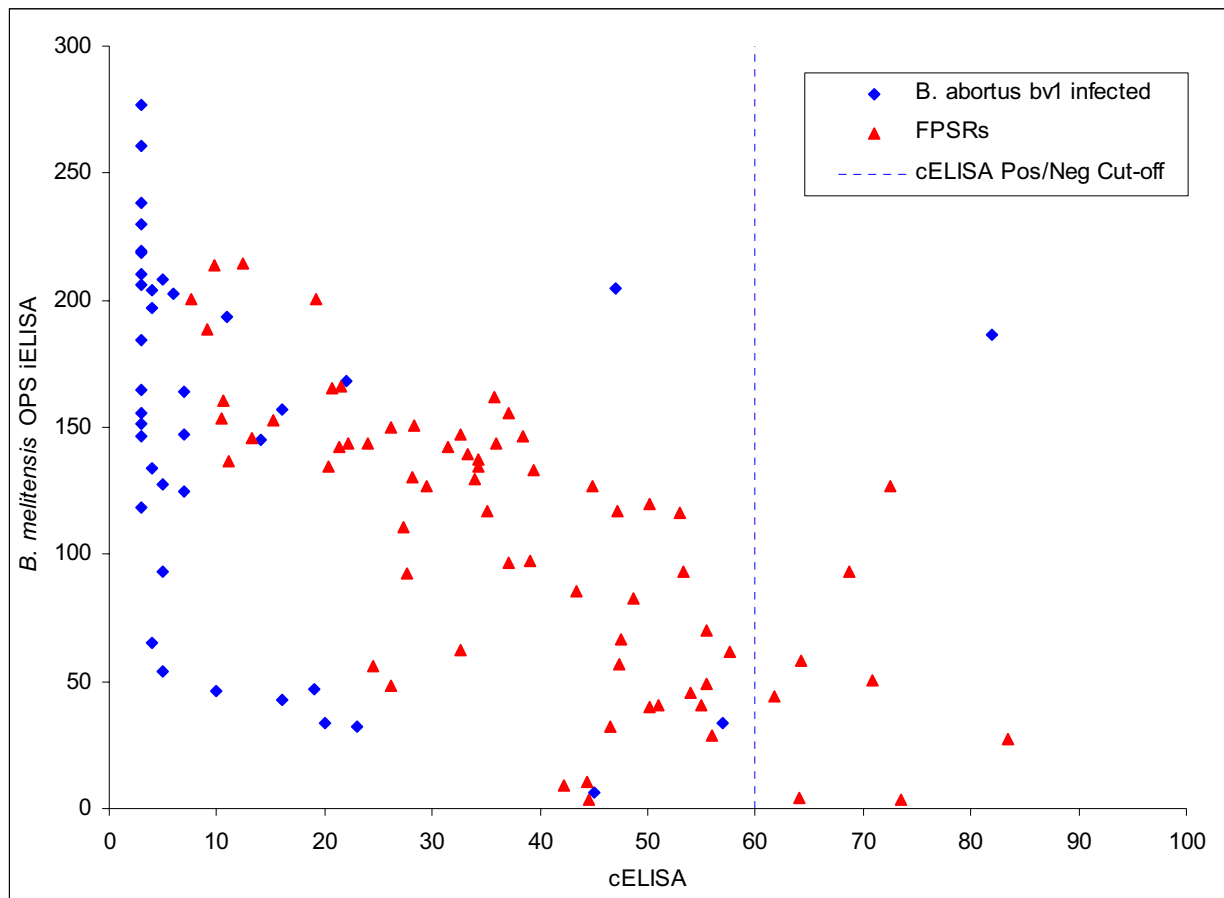


Figure 3.23. cELISA against *B. melitensis* 16M OPS iELISA results for cattle sera

The results of iELISA with *B. melitensis* 16M OPS (x-axis) and cELISA are shown above. The blue diamonds are results from *B. abortus* infected cattle, the red triangles are results from non-*Brucella* infected FPSR cattle.

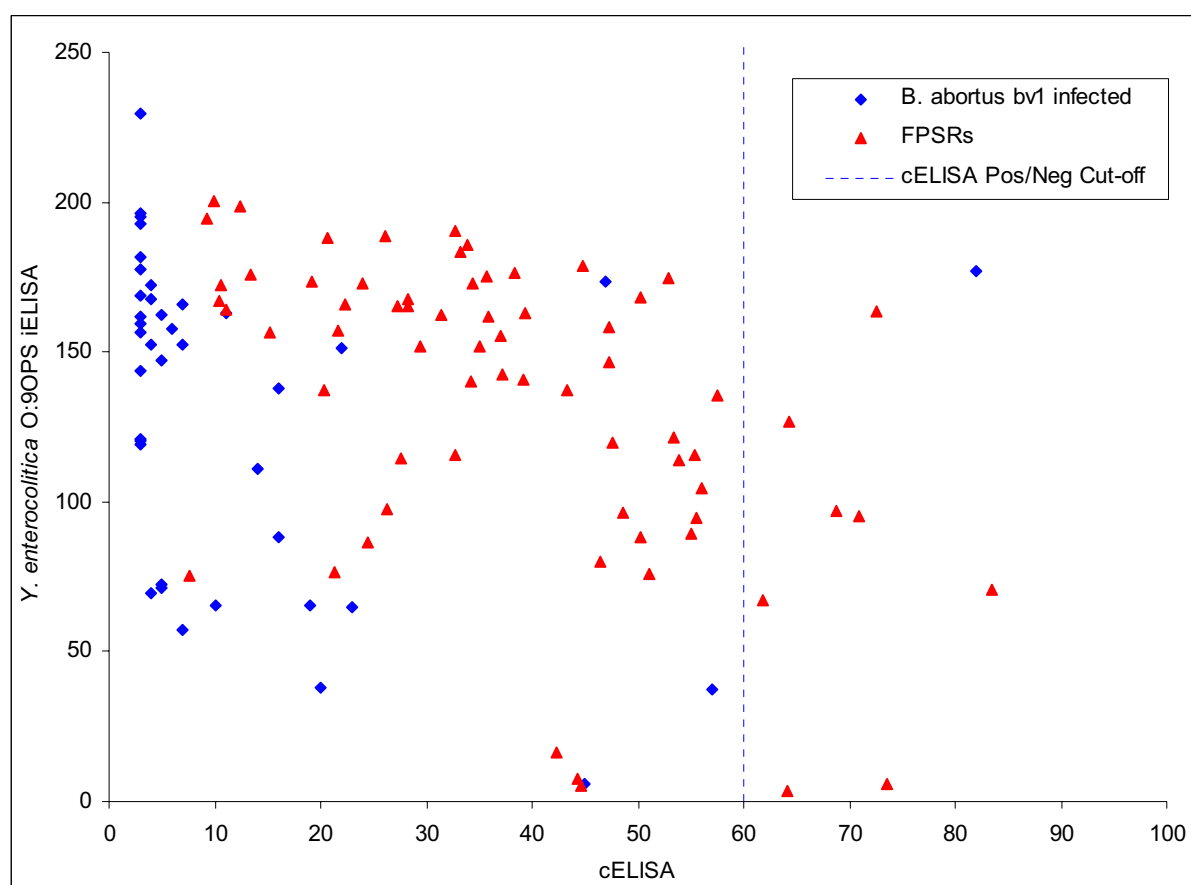


Figure 3.24. cELISA against *Y. enterocolitica* O:9 OPS iELISA results for cattle sera

The results of iELISA with *Y. enterocolitica* O:9 OPS (x-axis) and cELISA are shown above. The blue diamonds are results from *B. abortus* infected cattle, the red triangles are results from non-*Brucella* infected FPSR cattle.

3.9.2.3. Immunoassay: cattle sera, statistical analysis of OPS iELISA's diagnostic capability

As with the analysis of the pig sera, the results from both the *B. melitensis* and *Y. enterocolitica* O:9 OPS iELISAs performed on cattle sera were evaluated to find the positive/negative cut-off for each which generated the highest Youden Index ($YI = \text{diagnostic sensitivity [DSn]} + \text{diagnostic specificity [DSp]} - 1$). These optimised YI values with the associate DSn and DSp figures are shown in table 3.3. Also shown in this table are the AUC values derived from ROC analysis. The optimised YI values and the AUC values were of the order: Bm16M/YeO:9 > *B. melitensis* 16M OPS iELISA > *Y. enterocolitica* O:9 OPS iELISA. The ROC curves themselves are presented in figure 3.26.

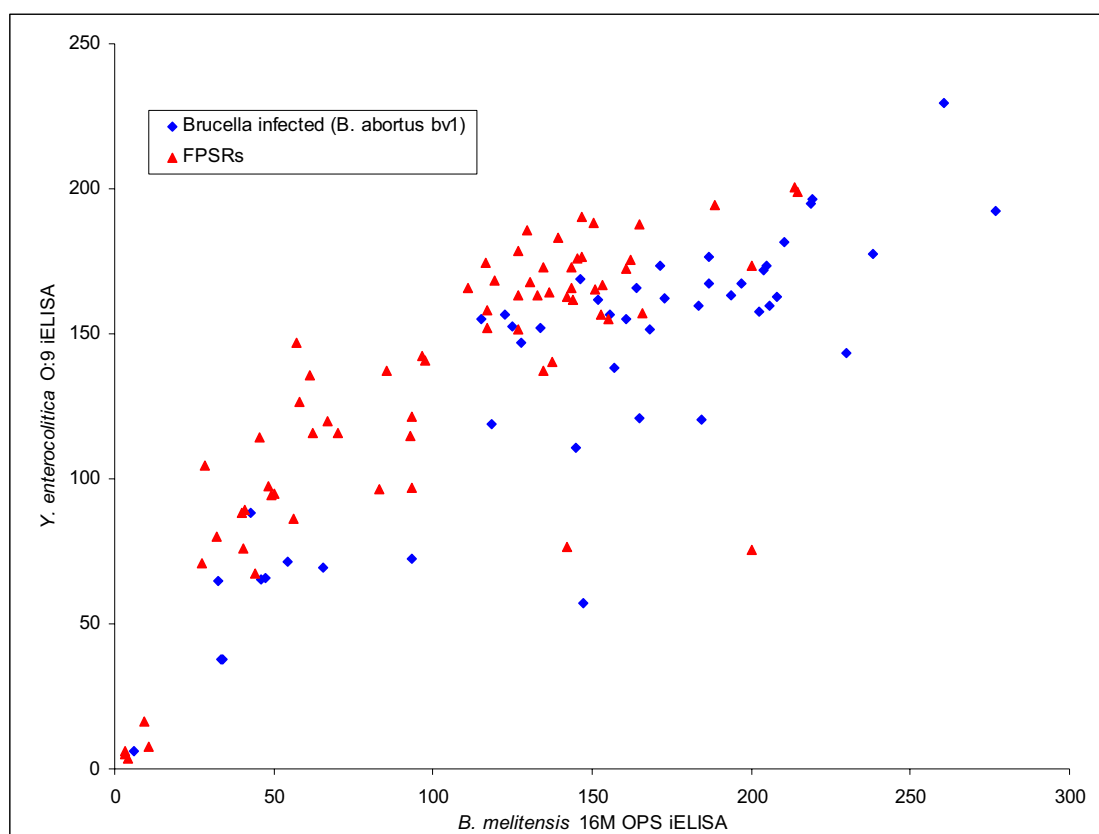


Figure 3.25. *B. melitensis* 16M against *Y. enterocolitica* O:9 OPS iELISA results for cattle sera

The results (expressed as a percentage of a common positive control) for *B. melitensis* 16M OPS iELISA (x-axis) and *Y. enterocolitica* O:9 OPS iELISA (y-axis) ELISAs; the solid blue diamonds represent the results for sera from cattle confirmed as infected with *B. abortus* biovar 1 (n=46) and the red triangles represent the results for sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

The results of significance testing for differences between AUC values is shown in table 3.4. This data shows that there is a highly significant difference ($P < 0.0001$) between the AUC values for the *Y. enterocolitica* O:9 and *B. melitensis* 16M OPS iELISAs and therefore the latter assay is significantly diagnostically superior. The improvement in diagnostic performance when using the Bm16M/YeO:9 ratio values is also demonstrated in Tables 3.3 and 3.4. The optimal YI value for this ratio is greater than that for both of the individual OPS iELISA assays. The DSp is equal to that of the *B. melitensis* 16M OPS iELISA but the DS_n is superior. The AUC is also greater, but not significantly so, compared to the AUC for the *B. melitensis* 16m OPS iELISA ($P = 0.202$).

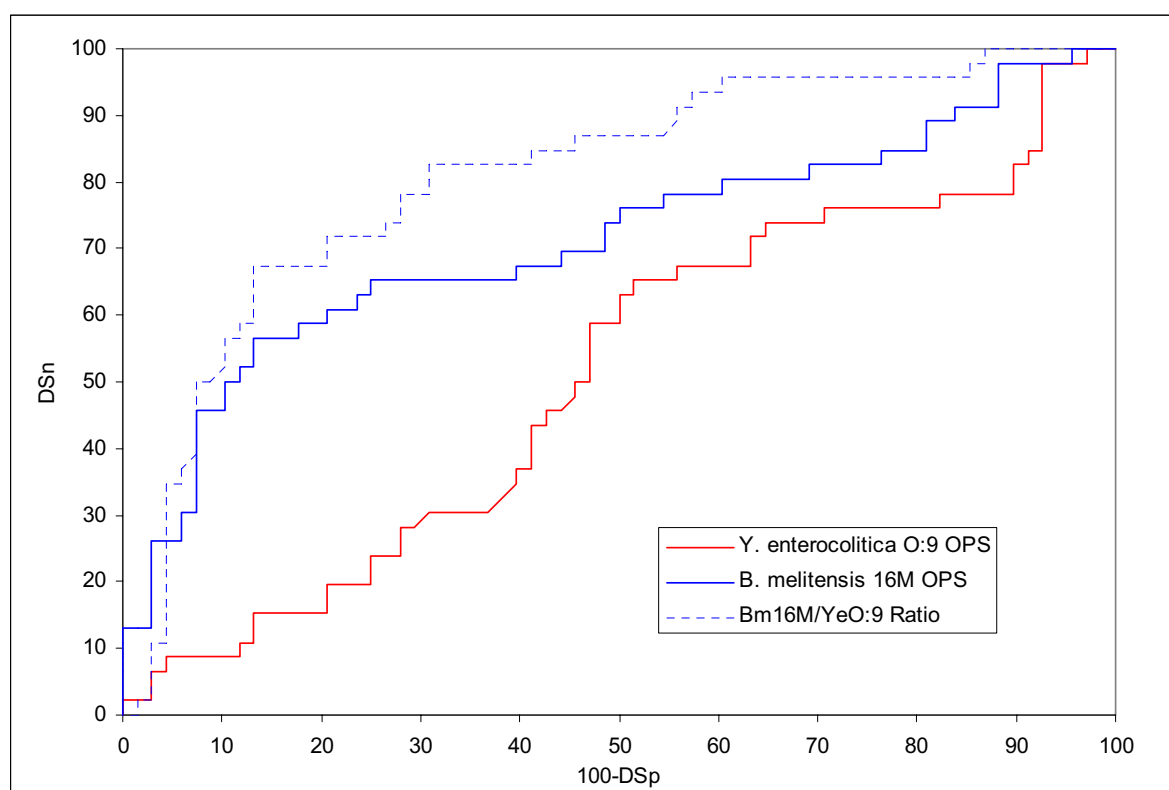


Figure 3.26. ROC curves for *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS iELISAs applied to cattle sera

ROC Curves generated from the results of the *B. melitensis* 16M OPS and the *Y. enterocolitica* O:9 OPS iELISAs, and the Bm16M/YeO:9 ratio as applied to sera from cattle confirmed as infected with *B. abortus* (n=46) and sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

Assay	Optimal Youden Index ($YI = D_{Sn} + D_{Sp} - 1$)			ROC - Area Under Curve	
	YI Estimate	D_{Sn} %	D_{Sp} %	AUC	95% Confidence Interval
<i>Y. enterocolitica</i> O:9 OPS iELISA	0.1375	65.22	48.53	0.5083	0.3990 - 0.6176
<i>B. melitensis</i> 16M OPS iELISA	0.4328	56.52	86.76	0.7123	0.6096 - 0.8149
Bm16M/YeO:9 Ratio	0.5415	67.39	86.76	0.8090	0.7264 - 0.8916

Table 3.3. Performance statistics for OPS iELISAs as tested on cattle sera

Assay	<i>B. melitensis</i> 16M	Bm16M/YeO:9
<i>Y. enterocolitica</i> O:9 OPS iELISA	< 0.0001	< 0.0001
<i>B. melitensis</i> 16M OPS iELISA		0.202

Table 3.4. Comparison of ROC AUC statistics for the OPS iELISAs as tested on cattle sera

3.9.2.4. Immunoassay: rLPS iELISA on cattle sera

The cattle sera was also evaluated by rLPS iELISA using methods analogous to those applied for the detection of anti-rLPS antibodies in pig, the results for which are shown above. The results are shown as a scatter plot against the results from the *Y. enterocolitica* O:9 OPS iELISA in figure 3.27. The rLPS iELISA results from the pig sera showed effective discrimination between true positive samples and those classified as FPSRs in conventional assays and appears to be a useful confirmatory serological assay. The results in figure 3.27 show that this is not the case in the cattle sera evaluated. Other than six of the true positive sera the rLPS data is well spread across both serum populations. This data suggests that the rLPS iELISA will not be the solution to the FPSR problem in cattle. These results do not replicate those published elsewhere (Nielsen et al., 2005; Nielsen et al., 2006b). This may be due to the nature of the populations under study. The sera from *B. abortus* infected cattle collected and evaluated in this study were all from Great Britain, and acquired during a period of intensive surveillance for brucellosis. These animals may have been in a relatively acute state of infection compared to the animals from which samples were taken for evaluation in other studies. Animals with a more prolonged and chronic infection may generate a more diverse antibody repertoire which includes the production of antibodies to antigens other than OPS.

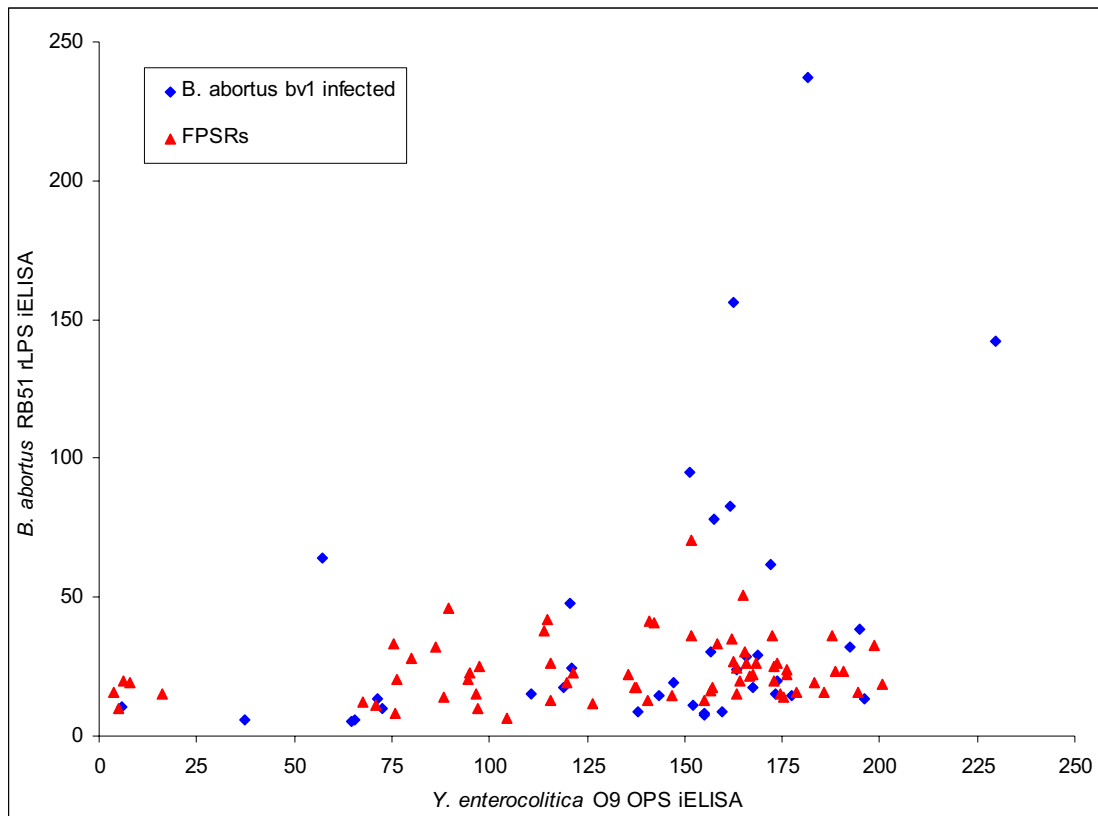


Figure 3.27. *B. abortus* RB51 rLPS against *Y. enterocolitica* O:9 OPS iELISAs for cattle sera

The results of iELISA with *Y. enterocolitica* O:9 OPS (x-axis) and *B. abortus* RB51 rLPS iELISA (y-axis) are shown above. The blue triangles are results from *B. abortus* infected cattle, the red triangles are results from non-*Brucella* infected FPSR cattle.

Nevertheless, the data from the rLPS assay serves to support the theory that the population dependent differences in antibody binding to the different OPS antigens is firmly related to structures present on the OPS rather than elsewhere. The results from the rLPS iELISA are shown in figure 3.28 where they have been plotted against the Bm16M/YeO:9 ratio. There is a positive relationship between the anti-rLPS titre and the Bm16M/YeO:9 ratio for the sera from the *B. abortus* infected cattle. However, there is also a positive relationship with the FPSR sera which is greater. As such the data demonstrates that antibody titres against the rLPS could make no net positive contribution to the average titre of the sera from the *B. abortus* infected animals in OPS iELISA.

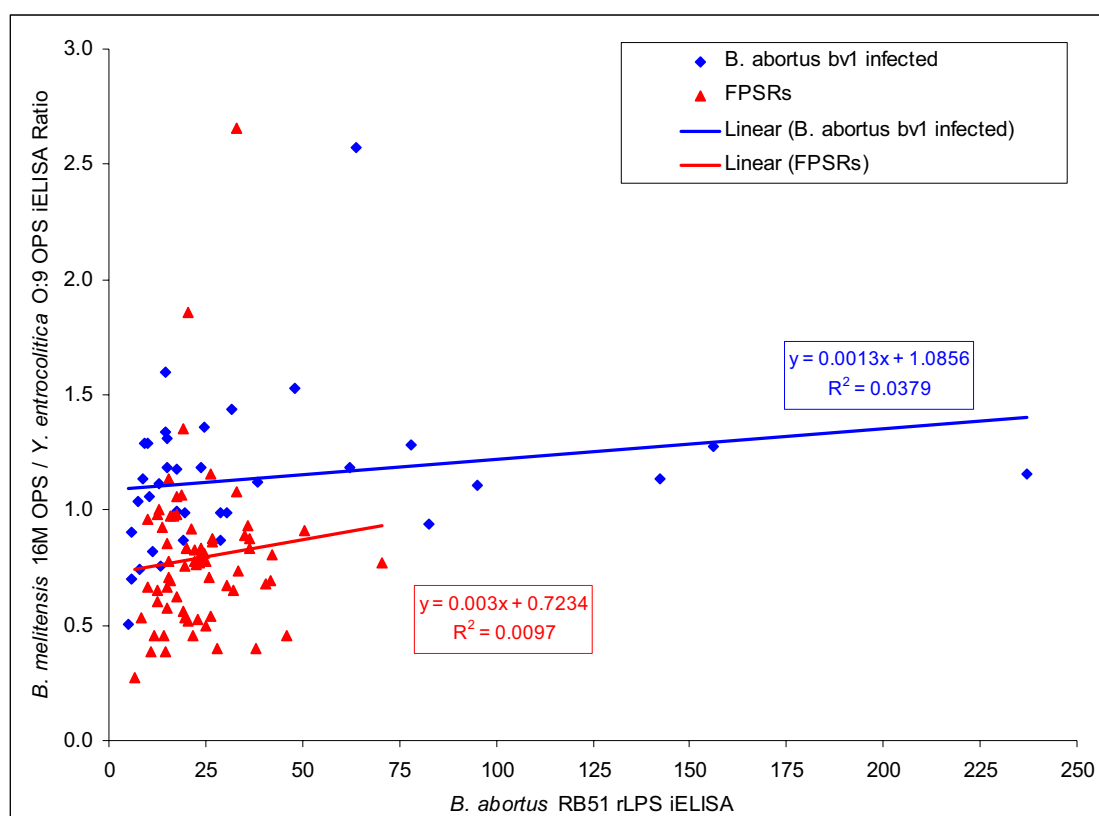


Figure 3.28. *B. abortus* RB51 rLPS iELISA against Bm16M/YeO:9 ratio for cattle sera

The results of iELISA with *B. abortus* RB51 rLPS iELISA (x-axis) are shown against the Bm16M/YeO:9 ratio. The blue diamonds triangles are results from *B. abortus* infected cattle, the red triangles are results from non-*Brucella* infected FPSR cattle. The data in the coloured boxes show the equations of the best fit lines and the coefficient of determination (R^2).

3.9.2.5. Summary of serological data from cattle sera

The results from the cattle sera reinforce the findings from the pig sera and reflect the potential universality of these findings to all host species. Once again, the discovery of the significance of the M epitope promotes further enquiries in order to more precisely define its role and relevance to serodiagnosis. It is also worth emphasising, as was the case with pigs, that the potentially important role in diagnosis for the M epitope has been demonstrated using sera from cattle infected with A dominant strains of *Brucella*.

3.10. Chapter 3: Summary and conclusions

The recent publication revisiting the structure of the *Brucella* OPS (Kubler-Kielb and Vinogradov) found that the M epitope, if present on the OPS, is always at the terminal end and will therefore be most exposed to the extracellular environment and host immune system. This may explain why even sera from animals infected with A dominant strains of *Brucella* react in the manner described above. This data refutes some of the preconceptions, described in the introduction, relating to the value of the M epitope structure to the universal diagnosis of brucellosis. An important caveat to this is the case of *B. suis* biovar 2 as this has no C specific or M epitopes.

The investigative work performed so far on the antigen-antibody interactions suggests that differences in the host antibody response to OPS as a result of infection with smooth *Brucella* or *Y. enterocolitica* O:9 are detectable in a number of experimentally and field infected animals. The ability of specific structures in the OPS to generate specific monoclonal antibodies has been known for many years but this is the first time that these structural differences have been shown to evoke specific antibody populations in naturally infected hosts or in any livestock species. The existence of these antibodies, one of the main hypotheses of this project, suggests that if the specific antigen(s) against which they are reacting can be isolated for diagnostic use then a more effective diagnostic assay to resolve FPSRs in cattle may be developed. This warrants further investigation as described in subsequent chapters. The methods and the data presented are novel and in contradiction of the current paradigm that for FPSRs the OPS antigen only represents the problem and has no part in the solution.

Chapter 4

CHARACTERISATION of OPS and OLIGOSACCHARIDES

4.1. INTRODUCTION

The data described in chapter 1 demonstrated that the OPS antigens derived from *B. abortus*, *B. melitensis* and *Y. enterocolitica* O:9 by hot phenol extraction, mild acid hydrolysis, centrifugation and desalting reacted vigorously with serum antibodies from *Brucella* and *Y. enterocolitica* O:9 infected cattle and swine. These antigens also reacted with sera of indeterminate aetiological origin that cross react in classical and contemporary serology for brucellosis. The methods deployed for the production of these antigens have been shown to lead to the purification of OPS. The comparative serology performed demonstrated that there is a significant difference between the reactions of antibodies to the homologous or heterologous OPS antigens in animals of known infection status. Furthermore, this pattern also exists when evaluating reactive sera from animals known not to be infected with or exposed to *Brucella*. However, although the binding profiles were significant at the population level, the method was not sufficient at the level of the individual animal to be a useful diagnostic. It may be that the presence on the native OPS of the common epitopes and their overlapping nature creates too much variance in the antibody binding interactions to be able to determine a sufficiently precise reaction to the specific OPS epitope antigens present.

4.2. Aims and Objectives

In order to reduce variance caused by the presence of common epitopes it is necessary to test the individual epitopes and evaluate them as individual, discrete serodiagnostic antigens. In order to do this, these discrete epitope antigens must first be produced. They may be developed as recombinant antigens through genetic engineering, chemically synthesised, or generated from the native antigen. A recombinant 4-N-formyl perosamine polysaccharide antigen has recently been produced (Iwashkiw et al., 2012) using the OPS synthesis genes from *Y. enterocolitica* O:9. This product will therefore not possess the specific epitopes of interest. The recombinant approach will not yield the antigens of interest until they, and the specific genes required for their synthesis, are identified. Synthesis is a highly skilled, specialised and expensive task and the structure of the specific epitopes of interest may not have been precisely defined. The final approach, to produce discrete epitope antigens from the native OPS, may allow for the generation of structural information about the epitope antigens. This was therefore the approach taken. A key element of the strategy to produce the individual epitope antigens from the native OPS was to use mass spectrometry as an analytical tool to evaluate the status of the native OPS antigen, monitor the generation of

oligosaccharides from partially depolymerised native OPS, and evaluate the structure of the oligosaccharides generated.

4.3. Gas Chromatography Electron Impact Mass Spectrometry (GC-MS)

GC-MS is a staple technique for the identification of monosaccharides within complex mixtures. The procedure can be used for composition analysis of polysaccharides after depolymerisation – which is most commonly conducted by acid hydrolysis. Trifluoroacetic acid is often favoured owing to its strength and the ease with which it may be removed by evaporation under dry nitrogen. After hydrolysis, the carbohydrates may be reduced to create alditols which may then be acetylated to produce alditol acetate derivatives of the monosaccharides that were present in the hydrolysed sample. These derivatives are more volatile than their precursors and variants may be reproducibly separated during gas chromatography on the basis of their size, conformation and chemical composition (Fox et al., 1989). At the end of the gas capillary the derivatives are ionised and fragmented by electron impact. This fragmentation occurs in a reproducible fashion and generates characteristic ions. By combining the peak retention time obtained from the chromatography and the information within the ion spectra relating to particular retention times it is possible to determine the identity of many common monosaccharides (although not all are completely resolved) and to some extent derive structural information of unknown monosaccharides.

It is also possible to predict the characteristic fragmentation ion spectrum from monosaccharides of known structure. In any event, an ion spectra of the derived alditol acetate from the *Brucella* and *Y. enterocolitica* O:9 OPS perosamine (D-Rha4NFO) has previously been described (Caroff et al., 1984a; Caroff et al., 1984b) and is shown in figure 4.1, with the predicted structure of the alditol acetate (which includes deuterium). The spectrum shows ions created due to fragmentation between C4-5 (m/z 289) and C3-4 (m/z 159). Other ions such as m/z 247 and 229 may be due to loss of ketene (m/z 42) and acetic acid (m/z 60) from the 289 m/z fragment and ion m/z 187 due to loss of both. Acetoxyl (m/z 59) may also be lost although resultant ions were not clear in this case.

GC-MS was therefore chosen as the first method to analyse the composition of the OPS within the antigen preparation though the interpretation of the EI mass spectra of alditol acetates derived from the hydrolysed antigen. Unfortunately the analysis by GC-MS did not provide data that identified D-Rha4NFO in the *B. abortus* S99 or *B. melitensis* 16M OPS samples. The successful identification of

the controls suggested that the process of carbohydrate reduction, conversion to alditol acetates, separation in the gas phase and fragmentation at the ionisation source were successful. The control process also included the use of an internal hydrolysis (hydrolysis was by 0.5 M and 2 M TFA) control (Lacto-N-hexaose, MW 1072.96, C₄₀H₆₈N₂O₃₁) whereby the constituent saccharides (Gal, Glc and GlcNAc) were individually identified in the subsequent mass spectrum by their characteristic EI mass spectra.

The inability to observe the D-Rha4NFO derivative from the OPS samples is therefore most probably related to: insufficient hydrolysis, over hydrolysis leading to decomposition, contamination with inhibiting substances (although internal monosaccharide controls were effectively derivatised and identified) or insufficient D-Rha4NFO content. Given that up to 100 µg of the OPS antigen preparation (derived from mild acid hydrolysis, centrifugation and desalting) was used for GC-MS analysis it is unlikely that the D-Rha4NFO content was insufficient. The quantity of the carbohydrate standards used varied from 7.5 – 14.5 µgs of monosaccharides and 20 µg of the oligosaccharide hydrolysis control. The OPS stocks evaluated by GC-MS had very high titres in antibody immunoassay not only suggesting that the material was sufficiently abundant but that it did indeed contain the D-Rha4NFO homopolymer. Unfortunately, the GC-MS analysis was unable to substantiate this.

Previous descriptions of the depolymerisation of *Brucella* OPS and similar saccharides describe some difficulties and are inconsistent. Complication may arise because although amino sugars generally possess more stable glycosidic linkages when subjected to acid hydrolysis, at the same time deoxy sugars are particularly susceptible to acid decomposition (Biermann, 1989). *Brucella* persoamine is both aminated (N-formamido) and deoxy (4,6-dideoxy). The loss of formyl groups so as to leave a primary amine of the 4th carbon and release formic acid is also a likely outcome of acid hydrolysis. As the N-formyl groups are such a critical component of the antibody interaction (Isshiki et al., 1995) with OPS its loss and replacement with a positively charged amine is of considerable consequence to this study. Additional evidence for this is that all of the bacterial OPS structures known to exhibit a degree of cross reaction against anti-*Brucella* antibodies possess carbohydrates within their sequence that have an N-formyl or other N-acyl group on the 4th carbon.

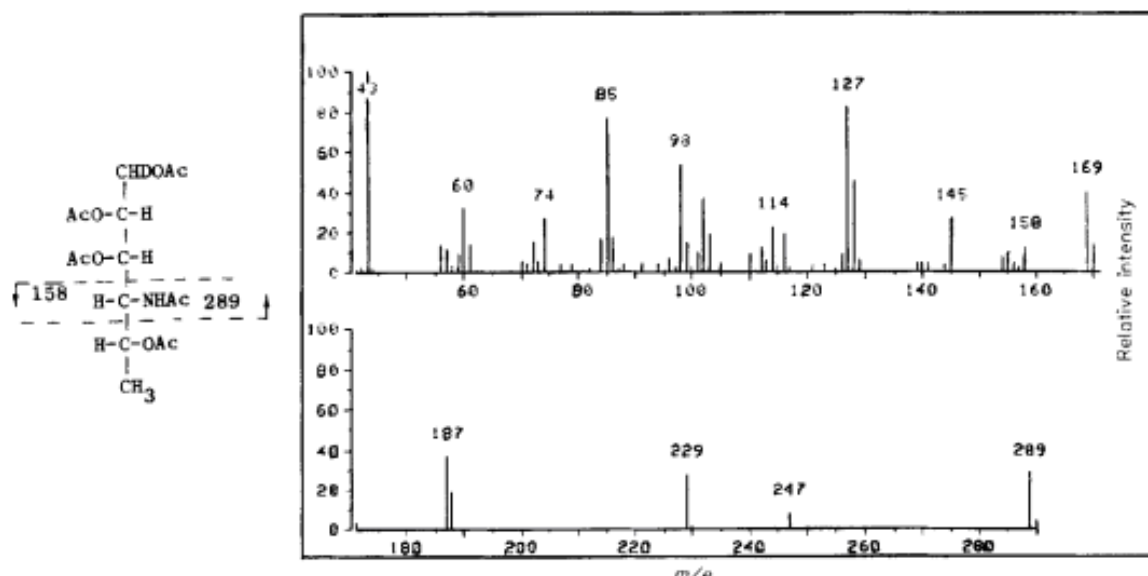


Figure 4.1 GC-MS of alditol acetate derivative of perosamine (D-Rha4NFo)

GC-MS ion spectrum of the alditol acetate derived from depolymerised, by solvolysis with hydrogen fluoride, *Y. enterocolitica* O:9 (Caroff et al., 1984a) and *B. abortus* S99 (Caroff et al., 1984b). Both were deacetylated prior to solvolysis.

4.4. Summary of depolymerisation methods for perosamine OPS

The OPS from *Vibrio cholerae* (serotype Inaba) is composed of a very similar repeating sugar unit to *Brucella* with N-acyl groups present on the fourth carbon. This OPS was reported as being resistant to standard acid hydrolysis methods including the use of 1 M sulphuric acid (100°C for up to 16 hrs) and 2 M trifluoroacetic acid (100°C for 16 hrs), however subsequent brief treatment with 10 M HCl at 90°C for 10 mins yielded only perosamine albeit with a great deal of decomposition (Redmond, 1978). In contrast studies on the OPS from *Brucella abortus* strain 1119-3 (A dominant) (Caroff et al., 1984b) note that it is completely degraded by hydrolysis with 2 M HCl or 1 M sulphuric acid and by methanolysis - but fail to describe the complete methods upon which this conclusion is reached.

Related work on the O-chain from *Y. enterocolitica* O:9 (Caroff et al., 1984a) describes hydrolysis conditions of 1 M sulphuric acid at 100°C for 15 hrs but without successful identification of the main OPS component (hydrolysis with 2 M HCl was reported to give the same negative result but specific conditions were not stated) but significant yields of hydrolytic products suggesting to the authors that the main component of the OPS was being degraded. Methanolysis with 2.5% methanolic HCl at

100°C (but unspecified time) was described to result in N-deacylation but otherwise leaving the OPS unchanged. However, the use of anhydrous hydrogen fluoride (HF) depolymerised both *B. abortus* and *Y. enterocolitica* O:9 N-acetylated (HF at 20°C for 16 hrs) or methylated (HF at 22°C for 3 hrs) OPS resulting in the identification of perosamine derived structures by GC-MS as shown in figure 4.1. This was also performed on the OPS from *B. melitensis* 16M (Bundle et al., 1987c), and used to identify 4-acetamido-4,6-dideoxy-D-manopyranosyl in the LPS OPS of *Escherichia coli* O:157 (Masoud and Perry, 1996).

The elucidation of the structurally similar OPS from *Francisella tularensis* also required the application of solvolysis by anhydrous HF to the native polysaccharide (2 hrs at room temperature) to identify the presence of 4,6-dideoxy-4-formamido-D-glucose (albeit with an apparent loss of 90%) as acid hydrolysis with 4 M HCl (100°C for 4 hrs) did not result in detectable depolymerisation (Vinogradov et al., 1991). More recently an investigation of the capsular polysaccharide from *Francisella tularensis* revealed that it also contained 4,6-dideoxy-4-formamido-D-glucose as part of a tetrasaccharide repeat (Apicella et al., 2010). Evidence for this structure came from NMR as there were no indicative derivatives visible by GC-MS after acid hydrolysis with 4 M HCl (100°C for 6 hrs) or methanolysis (1 M methanolic HCl at 80°C for 16 hrs).

It would seem most likely that the *Brucella* O-chain is both relatively resistant to acid hydrolysis and susceptible to decomposition once degraded. The favoured approach to depolymerisation is through solvolysis by HF either in the native or derivatised form (derivatised forms being potentially less susceptible to monomer degradation). Solvolysis progresses via a different reaction mechanism from acid hydrolysis owing to the reduction or absence of water. The glycosidic bonds are cleaved to yield glycosyl-halides which can be converted to monosaccharides by introduction into an aqueous environment. Depolymerisation through HF solvolysis is also more likely to leave N-acetyl or N-formyl groups intact than acid hydrolysis and as such is a useful tool in the compositional analysis of polysaccharides (Knirel et al., 1989; Yadav et al., 1994). HF is however exceptionally dangerous to work with as it is highly volatile and becomes extremely acidic upon contact with water.

A safer possible alternative solvolytic agent, trifluoromethanesulfonic acid ('triflic' acid), has more recently been described (Knirel and Perepelov, 2002). This has been used in structural studies of acid resistant bacterial OPS to identify aminated deoxysugars via depolymerisation (Kocharova et al., 2001; Kondakova et al., 2003; Perepelov et al., 2007; Perepelov et al.; Perepelov et al., 2000). The anticipated ion spectra of the *Brucella* perosamine would be different from that derived through

acid hydrolysis as the formamido groups are likely to be retained and the final mass of the C4 group would be 57 Da (HCNFO) rather than 71 (HCNHAc) (Vinogradov et al., 1991). Initial attempts to detect perosamine from *Brucella* OPS subjected to solvolysis with triflic acid were not successful. Although further optimisation of this approach, in particular refining the means by which excess triflic acid is removed from the sample prior to derivitisation, may have eventually proven fruitful. However, this study was set aside in order to pursue alternative methods of structural analysis.

Despite the attempts made the *Brucella* perosamine was not identified from the OPS used in the GC-MS experiments. It may be hydrolysed but subsequently rapidly degraded by the hydrolytic or solvolytic environments (Bundle et al., 1987c; Caroff et al., 1984a; Caroff et al., 1984b), it may also be resistant to hydrolysis or solvolysis within these environments (Redmond, 1975, 1978, 1979; Vinogradov et al., 1991). Given the poor status of the existing knowledge the number of samples that may need to be tested in order to identify suitable conditions for generation of intact perosamine monosaccharides and oligosaccharides may be large. Using GC-MS of derivitised samples as a tool to evaluate these samples would not be appropriate given the relative complexity of the post hydrolysis process and the inability to simultaneously monitor the status of the precursor polysaccharide. For example negative (blank) results would not indicate whether over or under hydrolysis had occurred.

4.5. Analysis of unhydrolysed OPS antigens by MALDI-ToF

Evaluation of the *Brucella abortus* S99 OPS by MALDI-ToF provided the potential to gain structural insight into the mass of the polymers present and their mass increments related to the perosamine units. However, this approach also presented some challenges. The OPS is a polar and non-volatile structure that is not particularly amenable to transmission into the gaseous state. It doesn't possess easily ionisable groups such as the primary amines and carboxylic acids found on peptides and proteins. It also has a wide mass range owing to the modal distribution of the number of sugars that make up the polysaccharide chain. Therefore, although considered as a single molecular type it is polymorphic and the mass, and therefore the m/z , will be distributed across the mass spectrum. Each ion will therefore only be a fraction of the total present and its abundance will be diluted accordingly leading to a relative loss of sensitivity when compared to ion abundance for a molecule with a single molecular mass.

4.5.1. MALDI-ToF of non-derivitised, non-hydrolysed *Brucella* OPS

The OPS was initially explored by MALDI-ToF in its native, unmodified form with DBH (2,5-dihydroxybenzoic acid) matrix. This matrix was used for all MALDI-ToF spectra presented in this thesis. A spectrum from the *B. abortus* S99 OPS material was obtained using a Voyager-DE STR mass spectrometer operating in the linear mode, this is shown in figure 4.2. Although the spectrum clearly demonstrates repeating mass units of approximately 173 m/z, which fits with repeating single unit increments of D-Rha4NFO, the spectrum is poorly resolved. In addition, the method appears to be very selectively sensitive as the mass units relating to the smaller polymers have higher intensity even though they are generally considered to be less abundant. The spectrum certainly does not conform to the perception of the polysaccharide as being evenly distributed around a modal length. The average number of perosamine residues in *Brucella* OPS is reported to be 96-100 (equal to a mass of 16,608 – 17,300 Da (Cardoso et al., 2006)). The data shown in figure 4.2 suggests a maximum mass of approximately 8,000 Da (equal to approximately 46 persoamine units). The difference may be due to a loss of sensitivity in the MALDI-ToF experiment as the length of the polymer increases and it becomes increasingly more difficult to ionise.

A high concentration of native OPS was required to obtain any visible peaks by MALDI-ToF. The spectrum in figure 4.2 was from a 20 mg/ml solution of OPS.

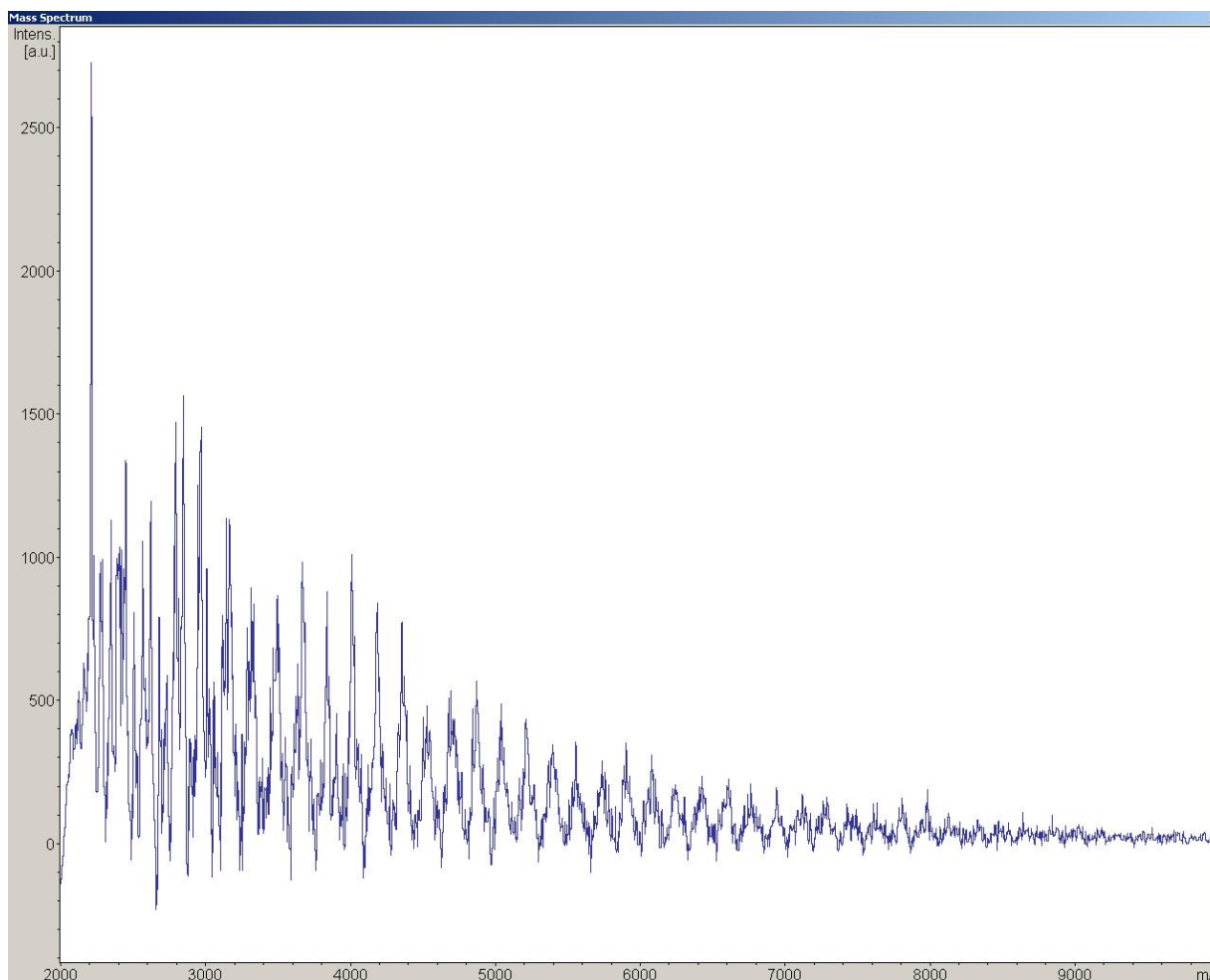


Figure 4.2. MALDI-ToF mass spectrum of native *B. abortus* S99 OPS

MALDI-ToF mass spectrum of native, non-permethylated, *B. abortus* S99 OPS from 2,000 to 10,000 m/z. Derived from the Applied Systems Voyager STR-DE MALDI-ToF. The individual peaks from the spectra were insufficiently resolved to satisfactorily annotate.

4.5.2. MALDI-ToF of permethylated non-hydrolysed *Brucella* OPS

A superior spectrum was obtained from the *B. abortus* S99 OPS that had been permethylated (Kang et al., 2005). This was obtained using a Bruker AutoFlex II MALDI-ToF with a DBH (2,5-dihydroxybenzoic acid) matrix. This is shown in figure 4.3. This, and all subsequent MALDI-ToF spectra presented, was acquired using a Bruker AutoFlex II. In this instance the equipment was operated in linear mode with matrix suppression enabled. The results from MALDI-ToF on the permethylated OPS demonstrated that this approach greatly improved sensitivity (Hung et al., 2012). Nevertheless it was still necessary to aggregate the spectra from multiple acquisitions from regions

of the sample spot on the source plate that provided the best data. The mass spectra from 2000 to 15,000 m/z (as shown in figure 4.3) provided greater intensity peaks across a wider m/z range and more consistent profiles from a lower quantity of material compared to the spectra from the underivatised OPS.

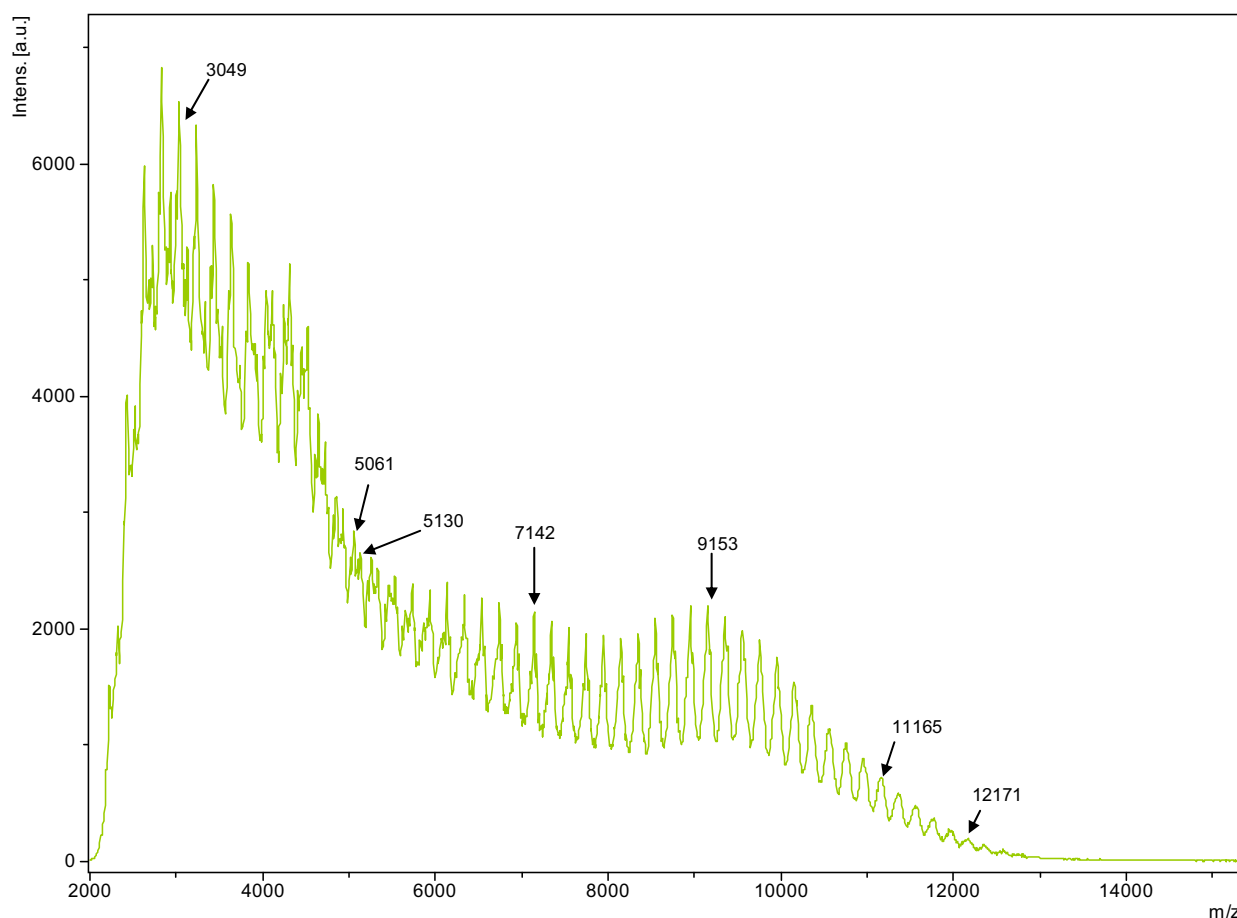


Figure 4.3. MALDI-ToF mass spectrum of permethylated *B. abortus* S99 OPS

MALDI-ToF mass spectrum of permethylated *B. abortus* S99 OPS from 2,000 to 15,000 m/z derived from a Bruker AutoFlex II. The ion represented by the peak at 12171 m/z corresponds to an ion with five more D-Rha4NFO units (permethylated mass within polymer = 201) than the ion at m/z 11165. The peaks at 9153, 7142 and 5130 correspond to ions with 15, 25 and 35 fewer permethylated D-Rha4NFO units respectively. The m/z difference between each peak is approximately 201. Double peaks appear at the lower m/z range of the spectrum, for example peaks at 5130 and 5061 m/z . The peak at 3049 m/z corresponds to an ion with 10 fewer D-Rha4NFO units than the peak at 5061 m/z .

4.5.3. Comparison of linear and reflection MALDI-ToF modes for evaluation of unhydroysed *Brucella* OPS (with and without matrix suppression)

Although the mass spectra of the OPS was improved, and quite striking, the resolution of the data was still poor but it is possible to discern that the profile consists of repeating evenly spaced peaks ranging from m/z of approximately 2,500 to 12,500. The gap between the peaks corresponds to the predicted mass of the permethylated D-Rha4NFO unit. This is 201 due to the addition of two methyl groups to the native sugar, one methyl group is added to the free hydroxyl present on either the 2nd or the 3rd carbon (dependent on the linkage) and the other methyl group is added to the nitrogen on the 4th carbon which is part of the N-formyl group (Hase and Rietschel, 1976). The spectrum suggests that, once the background signal has been taken into consideration, the most abundant high molecular mass species is at approximately 9,000 m/z corresponding to a polymer of approximately 44 units. The maximum length polymer estimated from this spectrum would be approximately 62 units. Given the current state of understanding related to the structure, this appears to be a credible modal length. However, a large amount of material was still required to generate this spectrum.

For the purposes of this project, the presence and detection of smaller oligosaccharides of the OPS is of critical interest. Therefore MALDI-ToF was also used to detect ions less than 2,000 m/z . The mass spectra in the top panel of figure 4.4 is the same as that shown in figure 4.3 and the spectra in all three panes have been derived from permethylated *B. abortus* S99 OPS. The spectrum shown in the top panel was acquired in linear mode with matrix suppression turned on. The same sample was also analysed in linear mode with matrix suppression disabled. This allowed for data to be collected at lower m/z values although, in this instance, the spectrum (shown in the middle panel) is dominated by the ions generated from the matrix. Nonetheless repeating peaks consistent with the structure of the OPS are demonstrable in this spectrum. As before, the increased sensitivity of detection for ions of smaller m/z is evident in the data.

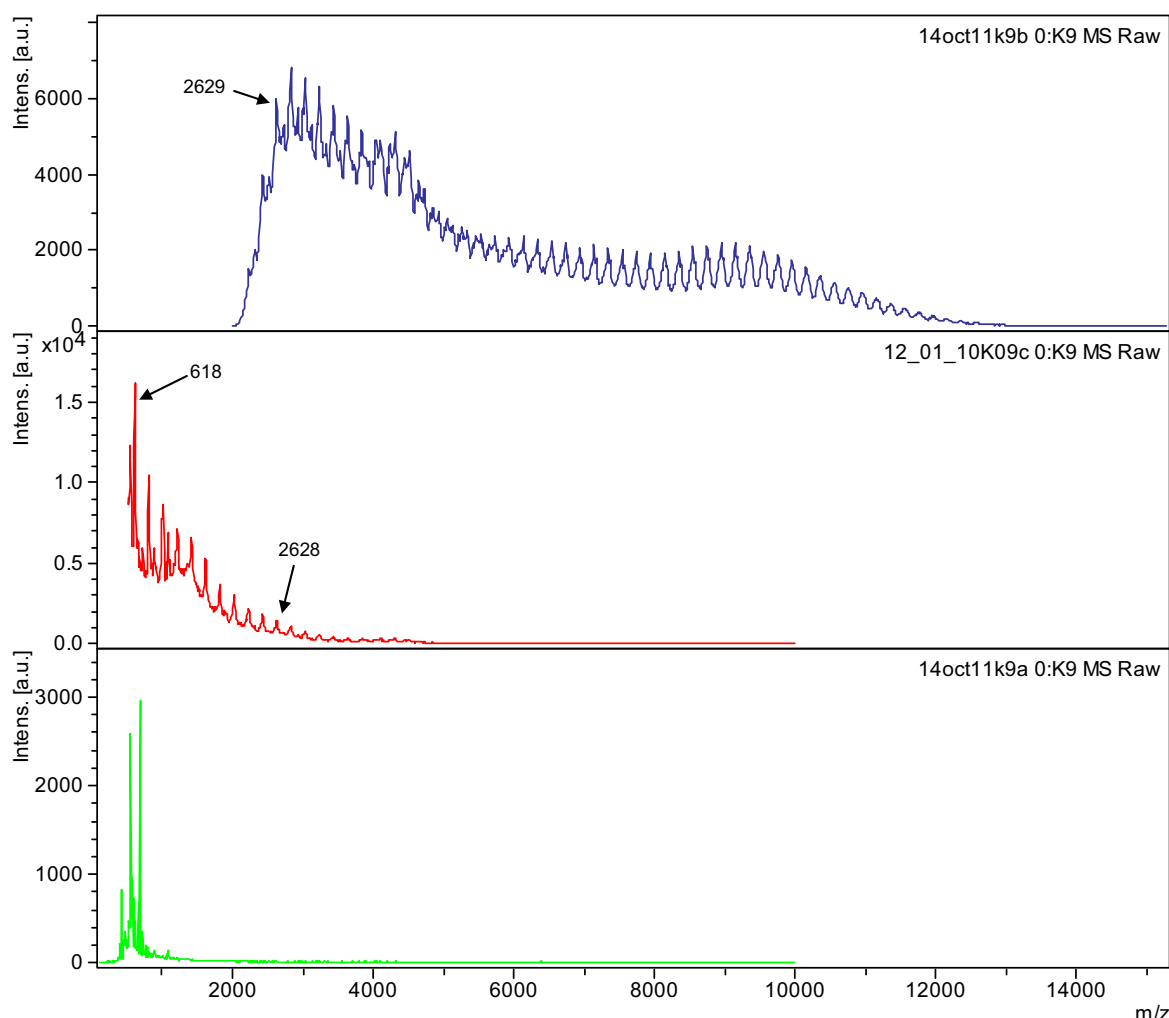


Figure 4.4 MALDI-ToF MS of permethylated *B. abortus* S99 OPS acquired in different modes: 0-15,000 m/z

MALDI-ToF Mass Spectra of permethylated *B. abortus* S99 OPS acquired in linear mode (top and middle) and reflectron mode (bottom). Spectra acquired with matrix suppression (top) and without (middle and bottom). A low m/z peak attributable to an ion of permethylated OPS has been annotated in the top spectrum. A peak of very similar m/z has been annotated in the middle spectrum. The peak at 618 m/z corresponds to an OPS polymer with 10 fewer D-Rha4NFo units. The two main peaks in the bottom spectrum do not correspond to the peak series observed in the top two spectra.

Disabling matrix suppression allows for the detection of smaller m/z ions yet it is evident that the use of matrix suppression improves the data acquisition for ions of greater m/z. This as shown by the difference between the top and middle panels of figure 4.4. where, although the samples are the same, the detection of ions above 4,000 m/z is greatly improved when matrix suppression is on.

The spectrum shown in the bottom panel was acquired in reflection mode with matrix suppression off. There are no peaks evident that would relate to ions generated (and that were detected in linear mode) from the *Brucella* OPS. No peaks were detectable in reflectron mode with matrix suppression on. Despite the benefits that the use of the reflectron brings, essentially more highly resolved data, the loss of sensitivity in this mode in this instance limits its practical value within this context.

The same three spectra presented in figure 4.4 are also presented in figure 4.5 with the scales altered to show more of the data relating to the smaller ions. This shows that the combination of data acquired from both linear modes (suppression on or off) does allow for evaluation of a greater range of m/z values compared to either one of the modes alone. Where the m/z ranges for the two spectra overlap the continuum of peaks related to the repeating units of the D-Rha4NFo within the OPS can be seen. One again, nothing is evident in the data acquired in reflectron mode. As a result of this, assigning accurate mass values to the peaks is not possible. The analysis software does allow for the assignment of m/z values to the peaks identified in linear mode although owing to the evident limitations in resolution, the accuracy of these values must be considered in moderation.

The peaks identified in the mass spectrum (taken in linear mode with matrix suppression off) shown in the middle panel of figure 4.5 have been annotated in figure 4.6. This shows m/z differences corresponding to a fully permethylated molecule incrementally increasing in size by successive D-Rha4NFo units. But the data does not correspond to a D-Rha4NFo homopolymer as the m/z values observed do not fit such a structure. A permethylated D-RhaNFo unit within the *Brucella* OPS would have a mass of 201. An OPS polymer would have additional hydroxyl groups at the reducing and non-reducing terminals, adding a mass of 18 and these would both be methylated adding a further mass of 2×14 . Therefore a D-RhaNFo homopolymer would have a mass according to the formula $201x$ (where x is the number of persamine units) + 18 (H_2O) + 28 ($2CH_2$). A disaccharide would therefore have a mass of $402 + 46 = 448$. A protonated or sodiated ion would have an m/z of 449 or 471 respectively. A trisaccharide would have mass of 649 and protonated and sodiated ions of m/z 650 and 672 respectively, and so on. Ions of either the protonated or the sodiated series are not evident in the spectrum from the permethylated OPS. It is also not possible to attribute the series of ions observed to the presence of proposed non-D-Rha4NFo units at the reducing end of the OPS. Two mannose sugars are proposed as being joined to the reducing end of the D-Rha4NFo homopolymer series (Kubler-Kielb and Vinogradov, 2013b). Therefore the precise structure relating to the observed ion series from the spectrum shown in figure 4.6 is not clear.

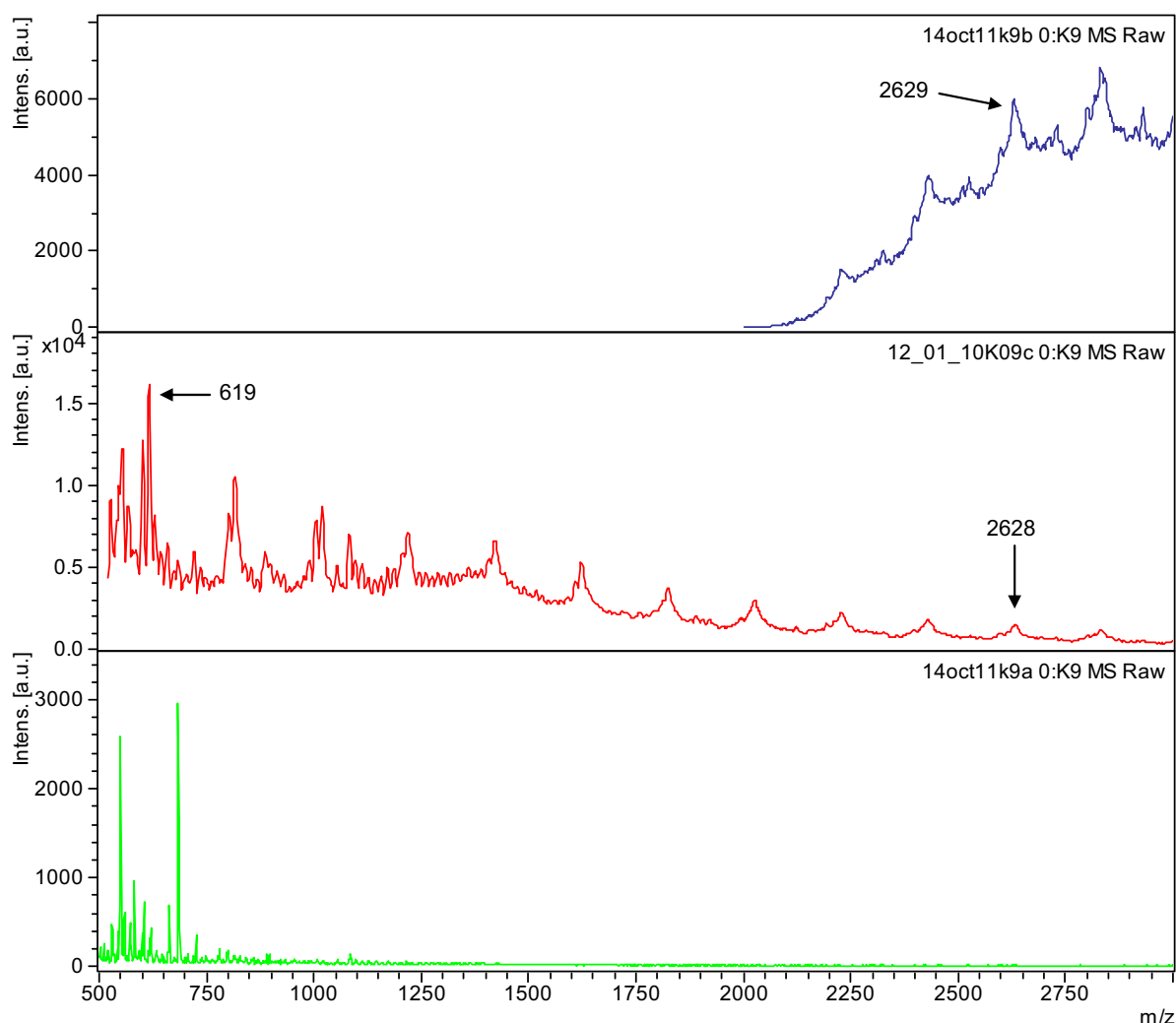


Figure 4.5. MALDI-ToF MS of permethylated *B. abortus* S99 OPS acquired in different modes: 0-3000 m/z

MALDI-ToF Mass Spectra of permethylated *B. abortus* S99 OPS acquired in linear mode (top and middle) and reflectron mode (bottom). These spectra were also presented in figure 4.4 over a different m/z range. Spectra acquired with matrix suppression (top) and without (middle and bottom). A peak attributable to an ion of permethylated OPS has been annotated in the top spectrum. A peak of very similar m/z has been annotated in the middle spectrum. The peak at 618 m/z in the middle spectrum corresponds to an OPS polymer with 10 fewer D-Rha4NFo units. The two main peaks in the bottom spectrum do not correspond to the peak series observed in the top two spectra.

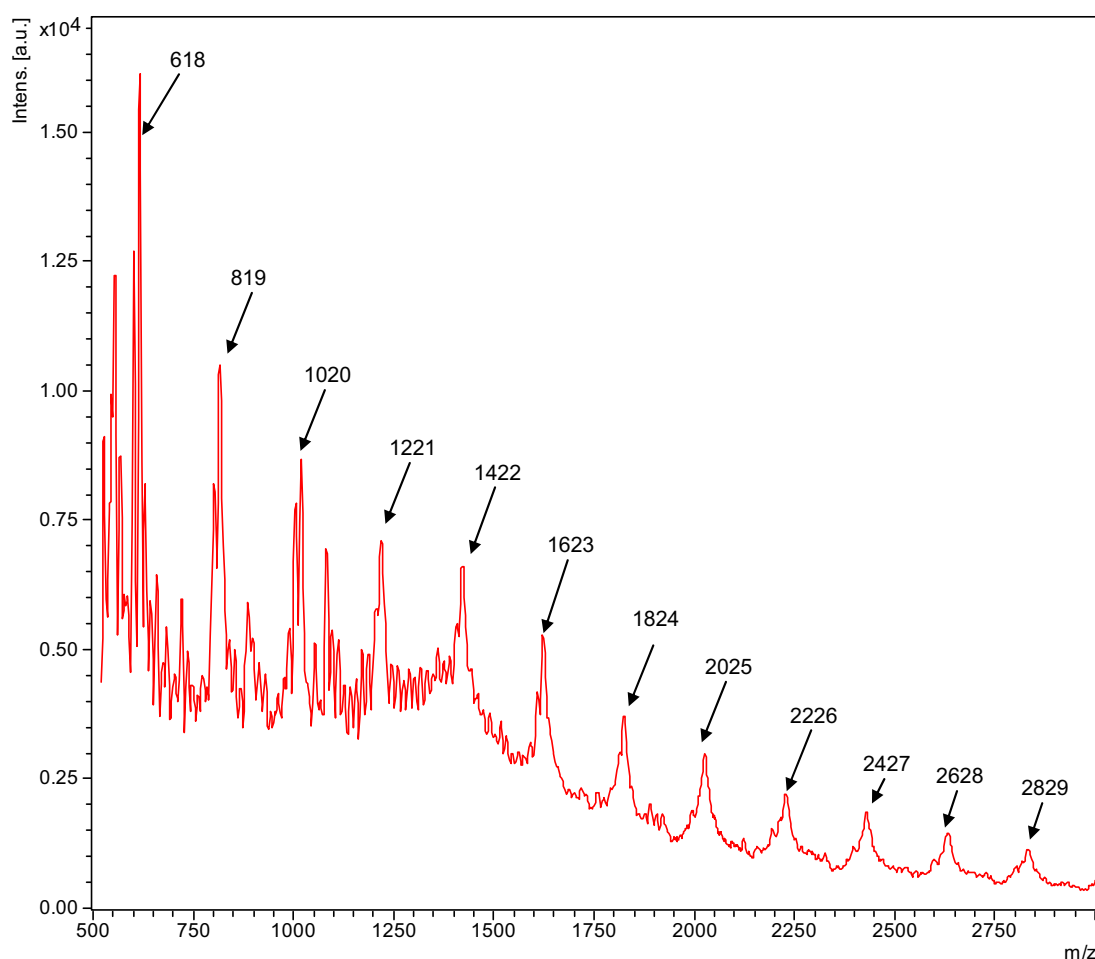


Figure 4.6. MALDI-ToF MS of permethylated *B. abortus* S99 OPS acquired in linear mode: 500-3000 m/z Spectrum acquired without matrix suppression as also shown in the middle panel of figure 4.5. The peaks have been annotated to show their m/z values which are 201 m/z values apart which correspond to a series of singly incrementing D-Rha4NFO residues.

The non-methylated OPS was also evaluated by linear MALDI-ToF. The spectrum acquired in the linear mode with matrix suppression off, shown in figure 4.7, did not contain a series of ions that related to those seen in the permethylated OPS sample. Permethylation of the sample clearly increases the analytical sensitivity of the investigative process. The spectrum did not contain any 173 m/z incrementally increasing series of ions as might be due to shorter members of the *Brucella* OPS polysaccharide. The annotated m/z values on the figure relate to the expected m/z values for a series of D-Rha4NFO homo-oligosaccharides, the values are equal to: $(173x) + 18 (\text{H}_2\text{O}) + 23 (\text{Na}^+)$, where x = D-Rha4NFO units (as can be seen in later spectra, the sodium adducts are the dominant species). There are no clearly distinguishable peaks at these values in the non-hydrolysed *B. abortus* S99 OPS.

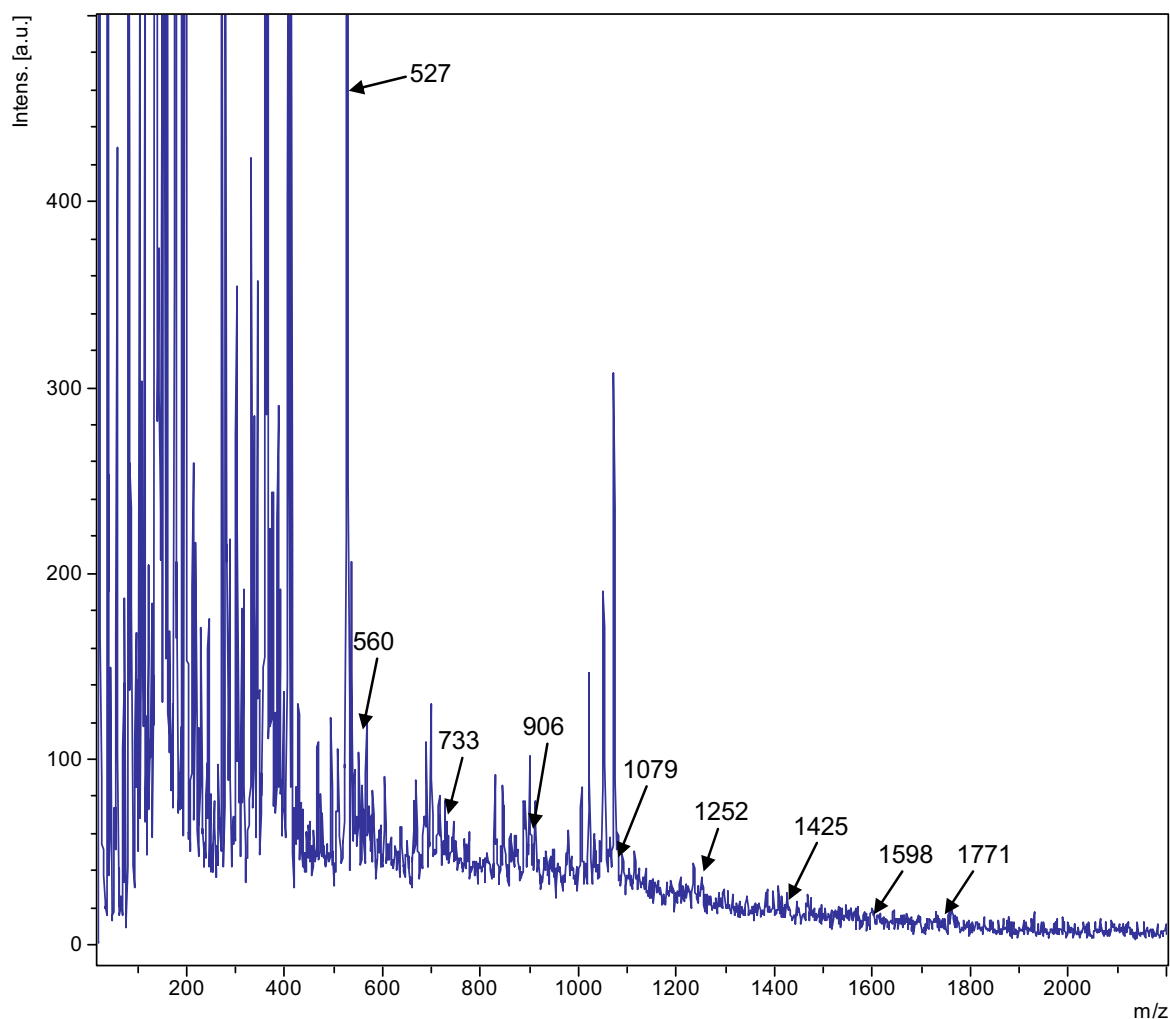


Figure 4.7. MALDI-ToF MS of non-permethylated *B. abortus* S99 OPS acquired in linear mode: 500-3000 m/z

Spectrum acquired without matrix suppression. The peak at 527 m/z is a maltitriose standard. The spectrum has also been annotated with m/z values that would correspond to a series of sodiated D-Rha4Nfo homo-oligosaccharides (560 corresponding to the trisaccharide). The spectrum does not show a series of ions that matches these values.

In summary the MALDI-ToF data on the *B. abortus* OPS gave some strong evidence for the presence of OPS by way of a repeating series of ions in the permethylated sample and some of these ions represented molecules short enough to present a very limited number of antibody epitopes. However these ions were not visible in reflectron mode or in the native (non-permethylated) OPS. The data from the linear mode indicated that these oligosaccharides were not homopolymers and without additional resolution it was not possible to further resolve their structure. Consequently, due to this structural uncertainty, they were not suitable as substrates for antibody

affinity selection. As anticipated, in order to generate D-Rha4NFo homo-oligosaccharides depolymerisation was necessary. Owing to the relatively acid resistant nature of the *Brucella* OPS it was predicted that non-D-Rha4NFo sugar units may be more readily hydrolysed and this may prove beneficial to the generation of the desired structures.

Owing to the length of bacterial OPS it is commonly considered too long to be examined directly by mass spectrometry. The usual analytical approach is for the OPS to be hydrolysed into more readily detectable oligosaccharides, especially when the OPS is composed of repeating units containing different sugars (this process may be expedited by the use of different hydrolysis conditions that selectively attach glycosidic bonds between specific sugar residues). If such units can be obtained in sufficient quantity and purity from the *Brucella* OPS then these may also be analysed by NMR.

4.5.4. MALDI-ToF analysis of *B. melitensis* and *E. coli* O:157 OPS

OPS from *B. melitensis* 16M and from *E. coli* O:157 was extracted, purified and permethylated as described for *B. abortus* S99. These samples were then analysed by MALDI-ToF and the spectra are shown below in figure 4.8. It is interesting to observe the difference between the three antigens. The *B. abortus* S99 OPS antigen provides a relatively clean, if not particularly well resolved mass spectrum, shown in figure 4.3, that clearly shows the repeating units (of 201 m/z difference according to the expected permethylated mass). The mass spectrum of the *B. melitensis* 16M OPS, shown in the middle panel of figure 4.8, is far less defined than that for the *B. abortus* S99 and this may, possibly, have something to do with the difference in the structure related to the frequency of the linkage types, it contains a 20% proportion of $\alpha(1\rightarrow3)$ links. Other issues including the presence of impurities may also be a factor. However it is still possible to determine a profile that relates to repeating peaks separated by approximately 201 m/z. However, it is noted that a published MALDI-ToF mass spectrum for *B. melitensis* 16M (Kubler-Kielb and Vinogradov) does contain more finely resolved peaks which are separated by the expected mass of the D-Rha4NFo unit and range from 3 to 33 units in length after which the peaks are undistinguishable from the noise. It is worth noting that the OPS analysed had been de-N-formylated by NaOH prior to MALDI-ToF potentially making them more amenable to ionisation through proton acquisition although the OPS had not been permethylated. Although even in this spectrum all peaks are of relatively low intensity compared to the background noise.

Both spectra, that published and that presented in figure 4.8, for the *B. melitensis* 16M OPS indicate that the OPS length increments in single D-Rha4NFo units as there is no obvious consecutive addition of discrete tetrasaccharide units as is the case with *E. coli* O:157, the MALDI-ToF spectrum for which is shown in the top panel of figure 4.8. However, as was commented upon (Kubler-Kielb and Vinogradov), the nature by which the *Brucella* OPS is constructed - with an initial series of $\alpha(1\rightarrow2)$ linked D-Rha4NFo units – means that it is not possible to rule out the possibility that the OPS subsequently extends by addition of tetrasaccharides after single unit heterogeneity has taken place. Although from what is understood of the OPS synthesis process this is unlikely (Greenfield and Whitfield, 2012).

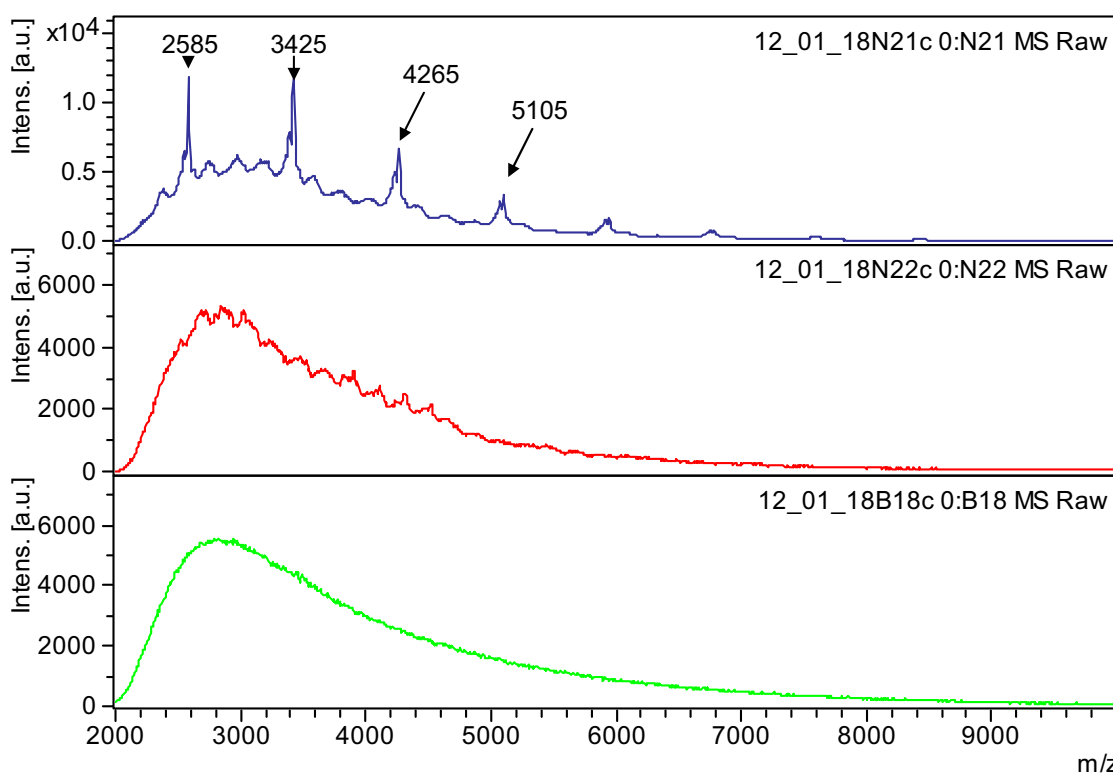


Figure 4.8. MALDI-ToF MS of permethylated OPS from *E. coli* O:157 (top) and *B. melitensis* 16M

The spectra above were acquired in linear mode with matrix suppression on. The m/z values of the significant peaks in the top panel have been annotated, the difference between each is 840 m/z . This is the mass of the permethylated repeating oligosaccharide unit. The bottom panel is a negative control sample (matrix only).

The MALDI-ToF mass spectrum for the *E. coli* O:157 OPS, figure 4.8, shows a clear, although not particularly well resolved, mass spectrum that corresponds to the mass of the tetrasaccharide repeating unit (Perry et al., 1986). The annotated mass spectrum for *E. coli* O:157, presented in figure 4.9, shows this information in more detail. As well as major peaks representing the tetrasaccharide repeats there are minor peaks below that correspond to the mass of the individual

sugars. The data suggests that the sequence of the repeating unit is not quite as described in the original paper. The sharper peaks for the *E. coli* O:157 OPS, compared to those in the *B. abortus* OPS mass spectrum probably reflects the greater relative abundance of the specific polymers as the repeats are in groups of 4 sugars rather than one (as in the *Brucella* OPS). The *E. coli* O:157 was chosen for analysis as an interesting example to compare between OPS synthesised by the ABC transporter mechanism and by the Wzx/Wzy mechanism. The MALDI-ToF mass spectrum clearly shows the existence of the tetrasaccharide unit. *E. coli* O:157 was chosen in particular owing to reports of its potential to raise antibodies cross reactive to *Brucella* OPS owing to the presence of D-Rha4NAc in its OPS (Nielsen, 2004; Perry and Bundle, 1990).

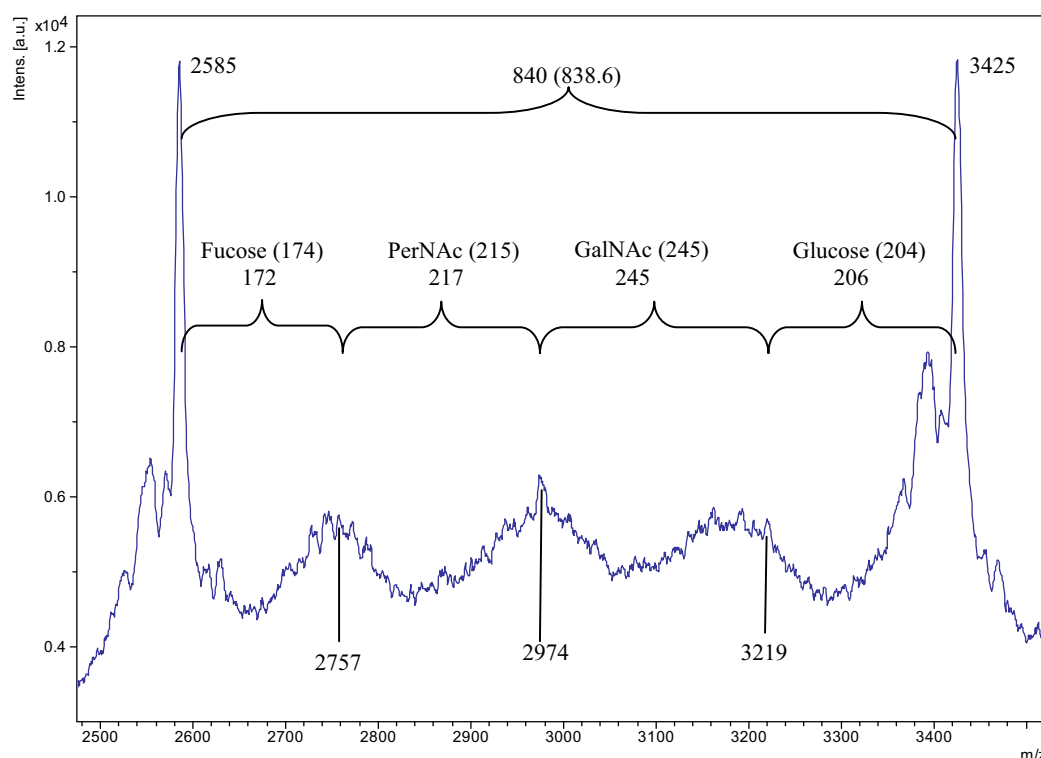


Figure 4.9. MALDI-ToF MS of permethylated *E. coli* O:157 OPS: m/z 2,400 – 3,100

E. coli O:157 OPS annotated with m/z values and sugars identified within the previously described structure (Perry et al., 1986) showing the tetrasaccharide repeating unit that makes up the molecule.

The analysis of the OPS samples by MALDI-ToF provided strong evidence that there was abundant OPS in the extracted antigens evaluated. The evidence pointed toward a repeating polymer containing incremental units of D-Rha4Nfo that varied in length from tri or tetrasaccharides to those that are 62 sugars long. Despite delivering this information, the suitability of MALDI-ToF as the main tool to monitor the progression of OPS depolymerisation was hampered by several factors. The

sensitivity of the method was relatively low even after sample permethylation, and the resolution was also relatively poor. In addition the ability to detect mono- and disacchrides is significantly impaired due to confounding ions originating from the matrix. As the hydrolysis reactions for *Brucella* OPS have not previously been effectively studied or understood (as described above) it was considered that the different hydrolysis conditions that will require investigation may vary in effectiveness from no effect to complete decomposition of all D-Rha4NFO. Given this range of uncertainty it was considered critical that any front line monitoring tool was able to readily evaluate any increase in monosaccharide abundance during hydrolysis so as to be able to help find the optimal balance between depolymerisation and decomposition.

4.6. Partial acid hydrolysis of OPS

As described above, the most successful approach to the depolymerisation of *Brucella* OPS is via the use of HF. As also described, this is a highly dangerous chemical. Although acid hydrolysis is of questionable value in this context, paradoxically, the successful depolymerisation of *Vibrio cholerae* OPS into perosamine monosaccharides had been achieved using concentrated (10 M) hydrochloric acid (Ito et al., 1994; Redmond, 1978). Furthermore concentrated HCl is also used to partially depolymerise chitin, an abundant structurally related amino polysaccharide – a GlcNAc homopolymer, extracted from the exoskeletons of crustaceans, insects, fungal cell walls etc., into chitosan oligosaccharides with variably retained N-acetyl groups (Cabrera and Van Cutsem, 2005; Domard and Cartier, 1989; Jung and Zhao, 2011). A primary consideration of the hydrolysis is the degree to which depolymerised *Brucella* OPS would be de-formylated as this is likely have a major impact on antibody binding. Specific studies on the degree of de-N-acylation during the production of chitin oligomers demonstrate that at high concentrations of HCl (12 M) depolymerisation occurs with greater rapidity compared to de-acetylation (130 times), whereas at 3 M HCl concentrations the rate is only 2.3 times greater (Einbu and Vårum, 2006). Therefore partial depolymerisation of *Brucella* OPS was attempted using 10 M HCl.

4.7. Modified FACE

The difficulties presented by the OPS D-Rha4NFO polymer found in *Brucella* and *Y. enterocolitica* O:9 required a significant investigation into the optimal conditions for its partial hydrolysis so that a

fair yield of intact (fully N-formylated) homo-oligosaccharides could be generated. A critical requirement to facilitate this investigation was a simple and sensitive means to simultaneously evaluate the status of the substrate – native OPS – and the production of mono and oligosaccharides. As described above, neither GC-MS nor MALDI-ToF was well suited to this particular task.

4.7.1 Development and evaluation of modified FACE method

Instead previously described methods for fluorophore assisted carbohydrate electrophoresis (Buzzega et al., 2010; Oonuki et al., 2005) were adapted. By altering the type of gel, from acrylamide to agarose, and optimising its percentage it was possible to observe the migration of monosaccharides and oligosaccharides as well as observe transformations in the original OPS. Briefly, the hydrolysed, or not, *Brucella* OPS antigen was conjugated via reductive amination to a fluorescent and negatively charged fluorophore enabling it to migrate through the gel towards an anode in a size, conformation and charge dependent manner. The D-Rha4NFo is otherwise neutral at physiological pH. De-N-formylated saccharides will become positively charged by virtue of their new primary amine group which will affect the net charge of the fluorophore conjugated molecule. The modified FACE method was developed and evaluated using maltotriose, maltose, glucose, mannose, glucosamine and N-acetyl-glucosamine standards. The hydrolysis of mannan into its maltose units was also evaluated by the modified FACE method.

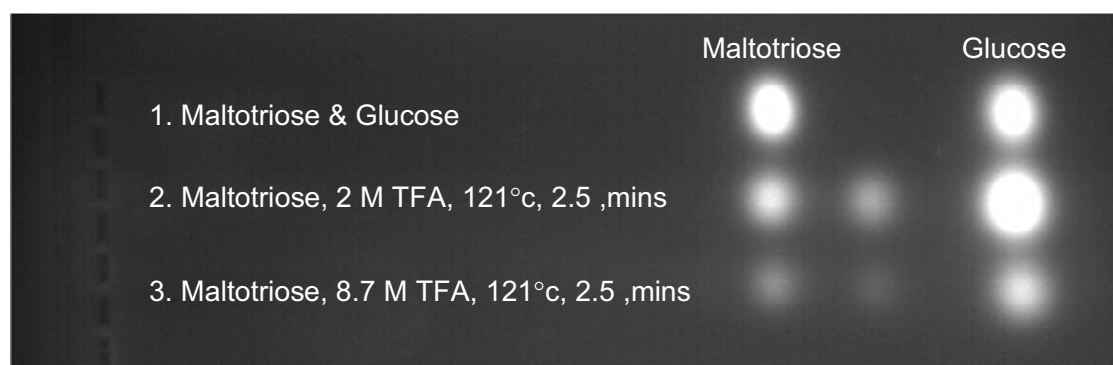


Figure 4.10. Modified FACE gel showing maltotriose, maltose and glucose

Modified FACE gel showing ANTS conjugated standards containing 16 µg of maltotriose and glucose (lanes 1 and 2). Maltotriose was subjected to acid hydrolysis as described in the image. The hydrolysis products were conjugated with ANTS and a volume corresponding to 80 µg of maltotriose pre-hydrolysis was added to lanes 3 and 4.

As simply shown in figure 4.10, the modified FACE method demonstrated its capability for simultaneous evaluation of an intact polymer, oligaccharides and monosaccharides. It also demonstrated the concept of partial acid hydrolysis – as the occurrence of a florescent spot (attributed to maltose) between the maltotriose and the glucose controls in the TFA hydrolysed maltotriose samples demonstrated. Hydrolysis of mannan by TFA also liberated plenty of monosaccharides although oligosaccharides indicative of partial acid hydrolysis were not readily visible (data not shown).

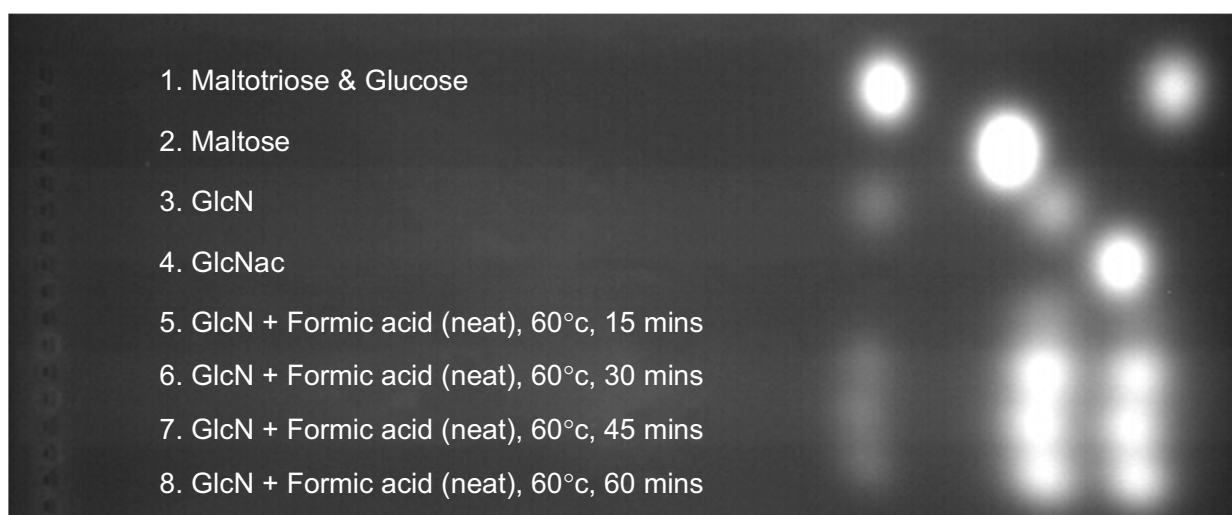


Figure 4.11. Modified FACE gel showing GlcN, GlcNAc and GlcNFo

Modified FACE gel showing ANTS conjugated standards containing 16 µg each of glucose, maltose, maltotriose, GlcN and GlcNAc as indicated above. The GlcN was incubated in formic acid (as described) and the ANTS conjugated product corresponding to 80 µg of GlcN pre-incubation was added to the gel in lanes 5-8 as shown.

Figure 4.11 shows the migration pattern of GlcN and GlcNAc relative to glucose, maltose and maltotriose. As can be seen from lanes 3 and 4, the GlcNAc – despite its larger size – migrates through the gel faster than GlcN (from left to right). This would be due to the positive charge on the GlcN (there are two spots in the GlcN lane, the one on the right is the GlcN monosaccharide). The GlcNAc does not migrate as fast as glucose (the spot in the top right hand corner of figure 4.11) and this is most probably due to its larger size. After heating GlcN in neat formic acid at 60°C for between 30 to 60 minutes an additional, faster migrating spot appears within the sample. This is most probably GlcN_{Fo} (Bandgar et al., 2011) and it appears to migrate slightly faster than GlcNAc which would be anticipated based on the molecular weight.

4.7.2 Partial acid hydrolysis of OPS: evaluation by modified FACE

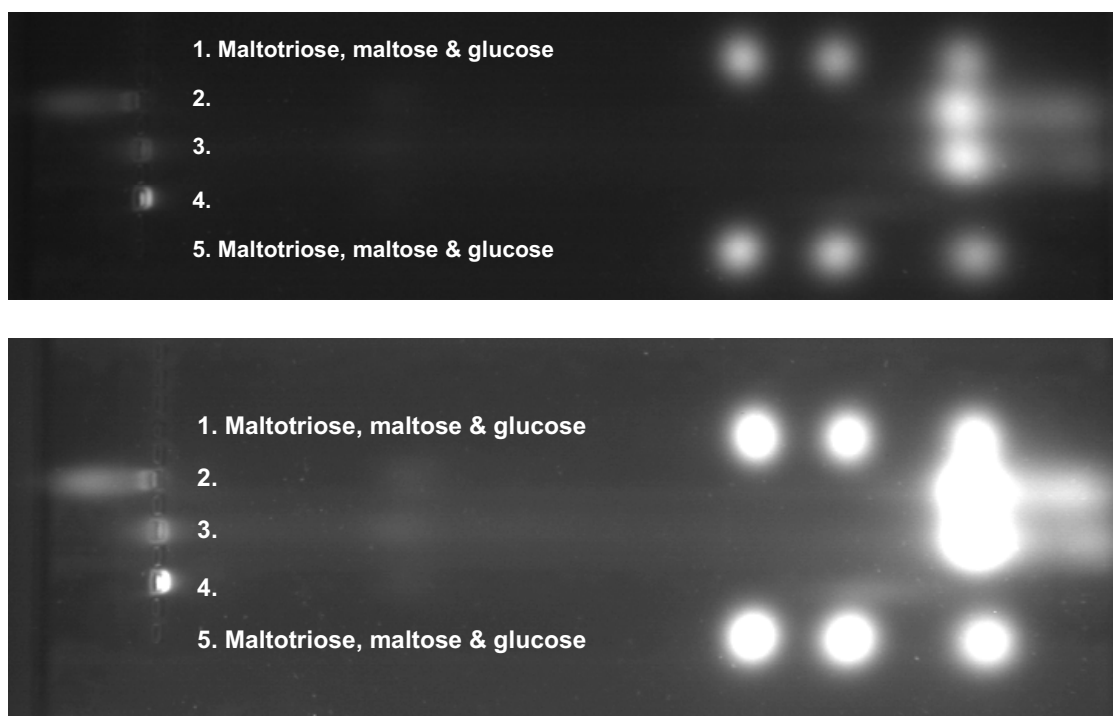


Figure 4.12. Modified FACE gel showing TFA and HCl hydrolysed *B. abortus* S99 OPS

Lanes 1 and 5 contain 16 µg each of standards, maltotriose (left), maltose (centre), glucose (right). Lane 2, trifluoroacetic acid hydrolysed *B. abortus* S99 OPS (140 µg). Lane 3, HCl hydrolysed *B. abortus* S99 OPS (140 µg). Lane 4, unhydrolysed *B. abortus* S99 OPS (140 µg). The top image was captured at low exposure and the low image captured at high exposure.

The images shown in figure 4.12 show the result of acid hydrolysis of *B. abortus* S99 OPS with TFA at 2 M and 121°C for 2 hrs (lane 2) and HCl at 10 M at 60°C for 15 minutes (lane 3) and the non-hydrolysed sample in lane 4. The unhydrolysed sample has no visible monosaccharides present. After hydrolysis with either TFA or HCl monosaccharides are detectably released that migrate at a similar speed to glucose, perhaps a fraction slower. The bottom panel in figure has been exposed for longer than the top and this helps to visualise the fluorescence emanating from near the sample addition wells. This shows the unhydrolysed OPS as a spot that has hardly migrated from the sample well at all. In the HCl hydrolysed sample, this spot has faded significantly and has migrated, ever so slightly, towards to anode. In the TFA sample, there is little fluorescence left in the sample well but there is a smear of fluorescence that has migrated towards the cathode. This suggests that this is material with a net positive charge and is quite likely to be polymers of OPS which have been de-formylated. Whereas each polymer will only be conjugated to a single negatively charged

fluorophore, each polymer may have lost multiple formyl groups. Given the importance of these N-formyl groups to antibody binding, this is quite a significant finding. The conjugation of a single fluorophore per molecule also explains why monosaccharides may fluoresce with higher intensity than polysaccharides for any given mass, rather than molarity.

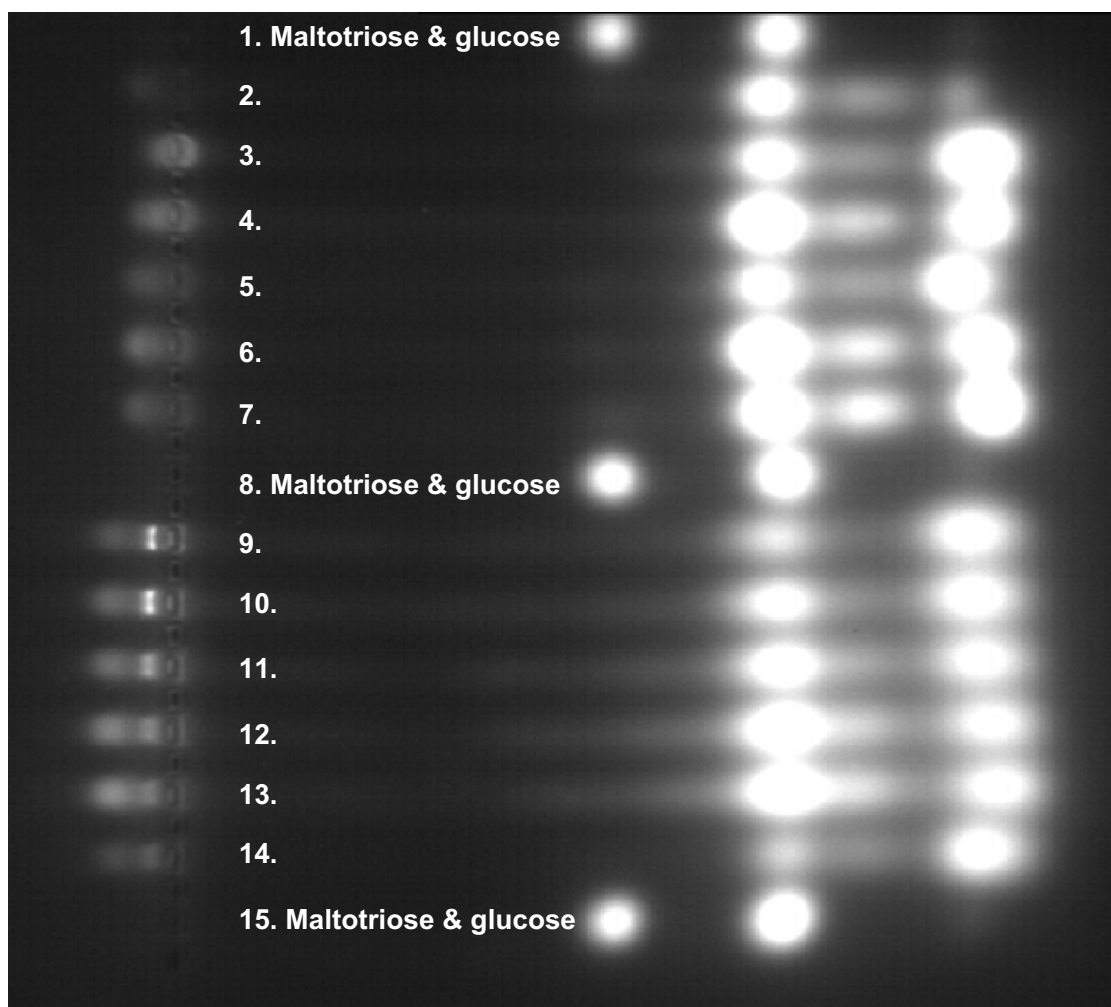


Figure 4.13. Modified FACE gel showing partially acid hydrolysed *B. abortus* S99 OPS: 1 of 2

Modified FACE 3% agarose gel showing the results of hydrolysis of *B. abortus* S99 OPS. Lanes 1, 8 and 15 show 16 µg each of maltotriose (left) and glucose (right) standards. Lanes 2-7 show the results of hydrolysis in 10 M HCl. Lane 2, 10 mins incubation at 80°C (70 µg). Lanes 3-7 have been incubated at 60°C for 7.5, 15, 30, 60 and 120 minutes respectively and contained approximately 140 µg of starting material. Lanes 9-13 show the results of hydrolysis with 2 M TFA at 121°C at 15, 30, 60, 120 and 240 minutes respectively (140 µg). Lane 14 shows the result from an archived sample hydrolysed with TFA for 120 mins at 121°C (70 µg).

Figure 4.13 shows the results from a time series of incubations in HCl and TFA. Both methods generate intense spots similar in migration speed to the glucose control. Dependent upon the sample there are either one or two spots further to the right. The furthest spot is probably too small to be a monosaccharide and may be due to fluorophore conjugation to decomposition products. The spot in the middle (most visible in lanes 6 and 7) may be a monosaccharide and may be D-Rha4NFo. Both formylated and de-formylated (in lower quantities) D-Rha4NFo could be present with the formylated form migrating faster as it is not positively charged. The addition of a Rha standard may have assisted in the resolution of this matter but this was not done. There are other sugar units present in the OPS sample besides D-Rha4NFo that may also appear on the gel, such as mannose, and this modified FACE method leaves uncertainty about the precise events unfolding during acid hydrolysis.

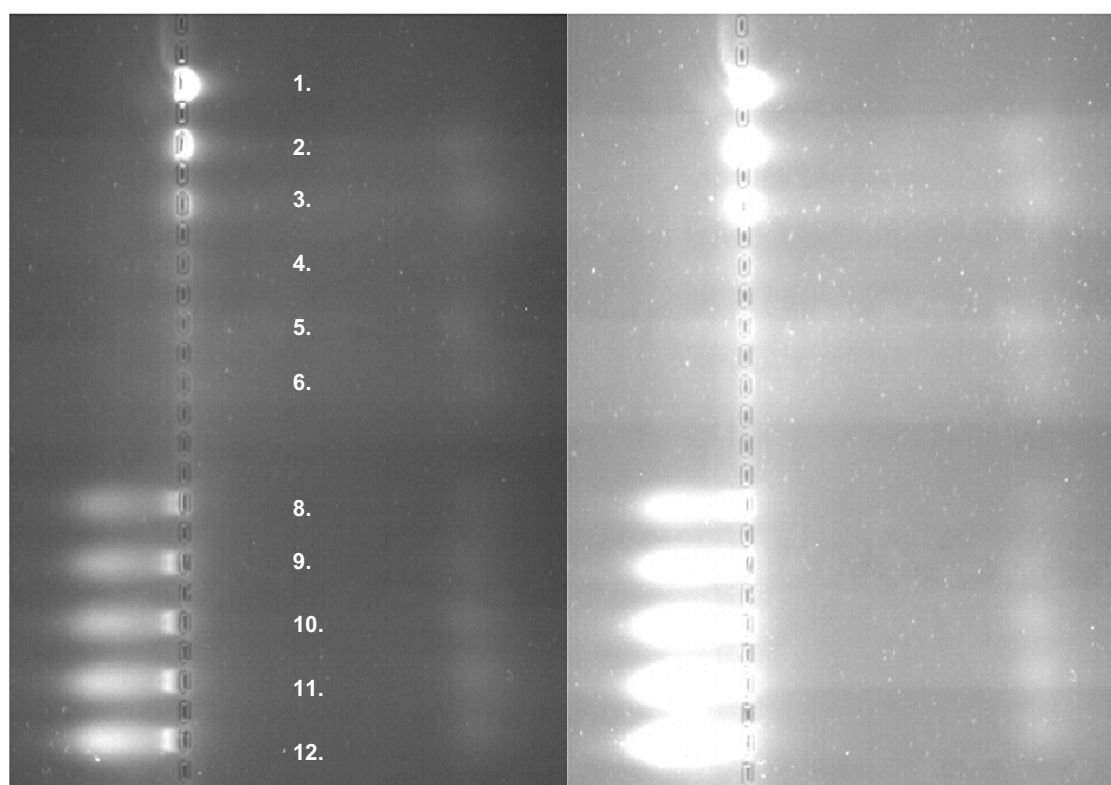


Figure 4.14. Modified FACE gel showing partially acid hydrolysed *B. abortus* S99 OPS: 2 of 2

Modified FACE 1.5 % agarose gel showing the results of hydrolysis of *B. abortus* S99 OPS. Lane 1, unhydrolysed OPS (70 µg). Lanes 2 - 6 show the results of hydrolysis in 10 M HCl at 60°C for 7.5 (lane 2), 15 (lane 3), 30 (lane 4), 60 (lane 5) and 120 (lane 6) minutes respectively each containing approximately 140 µg of starting material. Lanes 8 - 13 show the results of hydrolysis with 2 M TFA at 121°C at 15 (lane 8), 30 (lane 9), 60 (lane 10), 120 (lane 11) and 240 (lane 12) minutes respectively, each containing 140 µg. The panel on the left and right show the same samples with the image on the right captured using a longer exposure time.

The samples shown in figure 4.13 were run on a 3% agarose gel. There appeared to be some interesting detail relating to the fluorescence closer to the sample addition wells. This was explored further by running the samples again on a 1.5% gel to allow the larger molecules to migrate more freely. The image obtained from this 1.5% gel is presented in figure 4.14. This shows that HCl incubation (top) eliminates fluorescence due to the largest molecules whereas this is not the case for TFA hydrolysis (bottom). In addition to the reduced inter-glycosidic hydrolytic capability of the TFA method the material has become positively charged as it is migrating towards the cathode suggesting that significant deformylation has taken place.

There is also some evidence for the production of oligosaccharides produced by hydrolysis with both methods as there are faint spots visible to the right hand side of both images in figure 4.14 (although more apparent in the image on the right). These have not migrated as far as the maltotriose standard (which is not shown in this image but was run on the gel as one of the standards).

The data from the modified FACE on the hydrolysed OPS samples demonstrated that monosaccharides are liberated by hydrolysis with TFA and HCl. It is notable that even after incubation in 2 M TFA at 121°C for 4 hours a substantial quantity of high molecular weight (or highly positively charged) material remains in the sample. The increased intensity of the light emission and the small shift to the cathode that occurs with increasing TFA incubation time indicates that the balance of hydrolysis that is taking place is unfavourably shifted towards de-N-formylation rather than depolymerisation of the OPS. In contrast, hydrolysis by 10 M HCl appears much more straightforward. Hydrolysis appears to be more complete with the generation of fewer positively charged elements, either as polysaccharides or monosaccharides. However, neither method appeared to conclusively generate oligosaccharides that were detectable by modified FACE.

4.8. Partial acid hydrolysis of OPS: evaluation by MALDI-ToF

The evaluation of acid hydrolysis methods by modified FACE provided evidence that warm incubation with 10 M HCl was liberating intact sugars, potentially oligosaccharides and with limited degrees of deformylation. The pattern of migration within the gel suggested that some of these were intact D-Rha4NFO units although the depth of data imparted by this method limited the confidence in this conclusion. Although MALDI-ToF had previously demonstrated limited sensitivity the potentially

increased abundance of oligosaccharides as a consequence of partial hydrolysis may make them detectable by this method. The hydrolysed samples were therefore evaluated by MALDI-ToF.

The *B. abortus* S99 OPS was subjected to acid hydrolysis with 2 M TFA (121°C for 2 hrs) and 10 M HCl (60°C for 3 mins 45 secs hrs) and then permethylated. The MALDI-TOF spectra, acquired in linear mode matrix suppression enabled, for the samples is shown in figure 4.15. The top panel shows the spectrum from the HCl hydrolysed OPS. By comparing this to the equivalent spectrum from the unhydrolysed permethylated OPS shown in the bottom panel it is immediately apparent that there is a similar pattern of repeating peaks but that the range is curtailed to below 6,000 m/z in the hydrolysed material. In the unhydrolysed material, the peaks appeared to be reaching maximum intensity at 9,000 m/z. Below 4,000 m/z the peaks for the hydrolysed sample appear to be better resolved and more regular than the equivalent peaks in the unhydrolysed sample. These features would be consistent with populations of, on average, shorter polysaccharides as would be expected from successful hydrolysis. The sharpness of the peaks in the spectrum from the HCl hydrolysed sample also indicates that the hydrolysis has not lead to wholesale de-formylation. If this were the case considerable peak broadening and loss of resolution might be expected.

The spectrum from the TFA hydrolysed sample is shown in the middle panel of figure 4.15. Despite the presence of much material, as demonstrated by FACE, there are no discernable regular peaks present. No relevant ions were detectable at lower mass ranges using data acquisition without matrix suppression. A plausible explanation for this is that whatever hydrolysis has taken place has acted on the N-formyl group as well as, or in preference to, the glycosidic linkage. The effect of this would be to create a very diverse range of molecular weight structures each of which may become so dilute as to become undetectable. The MALDI-ToF and FACE analysis indicated that HCl rather than TFA hydrolysis was a preferable method for depolymerisation.

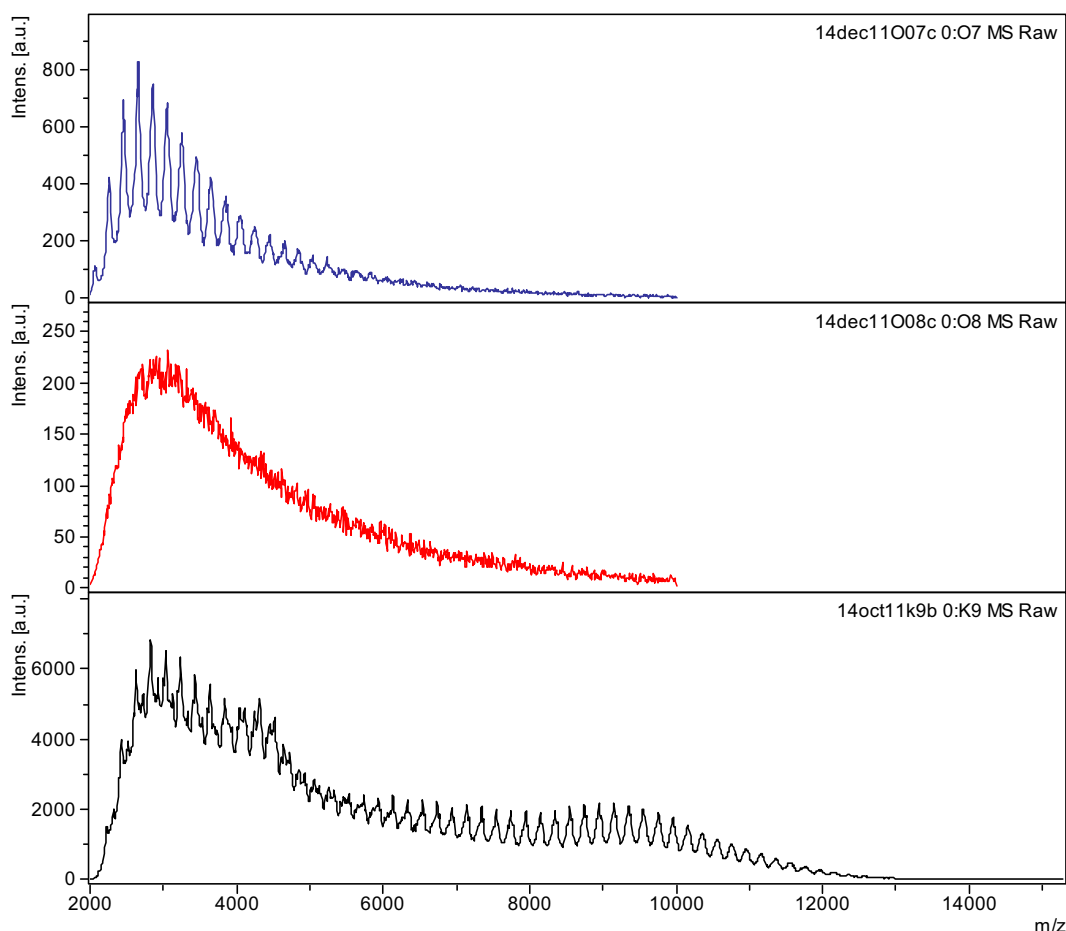


Figure 4.15. MALDI-ToF MS of partially hydrolysed permethylated *B. abortus* S99 OPS: 2,000 – 12,000 m/z

The top panel shows the OPS that has been hydrolysed by HCl (10 M, 60°, 3 mins 45 secs). The second panel shows the OPS that has been hydrolysed by TFA (2 M, 121°C, 2hrs). The bottom figure is the spectrum of the OPS pre-hydrolysis (as shown previously in figure 4.3). Methylation was performed post hydrolysis.

Figure 4.16 shows the spectra from the permethylated *B. abortus* S99 OPS acquired with matrix suppression disabled. The top panel shows the spectrum for the unhydrolysed OPS (as shown above in figure 4.6) as acquired in linear mode. The middle panel shows the spectrum for the hydrolysed OPS as acquired in linear mode, the bottom panel shows the same sample acquired in reflectron mode. All spectra show a series of peaks at intervals consistent with the mass of permethylated D-Rha4NFO but the peaks derived from the hydrolysed and non-hydrolysed OPS do not share the same m/z and must, therefore, have different composition. The uncertainty over the particular structure related to the series of ions within the spectra for the non-hydrolysed OPS has been described above. The same situation applies to the series of ions observed from the permethylated hydrolysed OPS in that the m/z values are not consistent with a D-Rha4NFO homopolymer.

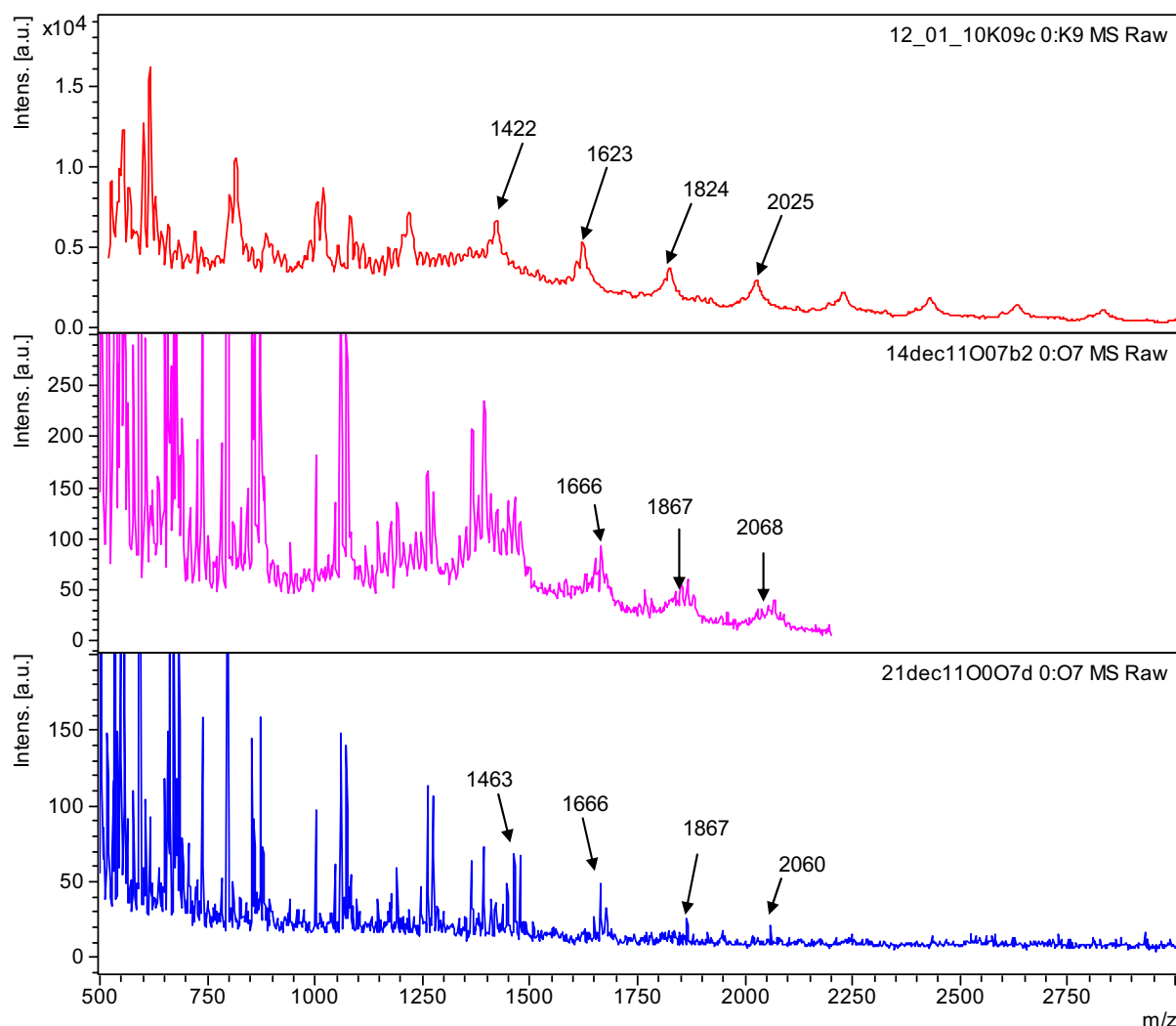


Figure 4.16. MALDI-ToF MS of partially hydrolysed permethylated *B. abortus* S99 OPS: 500 – 3,000 m/z
 The top panel shows the permethylated OPS pre-hydrolysis (as also shown in figure 4.6). The *B. abortus* S99 OPS was permethylated post hydrolysis (by 10 M HCl, 60°, 3 mins 45 secs) and evaluated in linear mode (middle) and reflection mode (bottom). All spectra were acquired with matrix suppression off. The m/z values of the most relevant peaks are indicated by the arrows.

A closer inspection of the data shown in figure 4.16 is shown in figure 4.17 where only the data from m/z range 1,500 – 2,000 is shown. This demonstrates that the reflectron mode (bottom panel) provides more highly resolved spectra. The peaks from this reflectron mode spectrum correspond to those within the spectrum of the permethylated hydrolysed *B. abortus* S99 OPS acquired in linear mode (middle panel) but provide an extra level of detail. This includes a series of ions that are 14 m/z units apart (1652, 1666 and 1680). This matches the mass difference between a methylated and non-methylated group. A difference of 28 m/z also corresponds to the loss of the formamido group to leave a primary amine. This may also be methylated. Thus, the possibility of de-formylation coupled

with permethylation creates some additional uncertainty that is unhelpful in the determination of structure.

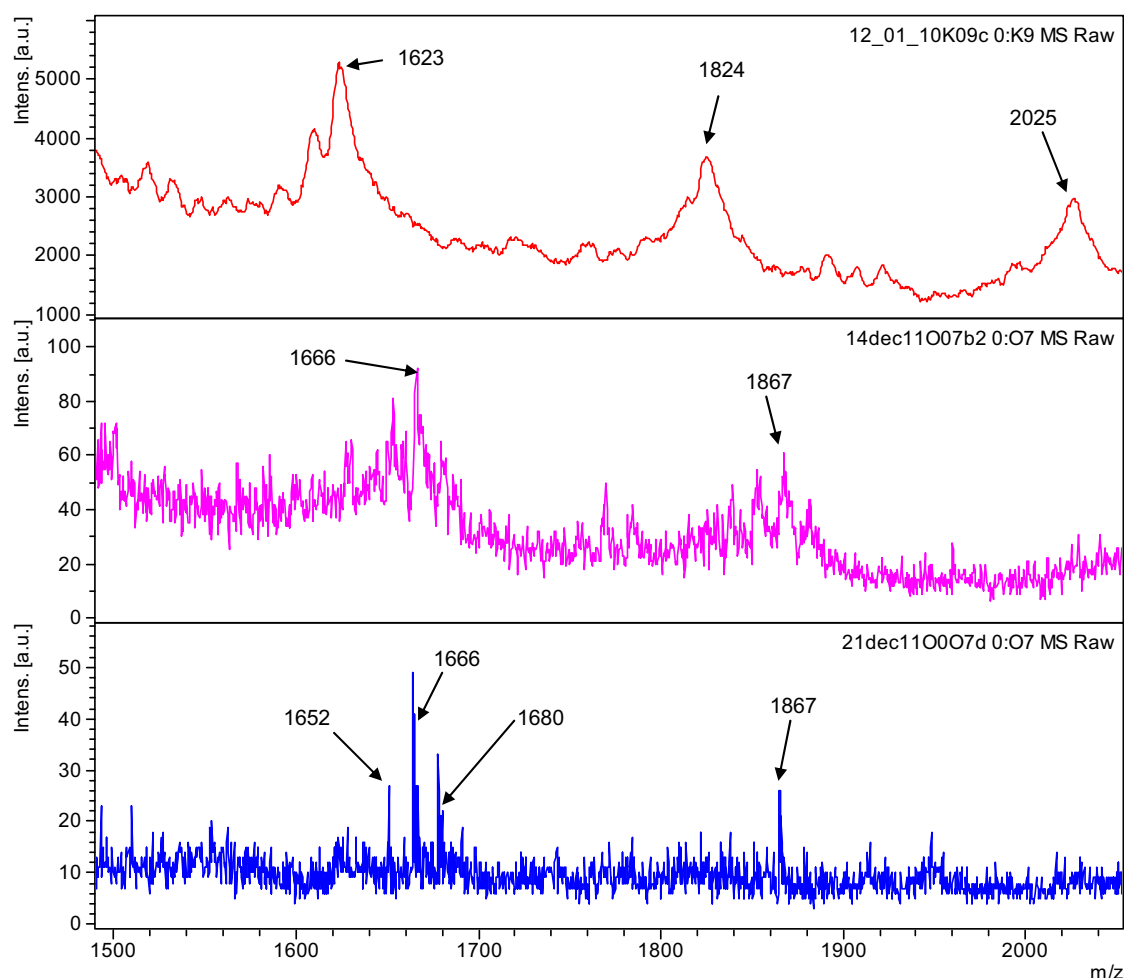


Figure 4.17. MALDI-ToF MS of partially hydrolysed permethylated *B. abortus* S99 OPS: 1,500 – 2,200 m/z

This figure shows data from the same spectra presented in figure 4.16 with a different m/z scale. The top panel shows the permethylated OPS pre-hydrolysis (as also shown in figure 4.6). The *B. abortus* S99 OPS was permethylated post hydrolysis (by 10 M HCl, 60°, 3 mins 45 secs) and evaluated in linear mode (middle) and reflection mode (bottom). All spectra were acquired with matrix suppression off. The m/z values of the most relevant peaks are indicated by the arrows.

4.8.1. Partial acid hydrolysis of OPS: evaluation of non-permethylated OPS by MALDI-ToF

The hydrolysed OPS was also analysed by MALDI-ToF without permethylation. The top panel in figure 4.18 is derived from the non-hydrolysed non-permethylated OPS as shown previously in figure

4.7 and is presented here for comparison with the spectra below. These spectra are from the partially hydrolysed (HCl at 60°C for 3 mins and 45 seconds) unmethylated *B. abortus* S99 OPS analysed in linear mode (middle panel) and reflectron mode (bottom panel). The middle panel shows an interesting series of ions that are shown in more detail in figure 4.19. This annotated series of ions, beginning at m/z 560, increase by 171 m/z and are readily distinguishable up to 1771 m/z (the sodiated deca-saccharide). Unlike the data from the permethylated samples, these ions fit to a series of sodium adducted D-Rha4NFO homopolymers. Their presence in the hydrolysed sample but not in the unhydrolysed sample also indicates that they have increased in abundance following hydrolysis. The peak at m/z 527 relates to a maltotriose standard added as an internal control after the hydrolysis period. The spectrum from acquisition performed in reflectron mode (bottom panel in figure 4.18 and separately in figure 4.20) shows the same series of ions albeit at lower intensities with detection limited up to the septasaccharide.

The ion intensities relative to the maltotriose standard (527 m/z) were used, in MALDI-ToF analysis of the non-permethylated sample, to determine the relative abundance of each oligosaccharide after subjection to a range of hydrolysis (10 M HCl at 60°C) incubation times. The results of this evaluation are presented in graphical form in figure 4.21. This clearly shows that after an initial increase in the abundance and mass range of (detectable) fully formylated oligosaccharides there is a swift reduction. There is some discrepancy in the data with a dip in the quantity of trimer and tetramer at 7.5 minutes that goes against the trend of the rest of the data. However, the overall picture is conclusive. It has already been shown that there are no detectable D-Rha4NFO homooligosaccharides in the unhydrolysed sample so the production of these fully formylated forms occurs rapidly under the hydrolytic conditions applied and the products are also short lived and scarce. Therefore hydrolysis needs to be carefully undertaken to ensure that such products are produced.

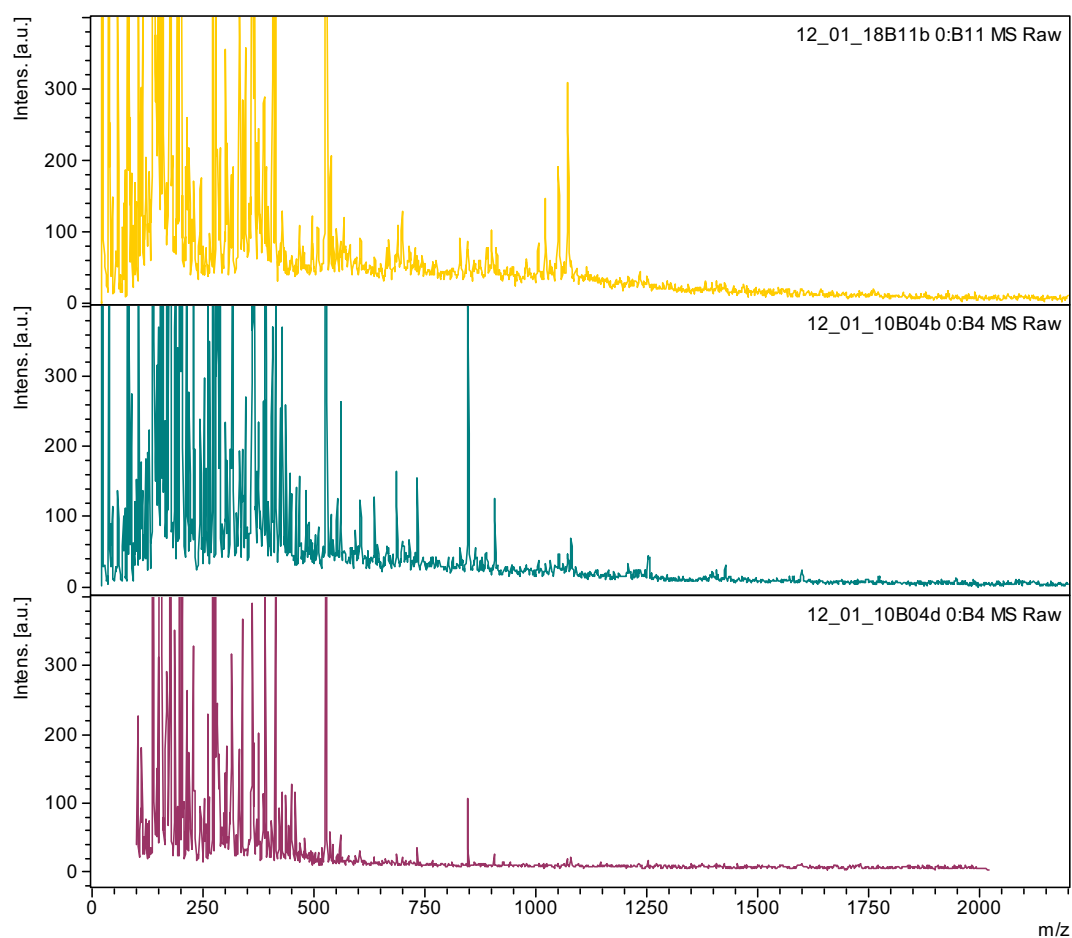


Figure 4.18. MALDI-ToF MS of partially hydrolysed non-permethylated *B. abortus* S99 OPS: 0-2,200 m/z
 The top panel shows the non-permethylated OPS pre-hydrolysis (as also shown in figure 4.7). The *B. abortus* S99 OPS was hydrolysed (by 10 M HCl, 60°C, 3 mins 45 secs) and evaluated without permethylation in linear mode (middle) and reflection mode (bottom). All spectra were acquired with matrix suppression off.

The graph shown in figure 4.22, relating to HCl incubated at 40°C, shows a slower increase in fully permethylated oligosaccharide abundance before over incubation leads to degradation of these structures. Although the lower temperature incubation leads to a more controlled hydrolysis process, incubation at 60°C was selected for large scale hydrolysis of OPS on the basis that: the experimental evidence shows that this is effective and, hotter methods are commonly employed in the partial hydrolysis of chitin (Cabrera and Van Cutsem, 2005).

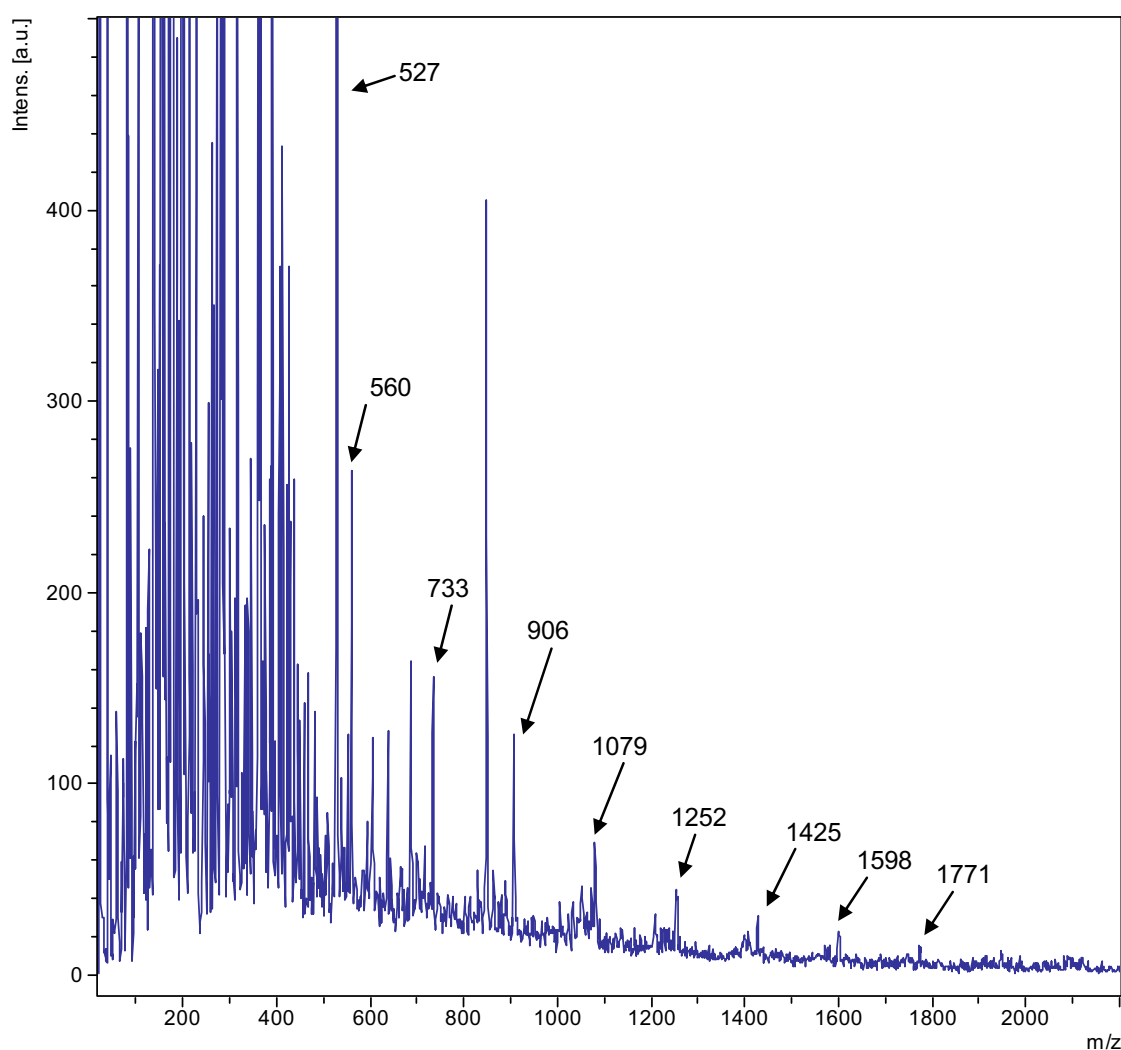


Figure 4.19. MALDI-ToF MS (linear mode) of hydrolysed non-permethylated *Brucella abortus* S99 OPS 0 - 2,200 m/z

MALDI-ToF mass spectrum, acquired in linear mode without matrix suppression, of hydrolysed (10 M HCl, 60°C, 3 mins 45 secs) *Brucella abortus* S99 OPS (as also shown in the middle panel of figure 4.18). The spectrum shows a repeating series of annotated peaks representing the intact fully formylated D-Rha4NFO homo-oligosaccharide (highest m/z in each series). The fully formylated oligosaccharides ions are visible at 560 (trisaccharide), 733 (tetrasaccharide), 906 (pentasaccharide), 1079 (hexasaccharide), 1252 (septasaccharide), 1425 (octasaccharide), 1598 (nonasaccharide) and 1771 (decasaccharide). The ion peak at m/z 527 is due to

the addition of a maltotrisose standard. The peaks at m/z just lower than the annotated peaks may be due to oligosaccharides with varying degrees of de-formylation (series as 1, 2 or more formyl groups ($m/z = 28$) are lost due to the hydrolysis).

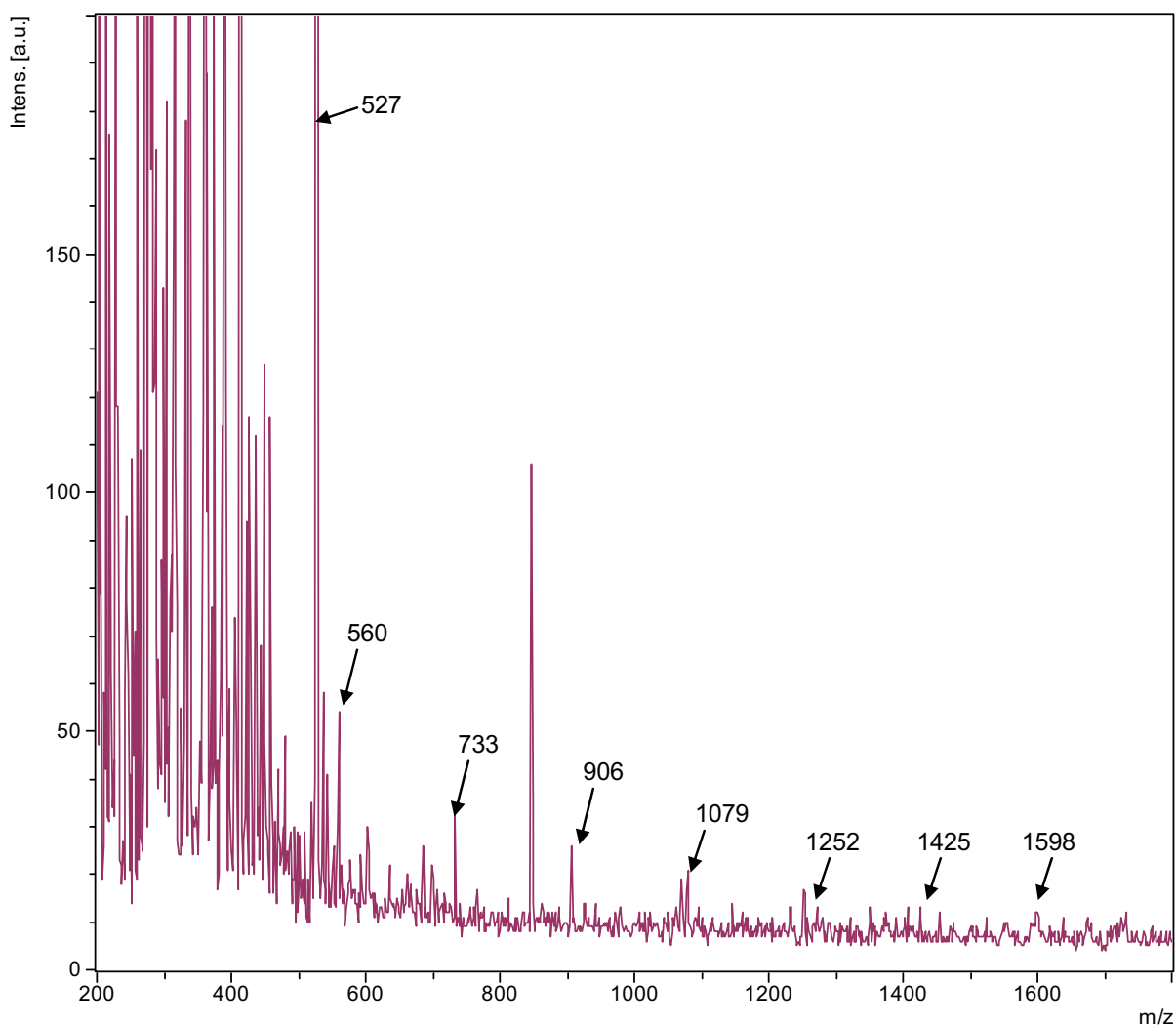


Figure 4.20. MALDI-ToF MS (reflectron mode) of hydrolysed non-permethylated *Brucella abortus* S99 OPS 0-2,200 m/z

MALDI-ToF mass spectrum, acquired in reflectron mode without matrix suppression, of hydrolysed (10 M HCl, 60°C, 3 mins 45 secs) *Brucella abortus* S99 OPS (as also shown in the middle panel of figure 4.18). The spectrum shows a repeating series of annotated peaks representing the intact fully formylated D-Rha4Nfo homo-oligosaccharide (highest m/z in each series). The fully formylated oligosaccharides ions are visible at 560 (trisaccharide), 733 (tetrasaccharide), 906 (pentasaccharide), 1079 (hexasaccharide), 1252 (septasaccharide). The ions corresponding to the octasaccharide (1425), nonasaccharide (1598), and decasaccharide (1771) are not clearly visible. The ion peak at m/z 527 is due to the addition of a maltotrisose standard.

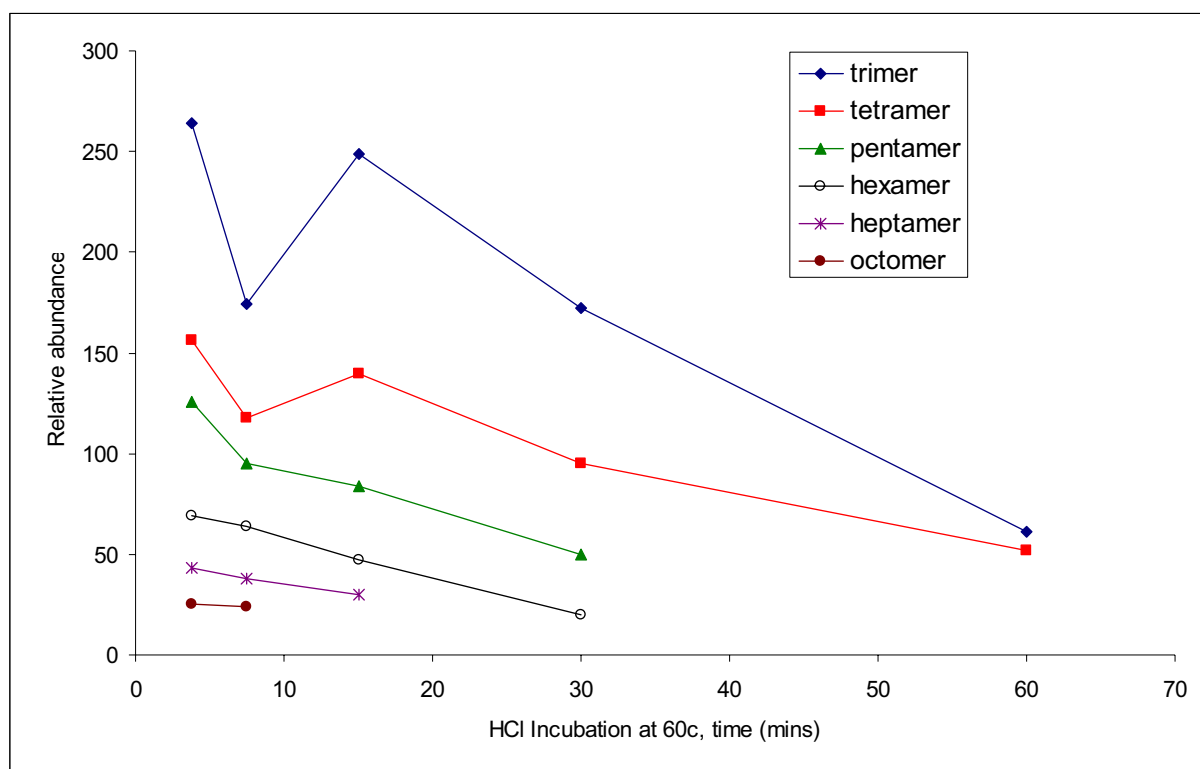


Figure 4.21. Relative abundance of D-Rha4NFO homopolymers post 10 M HCl hydrolysis at 60°C

The line graph in figure 4.21 shows the relative abundance (y-axis) of fully formylated D-Rha4NFO homopoligosaccharides (as shown in legend), as determined by MALDI-ToF, from *B. abortus* S99 OPS after incubation in 10 M HCl 60°C for a varied period of time (x-axis)

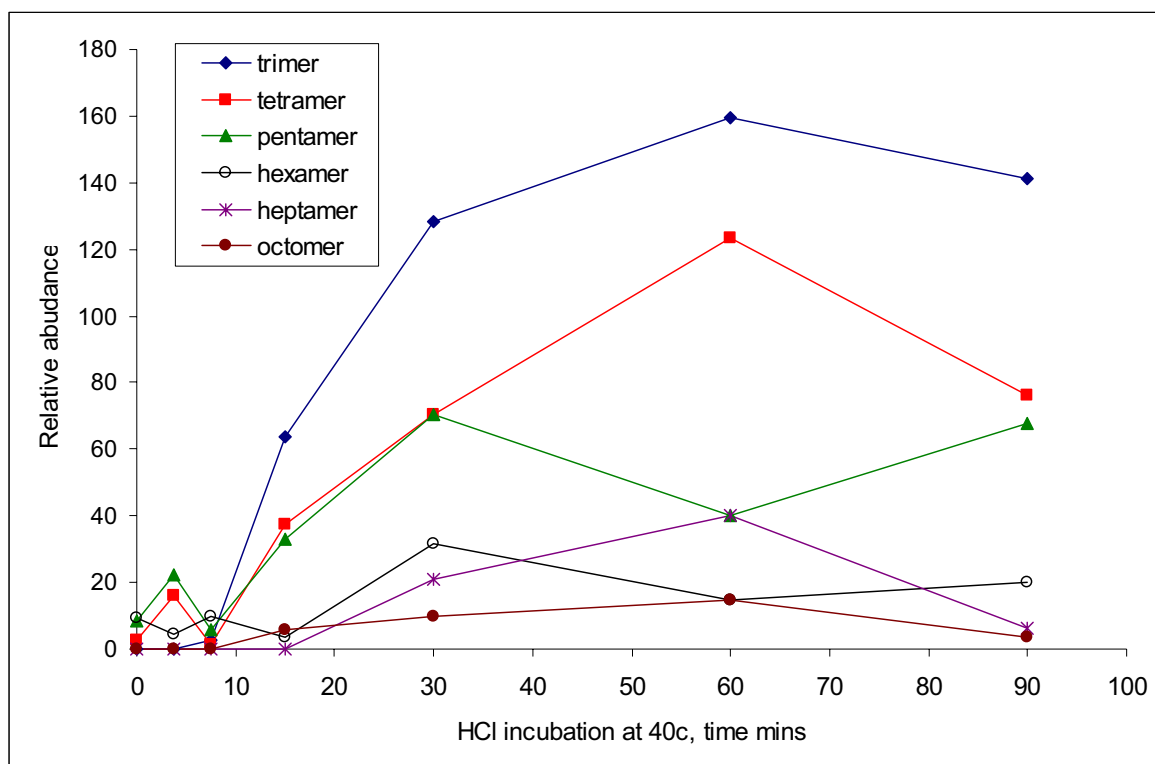


Figure 4.22. Relative abundance of D-Rha4NFO homopolymers post 10 M HCl hydrolysis at 40°C

The line graph in figure 4.21 shows the relative abundance (y-axis) of fully formylated D-Rha4NFO homo-oligosaccharides (as shown in legend), as determined by MALDI-ToF, from *B. abortus* S99 OPS after incubation in 10 M HCl 40°C for a varied period of time (x-axis).

4.8.2. Evaluation of deformylation of OPS oligosaccharides liberated by acid hydrolysis

The quality of the spectrum obtained in linear mode from the non-permethylated *B. abortus* S99 OPS sample after variable incubation time with HCl allowed for an interesting and informative analysis of the relative abundance of fully formylated and deformylated oligosaccharides. An example spectrum, for the *B. abortus* S99 OPS sample incubated for 30 mins at 60°C in 10 M HCl is shown in figure 4.23. The data for the pentasaccharide afforded the most illustrative analysis and a closer inspection of the data relating to the m/z range encompassing the D-Rha4NFO pentasaccharide and hexasaccharide (and the ion corresponding to the fully permethylated tetrasaccharide) is shown in figure 4.24. This presents compelling evidence for the successive loss of 28 m/z that is most probably due to the loss of the formyl group from the 4th carbon.

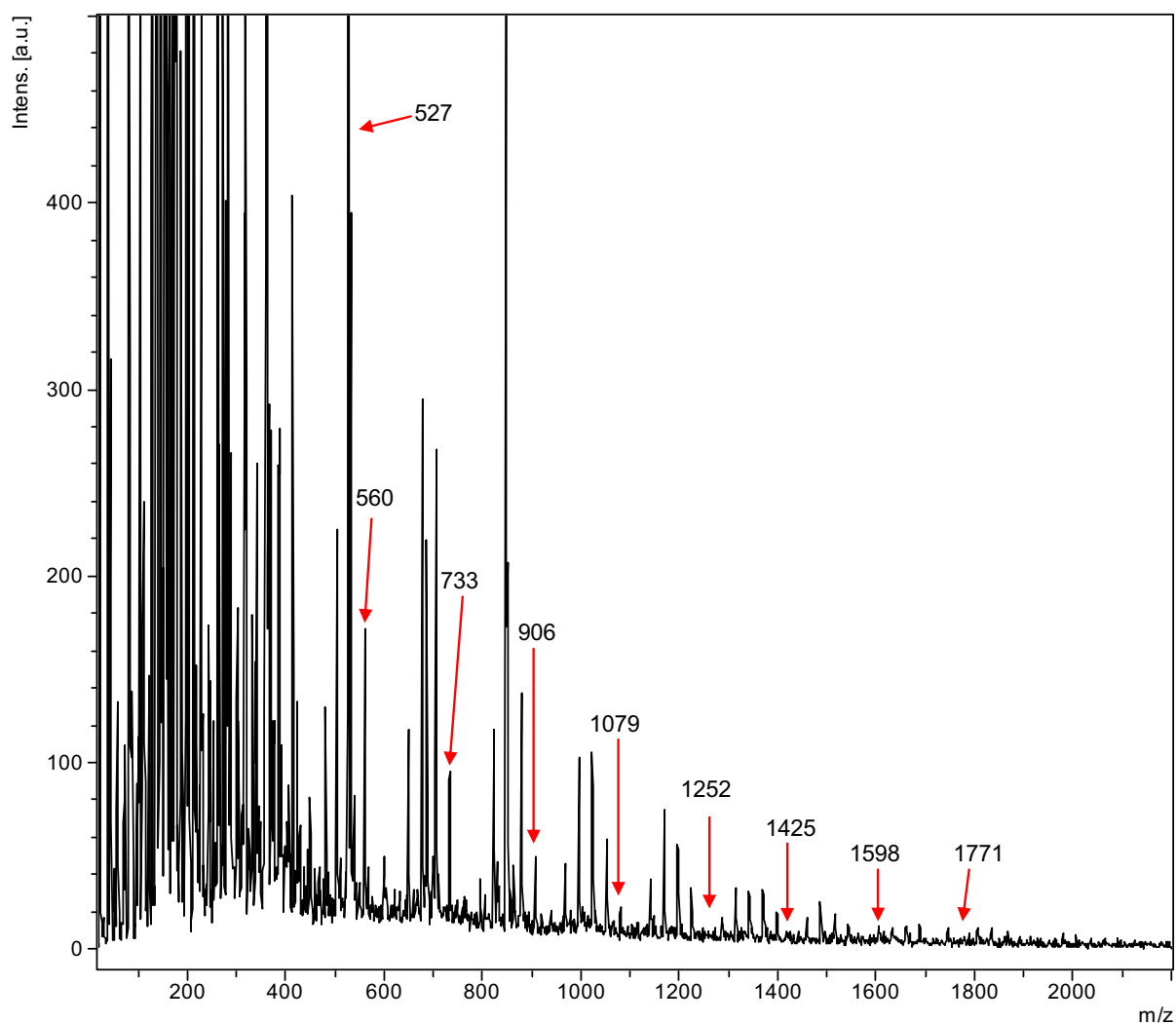


Figure 4.23. MALDI-ToF MS (linear mode) of hydrolysed non-permethylated *Brucella abortus* S99 OPS 0 - 2,200 m/z

B. abortus S99 OPS was hydrolysed by 10 M HCl at 60°C for 30 mins. The annotations in the above spectrum relate the the m/z values for a series of fully formylated D-Rha4Nfo homo-oligosaccharides beginning with a sodiated trisaccharide m/z 560 followed by fully formylated 733 (tetrasaccharide), 906 (pentasaccharide), 1079 (hexasaccharide), 1252 (septasaccharide), 1425 (octasaccharide), 1598 (nonasaccharide) and 1771 (decasaccharide). The ion peak at m/z 527 is due to the addition of a maltotriose standard. The series of peaks below the m/z values for the fully formylated groups may be due to oligosaccharides with varying degrees of de-formylation (series as 1, 2 or more formyl groups ($m/z = 28$) are lost due to the hydrolysis).

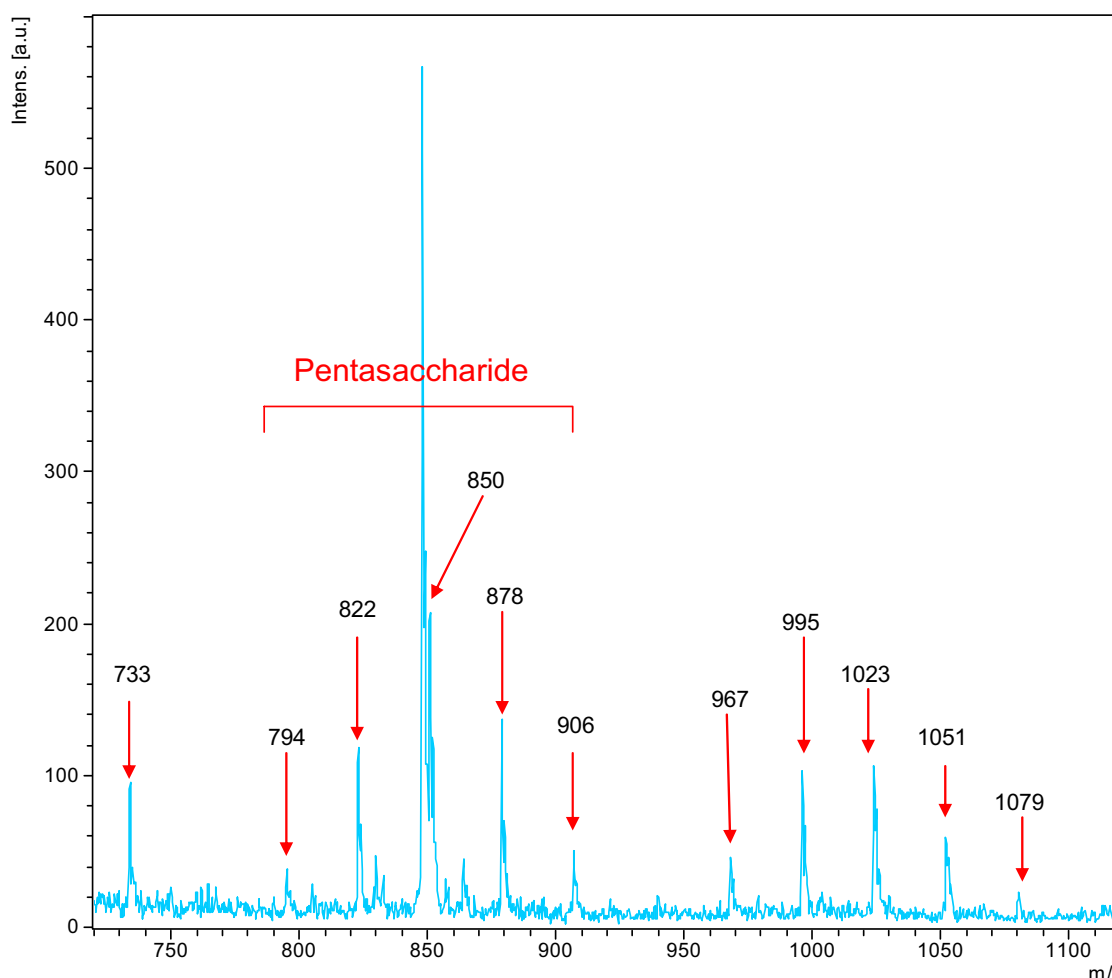


Figure 4.24. MALDI-ToF MS (linear mode) of hydrolysed non-permethyated *Brucella abortus* S99 pentasaccharide 670 – 1,120 m/z

B. abortus S99 OPS was hydrolysed by 10 M HCl at 60°C for 30 mins). The annotations in the above spectrum relate the the m/z values for a series of variably formylated D-Rha4N/Fo oligosaccharides. The peak annotated at 1079 m/z corresponds to a fully formylated sodiated hexasaccharide, the peaks successively 28 m/z lower result from loss of formyl groups ($m/z = 28$) from this structure. The ion series for the pentasaccharide is also shown. The fully formylated form has 906 m/z. The ion related to the fully formylated form of the tetrasaccharide ($m/z = 733$) is also visible.

The data for the generation and degradation of the D-Rha4N/Fo pentasaccharide over the course of the different HCl (10 M, 60°C) hydrolysis incubation times studied is presented graphically in figure 4.25. This shows how the fully formylated pentasaccharide is at its peak in the shortest incubation period evaluated (3 mins, 45 secs) and has disappeared after 60 minutes have elapsed. The singly and doubly deformylated pentasaccharides peak increased after 30 minutes incubation and then decrease in abundance. The triply, quadruply and fully deformlyated forms are all present after 60 minutes incubation with no evidence that their abundance has peaked. The time frame over which

the forms appear and disappear indicate that, whilst the yield of oligosaccharides might increase with more time, fully formylated forms are relatively short lived.

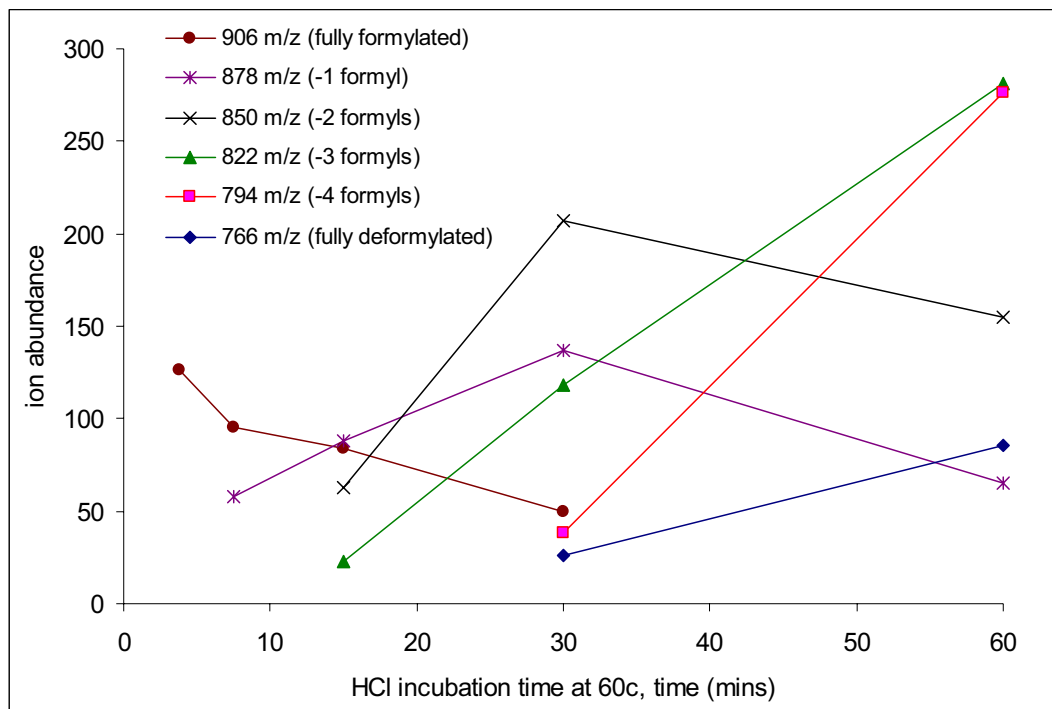


Figure 4.25. Relative abundance of deformylated pentasaccharides post 10 M HCl hydrolysis at 60°C

The line graph in figure 4.25 shows the relative abundance of different forms of pentasaccharide derived from HCl acid hydrolysis at 60°C. The different forms, corresponding to the m/z figures shown in the legend, relate to the loss of one (m/z = 878), two (m/z = 850), three (m/z = 822), four (m/z = 794) and fully deformylated forms.

4.8.3. Determination of optimal conditions for partial acid hydrolysis of OPS

Once the basic hydrolysis method had been established using modified FACE it was further optimised using MALDI-MS to evaluate the outcome of each methodological permutation. The optimised hydrolysis protocol was a 3 min 45 sec incubation of OPS in 12 M HCl at 60°C (5 µg OPS/µl HCl) followed by 1:15 dilution in ice cold water with three rounds of freeze drying to remove all residual HCl from the preparation. This procedure gave the greatest quantity of completely formylated oligosaccharides in the 3-8 mer range. This is best shown in figure 4.22 which shows how (with the exception of three outlier points) the yield of fully formylated oligosaccharides decreases rapidly during HCl incubation. It also shows that the yield of the higher mass oligosaccharides is

lower than the shorted oligosaccharides (although this may be an artefact of the mass-spectrometry method). None of these products are visible in the unhydrolysed OPS sample.

4.9. Analysis of OPS and hydrolysed OPS by HPLC-ESI-MS

The addition of ESI-MS as an analytical tool was considered as the merits of this approach may overcome some of the limitations described for alternative methods of OPS analysis, such as GC-MS, MALDI-ToF and modified FACE. The data from MALDI-ToF strongly suggested that the yield of fully permethylated oligosaccharides would be small. The objective of hydrolysis was to obtain these oligosaccharides for subsequent application to antibody-affinity chromatography processes after which their abundance and concentration is likely to be reduced. A process that could detect, from within a mixture, low quantities of specific analyte with a high degree of m/z resolution and precision was required. Furthermore, the ambition was to evaluate the structural details of identified oligosaccharides, such as the type of glycoside linkages within. This may be achievable by cross-ring fragmentation during MS/MS but only if the MS platform is sufficiently sensitive to detect the fragmented ions. On the basis of the previous work, the MALDI-ToF would not have the sensitivity to perform this type of analysis. The use of ESI also enabled the option to perform on-line chromatography which may also help to separate and evaluate the analytes.

As described in the introduction, ESI-MS/MS methods have the capability to be both highly sensitive and highly resolved. They can also produce high data quality over a wide mass range owing to the propensity of the technique to induce multiple charges on larger molecules. However, this same property leads to convolution of the data with multiple ions, and multiple peaks on the mass spectrum generated for identical source molecules. When the analyte of interest is a polymer comprised of a distribution of molecular lengths and thus types, the degree of convolution may rapidly be increased. In addition, the presence of different adducts (such as protonated, sodiated and ammoniated) also increases the number of m/z values that may be observed for any given molecule – especially as different adducts may occur on the same ion. Therefore, whilst the use of ESI-MS/MS presents considerable opportunities, it also presents many potential challenges.

4.9.1. Analysis of *B. abortus* S99 and *E. hermannii* OPS by SEC-ESI-MS

In order to optimise the evaluation of the OPS by ESI-QqToF and ESI-QqQ a variety of conditions were tested using carbohydrate standards including monosaccharides such as glucose, GlcN and GlcNAc, trisaccharides such as maltotriose and chitotriose (a GlcNAc trisaccharide) and dextran. The main issue encountered was the selection of buffer for the mobile phase. The use of ammonium formate for example resulted in the excessive convolution of the data due to the formation of protonated, sodiated and ammonium adducts. This was particularly problematic in samples containing variably sized molecules – such as OPS. The most successful approach to minimising excessive convolution was to add sodium iodide to the buffer in order to promote sodiated adducts over other forms. This method has also proven successful for the identification of mixed length dextran ions in SEC-ESI-MS (Deery et al., 2001). However, in the presence of basic groups such as primary amines, protonated ions remained common.

4.9.1.1. SEC of *B. abortus* S99 and *E. hermannii* non-hydrolysed OPS

A Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column was used on-line with an ESI-QqToF for evaluation of the native OPS from *B. abortus* S99. The UV 210 nm spectrum obtained from the column is presented in figure 4.26. This shows two relevant peaks, one eluting at approximately 6.5 to 7.5 minutes and the second eluting at between 8.5 and 11.5 minutes. The comparable UV spectrum obtained using absorbance at 280 nm (not shown) only shows the first of these two peaks. Prior evaluation of the OPS antigen preparations has shown that the OPS absorbs readily at 210 nm but considerably less so at 280 nm. The data from the UV absorbance chromatograms therefore indicates that the OPS is eluting in the second peak and the first peak, corresponding to the solvent front, contains residual non-separated proteins from the OPS preparation. Silver staining of the OPS antigens (presented in chapter 1) demonstrated the presence of some large proteins in the preparations.

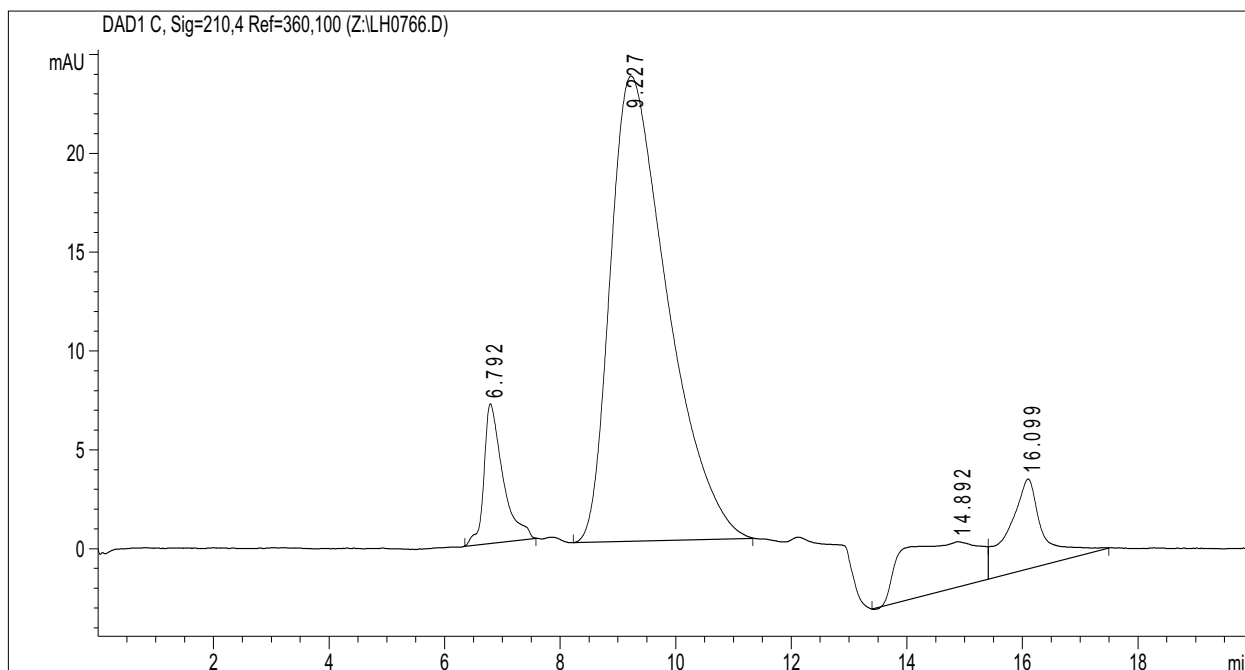


Figure 4.26. Size exclusion chromatograph (UV absorbance) of *B. abortus* S99 OPS

Figure 4.26 shows the size exclusion chromatograph (UV absorbance) of *B. abortus* S99 OPS antigen preparation using a Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column with absorbance (y-axis) against time (x-axis). The peak at 6.792 elutes at the solvent front. The peak at 9.227 represents the elution of the OPS.

4.9.1.2. SEC-ES-QqToF of *B. abortus* S99 and *E. hermannii* non-hydrolysed OPS

However, the total ion count from the ESI-QqToF over the time period corresponding to the second peak was low and did not reflect the presence of large quantities of ionisable material. The signals that were present matched the spectral profile of polyethylene glycol (PEG). As an ionisable surfactant, contamination with this material can cause considerable inhibition of other signals (Cech and Enke, 2000). Fractions eluted from the SEC column collected in 30 second intervals were evaluated for the presence of OPS by cELISA the results of which (data not shown) demonstrated that fractions collected over the time period corresponding to the second peak contained large quantities of OPS.

The SEC-ESI-MS output for the *Brucella* OPS contrasts with that obtained from analysis of a similar OPS antigen preparation from *E. hermannii*. This OPS is a variably $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ linked polysaccharide of D-Rha4NAc (Perry and Bundle, 1990) and is therefore highly similar to the OPS from *Brucella*. A preparation of sLPS from *E. hermannii*, strain NRCC 4299, was received via a

generous donation by Dr Andrew Cox from the National Research Council of Canada. An OPS preparation from this was prepared as described above for *Brucella* and *Y. enterocolitica* O:9 and was evaluated using a SEC-ESI-QqQ with the Tosoh TSK-GEL PWXL-CP G3000 column. The UV absorbance chromatograms obtained from the SEC column were almost identical to that obtained from the analysis of the *B. abortus* S99 OPS. Once again it contained two relevant peaks, one eluting at approximately 6.5 to 7.5 minutes and the second eluting at between 8.5 and 11.5 minutes.

Unlike the SEC elution profile from the *B. abortus* S99 OPS that from *E. hermannii* OPS did tie in with the total ion count chromatogram, shown in figure 4.27, albeit with a slight delay owing to extra length of the capillary that feeds into the ESI interface from the SEC column.

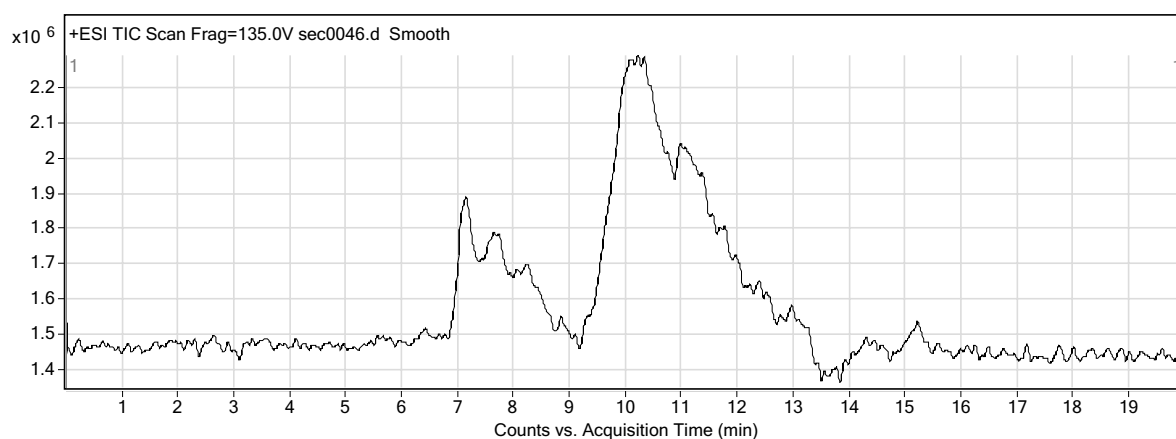


Figure 4.27. Size exclusion chromatograph (total ion count) from *E. hermannii* OPS

Figure 4.27 shows the size exclusion chromatograph of *E. hermannii* OPS antigen preparation using a Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column as the total ion count detected by the online ESI-QqQ. The peaks at 7-8 mins correspond to those observed in the UV chromatogram (not shown) and the second one, peaking at 10 minutes represents the OPS.

The ESI spectra obtained from the *E. hermannii* was complex and contained in excess of 200 separate ions that may be confidently attributed to the OPS. The reason for this complexity is due to the variation in polymer lengths that makes up the OPS and that each of these may be variably charged. The spectra are thus highly convoluted and attempting *de-novo* structural investigation would be exceedingly challenging. The analysis of OPS synthesised by the Wzx/Wzy pathway would offer some comparative relief owing to the construction of the OPS in repeating blocks of larger, oligosaccharide, units. This would result in a reduced number of molecular lengths within the OPS population with larger gaps between them. Despite the complexity the data is however useful to confirm the presence of known structures and to add some detail to the existing knowledge. The ions attributed to the *E. hermannii* OPS can be initially identified as prominent high frequency, peaks with

gaps corresponding to those anticipated given the known structure. After these have been identified other predicted ions in the particular series can be searched for.

An example ESI spectrum from the *E. hermannii* OPS sample, taken at 10.11 minutes at the top of the total ion count peak, is shown in figure 4.28. Some of the prominent high frequency ions have been annotated with their m/z . The mass of the repeating D-Rha4NAc unit is 187 and this would be the difference between peaks if the ions were singly charged. If the ions were quadruply charged the differences would be $187/4 = 46.75$. The difference between the peaks 822.5 to 916.1 is 93.6, between 1010.6 and 916.1 is 94.5, between 1104.2 and 1010.6 is 93.6, between 1244.0 and 1104.2 is 139.8, between 1430.9 and 1244.0 is 186.9 and the difference between 1477.7 and 1430.9 is 46.8. All of these differences are almost exactly divisible by 46.75. Therefore it can be concluded that these are part of a quadruply charged series of OPS ions. Multiplying the m/z values by 4 (due to the charge), then subtracting 92 (to account for four sodium adducts) provides the mass of the ion detected. By dividing this value by 187 it is possible to determine the length of the polymer. As discovered in the analysis of *Brucella* OPS by MALDI-ToF the total mass of the *E. hermannii* polymers are not divisible by the mass of the repeating units. Given what is now known about the different structural elements that make up OPS the remainder is probably due to the presence of non-D-Rha4NAc sugars at the reducing end, as appears to be the case with the OPS from *Brucella*. However it appears most likely that the 822.5 m/z ion is a 17 mer and the 1477.1 m/z ion a 31 mer.

Overall it was possible to detect ions with m/z values compatible with those that varied from being singly to dodecaply sodiated. Although the m/z range of the ESI-QqQ is limited, these multiple charges enabled polymers of up to 90 sugar units in length to be detected. As might be expected, the longer polymers tend to carry more charges. This is summarised in table 4.1. Also, the longer polymers are eluted from the SEC earlier than the shorter ones as would be expected. This is demonstrated by the single ion chromatograms shown in figures 4.29.

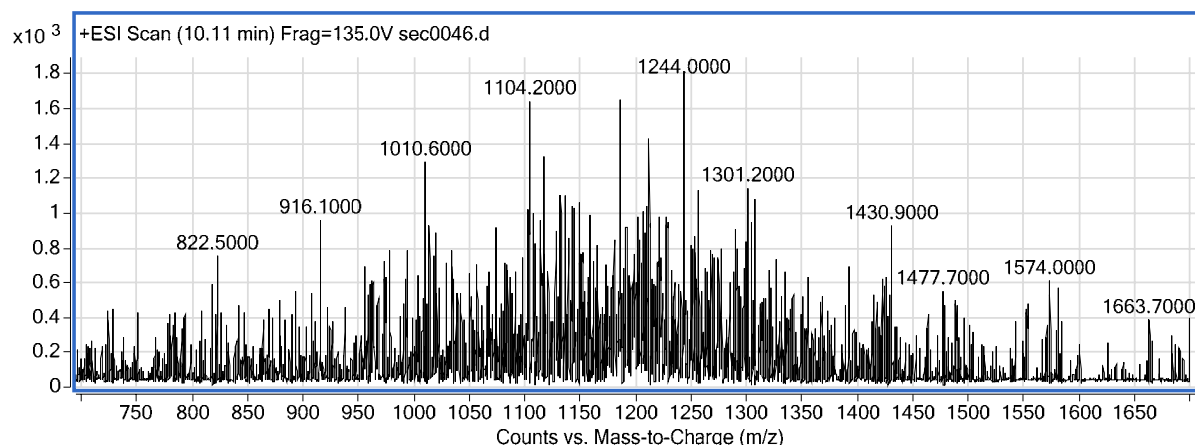


Figure 4.28. ESI-QqQ mass spectrum of *E. hermannii* OPS (10.11 mins)

Fig 2.28 shows the spectrum of *E. hermannii* OPS taken at 10.11min showing a series of quadruply sodiated polymers, the most abundant being the 17mer (m/z 822.5), 19mer (916.0), 21mer (1010.6), 23mer (1104.2), 26mer (1244.0), 30mer (1430.9) and the 31mer (1477.7).

No of adducted sodium ions (charge state)	dp size range	m/z range
1	1-8+	228.1-1538.4
2	2-16	219.1-1529.5
3	8-23	528.1-1464.1
4	14-35+	682.6-1665.4
5	24-44+	925.1-1673.8
6	28-53+	899.5-1679.5
7	40-58	1095.2-1576.6
8	45-65	1078.2-1546.2
9	(50-70)	1064.9-1480.9
10	(55-80)	1054.3-1522.3
11	(60-90)	1045.7-1556.2
12	(65-95)	1038.4-1506.4

Table 4.1. Degree of polymerisation of *E. hermannii* OPS

Degree of polymerisation of *E. hermannii* OPS (dp) found in ions with specific charge states (adducted sodium ions). The '+' signs indicate that the sequence may continue beyond the mass range of the QqQ, and the brackets indicate that the range has been deduced from only two or three extracted ions.

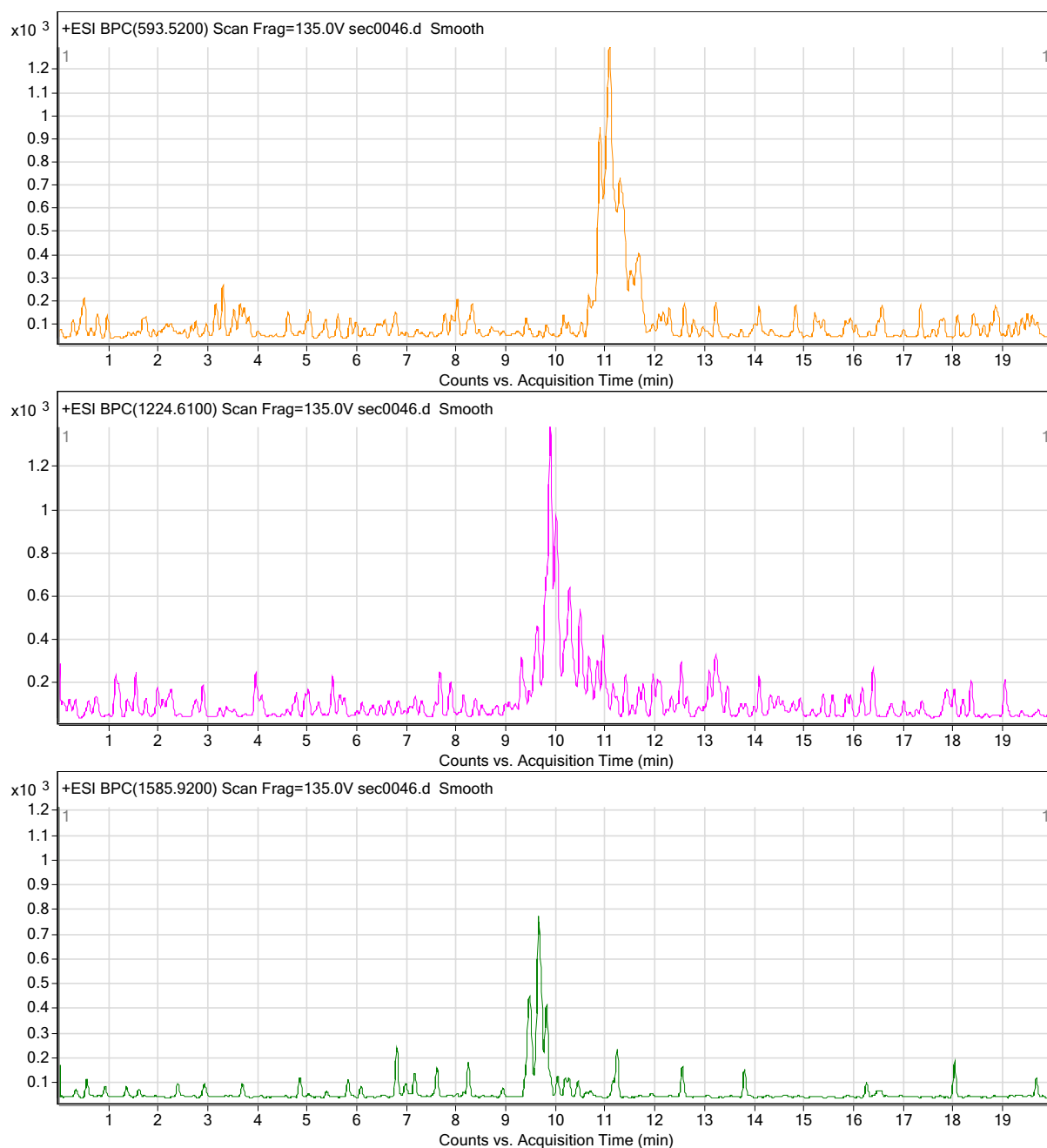


Figure 4.29. Size exclusion chromatographs (specified ions) for *E. hermannii* OPS

Fig 2.29: ESI-QqQ mass spectra of *E. hermannii* OPS eluting from Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column. Top panel: single ion chromatogram of doubly sodiated hexamer at m/z 593.5; second panel: single ion chromatogram of quintuply sodiated 32-mer at m/z 1224.6, and lower panel: single ion chromatogram of sextuply sodiated 50-mer at m/z 1585.9.

The conclusion of the analysis of *E. hermannii* OPS by online SEC-ESI-MS is that the method is effective for the analysis of OPS, for this type of structure at least, especially when some information about the structure is already known. The ions are highly resolved, the approach is highly sensitive and confirms that the OPS does separate by size on the SEC column. The OPS from *E. hermannii* is

extremely similar to that from *Brucella* and *Y. enterocolitica* O:9 OPS and ionises effectively under the described conditions. Therefore the *B. abortus* S99 O:9 OPS should also be detectable by ESI. That it is not is almost definitely due to ion suppression caused by PEG.

4.9.2. Analysis of non-hydrolysed *B. abortus* S99 OPS by Reverse Phase ESI-QqQ

4.9.2.1 Reverse Phase separation of *B. abortus* S99 OPS

The *B. abortus* S99 OPS was separated on the Tosoh TSK-GEL PWXL-CP G3000 column and the fraction containing the OPS was collected. This was then applied to a Vydac C18 column for reverse phase chromatography to determine if this would effectively separate the PEG from the OPS. The UV 210 and 280 nm chromatograms from the C18 column are shown in figure 4.30. These indicate that the OPS is eluting from the column at between 4 and 8 minutes, peaking at 6 minutes. The absence of a peak in the 280 nm chromatograph supports the notion that the 4 – 8 minute peak is the one for the OPS. This separation was done online with the ESI-QqToF and the total ion count chromatogram is shown in figure 4.31.

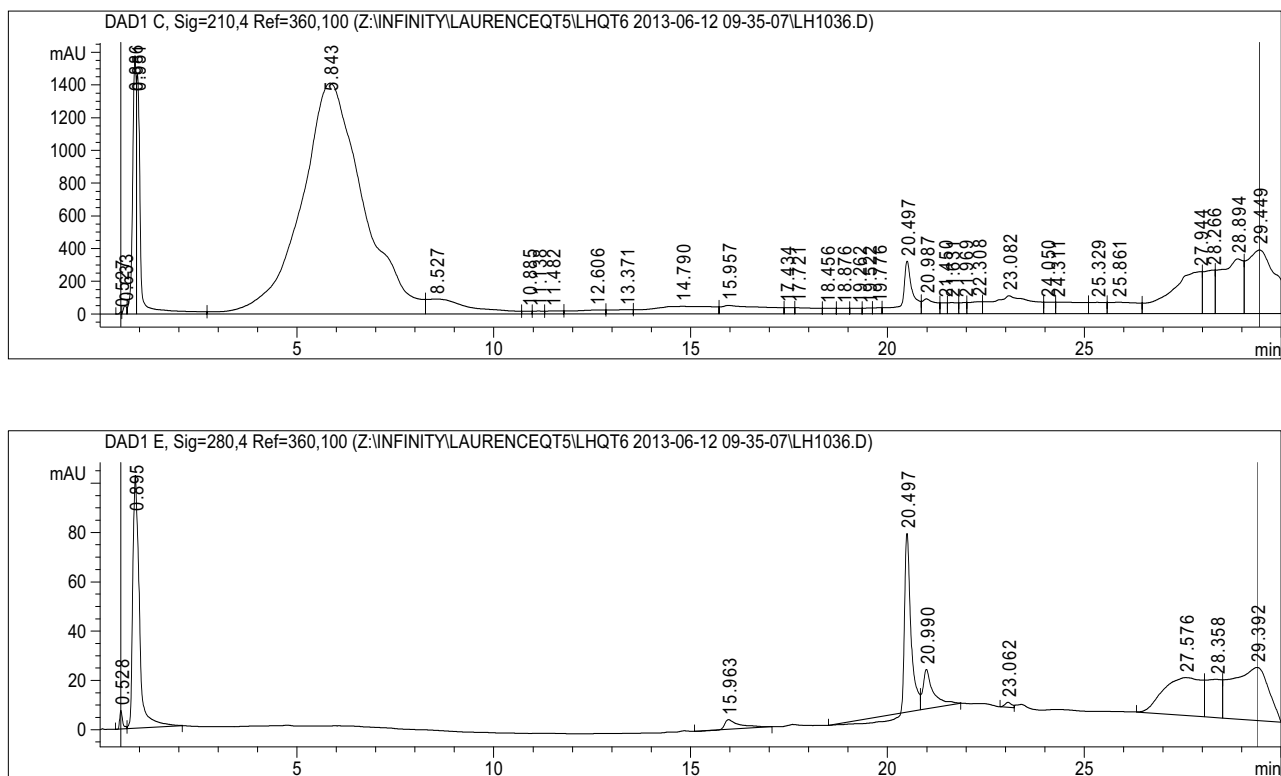


Figure 4.30. Reverse phase UV chromatographs *B. abortus* S99 OPS

Figure 4.30 shows the reverse phase UV chromatograms, 210 nm (top), 280 nm (bottom) of *B. abortus* S99 OPS antigen preparation using a Vydac C18 column with absorbance (y-axis) against time (x-axis). The peak at 5.8 minutes that absorbs at 210 nm but not at 280 nm represents the OPS.

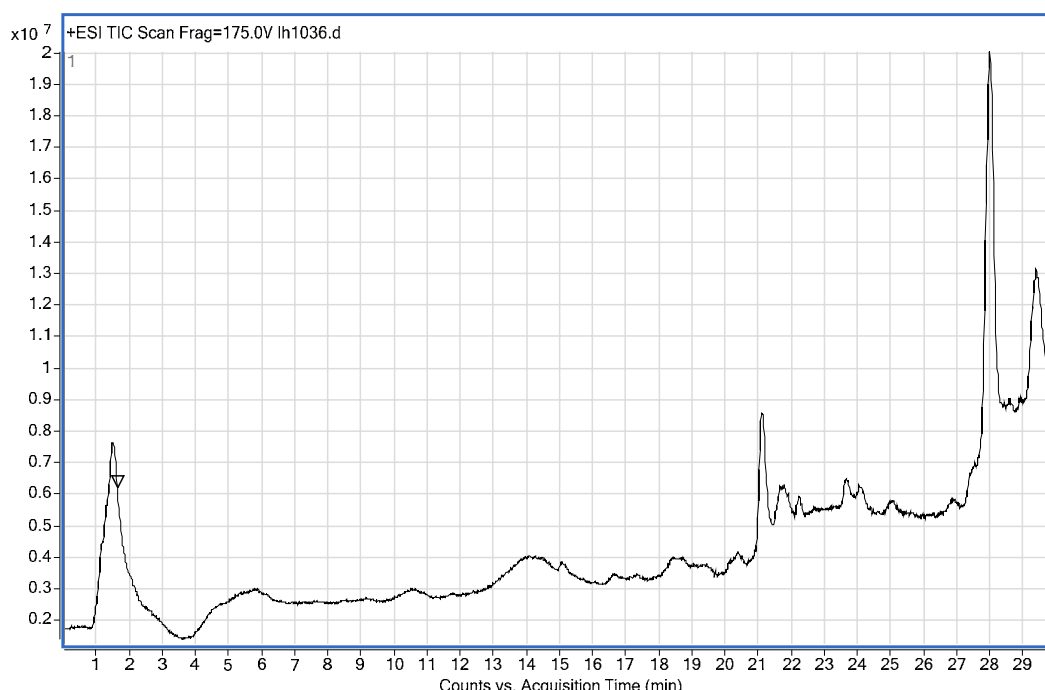


Figure 4.31. Reverse phase total ion count chromatogram for *B. abortus* S99 OPS

Figure 4.31 shows the reverse phase total ion count chromatograms of *B. abortus* S99 OPS antigen preparation using a Vydac C18 column showing total ion count (y-axis) against mins (x-axis). The 'peak' at 5.8 represents the OPS. The 'peak' at 12 – 16 minutes is due to PEG, which reappears again at 28 mins.

As before ions from PEG were detectable in the samples but eluted sufficiently later than the OPS that they were no longer a suppressant. In fig 4.31 the PEG ions appear coincident with the increased ion count observed between 12 and 16 min and again at 28min. This is sufficiently separated from the OPS elution which occurs between 5 and 7 minutes (there being a time delay between the analytes passing the UV detector and reaching the ESI interface). This resulted in the detection of from the OPS as will be shown in subsequent figures.

4.9.2.2. Reverse phase ESI-MS-QqQ of *Brucella* core oligosaccharides

Ions that could be attributed to the core oligosaccharide were also detected and eluted from the column at between 1 to 3 minutes. The spectrum at 1.64 minutes is shown in figure 4.33. This shows singly doubly and triply protonated ions of m/z 1027.4, 514.2 and 343.1 respectively. The structure of the core was recently elucidated (Kubler-Kielb and Vinogradov, 2013b) and is proposed to be that shown below in figure 4.32. This structure contains several GlcN residues which probably endows the structure with a net positive charge and this may partially explain why it was eluted before even the smaller components of the OPS.

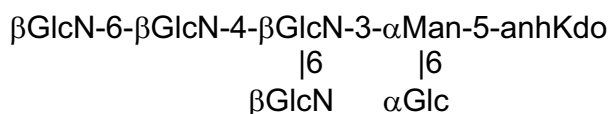


Figure 4.32. Published structure of the LPS core of *Brucella*

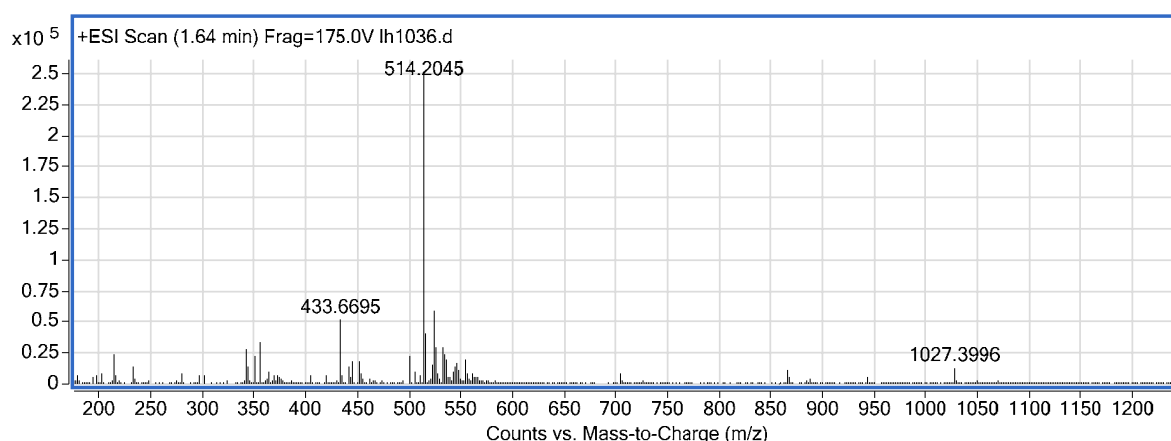


Figure 4.33. ESI-QqQ mass spectrum of *B. abortus* S99 OPS at 1.64 mins

Spectrum taken at 1.64 min (from the Vydac C18 column) showing ion count (y-axis) against m/z (x-axis). The annotated peaks show the singly, doubly and triply charged ions of the core polysaccharide at m/z: 1027.4, 514.2 and 343.1 respectively.

The mass of 1027.4 is consistent with this structure less one Glc. In the publication describing the core structure the authors detected ions at m/z 1028.7 relating to the proposed structure minus αGlc . The calculated mass for this structure ($\text{Hex}_1\text{HexN}_4\text{anhKdo}_1$) is 1026.8 Da therefore the ions depicted in figure 4.33 are a closer match for this than those published. It is interesting to note that the predominant ions are protonated even though NaI is present above trace levels in the mobile phase. This is consistent with presence of at least three basic groups, for example the primary amines on the glucosamine. The doubly protonated form, 541.2 m/z, is the most abundant.

4.9.2.3. Reverse phase ESI-MS-QqQ of *Brucella* OPS

Spectra obtained at 5 minutes and 7 minutes, shown in figures 2.34 and 2.35 respectively show a series of ions that correspond to the presence of the *B. abortus* S99 OPS. Most of these could be assigned to a series of quadruply and quintuply charged OPS ions by reason of the mass differences between adjacent ions in the series with 43.26 m/z units between quadruply charged and 34.61 m/z

units between quintuply charged ions (these differences being factors of the mass of the 173 Da D-Rha4NFo unit). For example, in figure 4.34, there is a series of ions that decrease by m/z 43.26 from the ion at 1335.74. In figure 4.35, there is a series of ions that decrease by 34.61 from the ion observed at 1752.32.

With a few exceptions the masses calculated for the polymers producing the ions in these series do not correspond to a whole number of D-Rha4NFo units. This was also the case with spectra from *E. hermannii* described previously. The recent discovery of the reducing end sugars in the *Brucella* OPS (Kubler-Kielb and Vinogradov, 2013b) provided an basis upon which to try and match up the observed ions series with this published structure. The reducing end sugar sequence is: Rha4NFo-3- α Man-3- α Man-3- β QuiNAc-4- β Glc-4-Kdo (and an extra mannose at an undefined position).

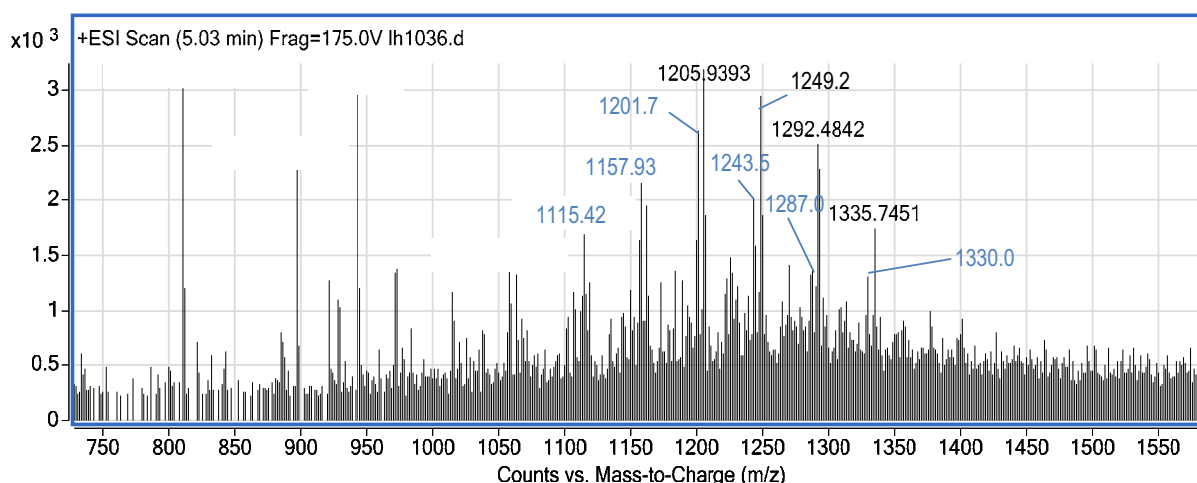


Figure 4.34. ESI-QqQ Mass Spectra of *B. abortus* S99 OPS eluting at 5 mins

Spectrum taken at 5.03 min (from the Vydac C18 column) showing ion count (y-axis) against m/z (x-axis). The prominent peaks are mainly repeating quadruply charged ions of the *Brucella* S99 OPS as indicated by their repeating m/z difference of 43.26. Prominent peaks from two ion series have been annotated in blue and black

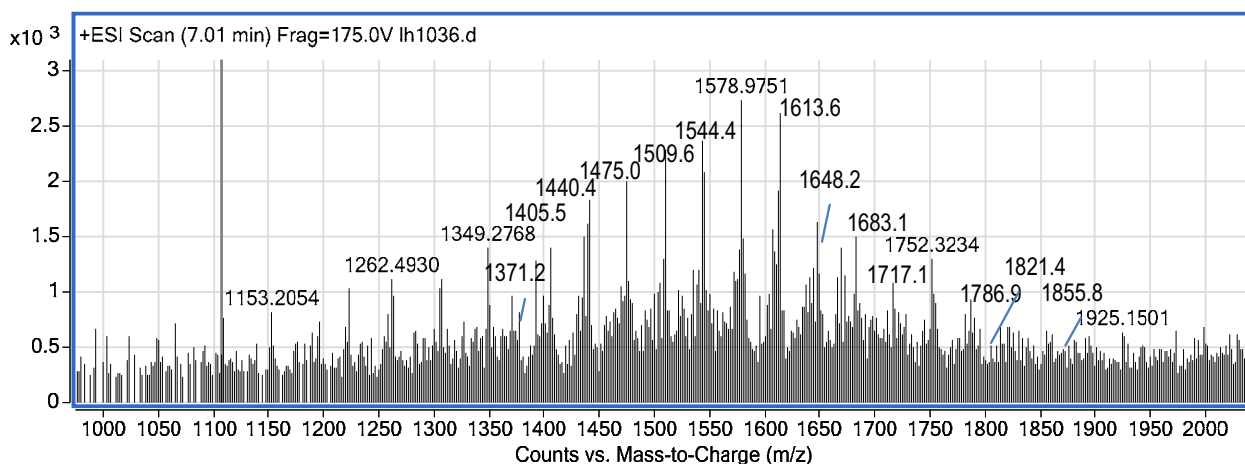


Figure 4.35. ESI-QqQ Mass Spectra of *B. abortus* S99 OPS eluting at 7 mins

Spectrum taken at 7.01 min from the Vydac C18 column showing ion count (y-axis) against m/z (x-axis). The prominent peaks are mainly repeating quintuply charged ions of the *Brucella* S99 OPS as indicated by their repeating m/z difference of 34.61.

Five series of ions were particularly apparent: a) 9 ions of m/z 1115.4 to 1461.5 each approximately 43.26 m/z units apart, b) 9 ions of m/z 1119.4 to 1465.5 also approximately 43.26 m/z units apart, c) 24 ions of m/z 1128.6 to 1924.6 each approximately 34.6 m/z units apart, d) 7 ions of m/z 1499.8 to 1707.4 each approximately 34.6 m/z apart and e) 5 ions of m/z 1548.0 to 1686.4 each approximately 34.6 m/z units apart. There appear to be many other ion series but these were most prominent. These five ion series are consistent with the following formula:

- a) Poly-D-Rha4NFo-Man-Man-QuiNAc-Glc-anhydroKdo – triply deformylated
- b) Poly-D-Rha4NFo-Man-Man-QuiNAc-Glc-Kdo – triply deformylated
- c) Poly-D-Rha4NFo-Man-Man-QuiNAc-Glc-anhydroKdo – quadruply deformylated
- d) Poly-D-Rha4NFo-Man-Man-QuiNAc-Glc-anhydroKdo – quintuply deformylated
- e) Poly-D-Rha4NFo-Man-Man-QuiNAc-Glc-Kdo - quadruply deformylated

All of these sequences contain an extra mannose at an undefined position (Kubler-Kielb and Vinogradov). Also described in the recent publication was the presence of Kdo in anhydro variants. A degree of deformylation in the native OPS was also described which was attributed to either the action of mild acid hydrolysis or to how the native form actually is. The deformylated ions each appear to have one sodium adduct and then, corresponding to the number of deformylations, the

rest of the charge is due to protonation. One formyl group appears to be lost for every, approximately, eight D-Rha4NFo residues.

Taking into account the non-D-Rha4NFo sugars the calculated number of D-Rha4NFo units within these ions is: a) 20-28, b) 20-28, c) 27-50, d) 38-44 and e) 39-43. Thus OPS molecules with chain lengths from 20 to 50 units long covering a molecular weight range from 4200 to 9600 Da are observed. The polymers appear to elute from the reverse phase column in order of increasing chain length.

Several simple series of ions could be seen which were consistent with the presence of short homopolymers of D-Rha4NFo. Two series of ions were prominent: 7 ions from m/z 464.7 to 983.9 at approximately 86.5 m/z units between ions, 6 ions from 605.9 to 893.4 m/z at approximately 57.5 m/z units apart. This is consistent with the presence of doubly and triply sodiated polymers from five to fifteen units long. There did not appear to be any deformylation within these homopolymers. Their origin is hard to rationalise as synthesis of the OPS begins at the reducing end with the addition of the non-D-Rha4NFo sugars. These structures may result from the mild acid hydrolysis used to prepare the OPS from the sLPS. There is substantial evidence that non-covalently bound OPS exists within the bacterial outer membrane and that it may be extracted without acid hydrolysis. It has been given various names but 'Native Hapten' is the widely recognised (Aragon et al., 1996). It is relatively easy to rationalise a process whereby ionic interactions between OPS molecules allow some to be carried to the outer membrane without attachment to the core. However, a route of synthesis that leads to the exclusion of the primer and adapter sequence of sugars remains somewhat mysterious.

The HPLC-ESI-MS data therefore confirmed, although the data was highly convoluted, that the OPS antigen was present in the prepared samples. Furthermore the desire to evaluate by ESI-MS drove a process whereby it was purified from protein, PEG and core sugars by HPLC. The conditions that facilitated this may constitute valuable information for future studies. The core was not identified in the MALDI-ToF analysis. The ESI-MS also identified some D-Rha4NFo homopolymers which were also not identified by MALDI-ToF. The sensitivity of the ESI-MS approach demonstrates that the OPS polymers ionise effectively. The ESI-MS approach also appears to be far less susceptible to decreases in sensitivity caused by increased polymer size. Therefore ESI-MS was used in preference to MALDI-ToF for the rest of the study. These results demonstrate the power and utility of the combination of HPLC and MS.

4.9.3. Evaluation of non-hydrolysed *B. abortus* S99 OPS by GC column – ESI-QqToF

As well as increased sensitivity the ESI-MS approach sits perfectly with liquid chromatography and this may help to resolve some of the conformational features of the oligosaccharides, such as linkage, generated by HCl acid hydrolysis. Graphatised carbon (GC) HPLC columns have been used for the separation of carbohydrates based on conformation as well as size prior to analysis by ESI-MS (Ruhaak et al., 2009). A nano-chip adaptor for the ESI-MS was used for analysis as there was no need for fraction collection. The OPS preparation of *B. abortus* S99 was added to the GC column chip and the total ion count from the ESI-QqToF used for this analysis is shown below in figure 4.36. This shows the OPS eluting between 12-15 minutes with sharp peaks at the beginning and end of this period that may relate to impurities within the sample (this was performed with the 'crude' OPS antigen rather than one 'polished' by the HPLC processes previously described).

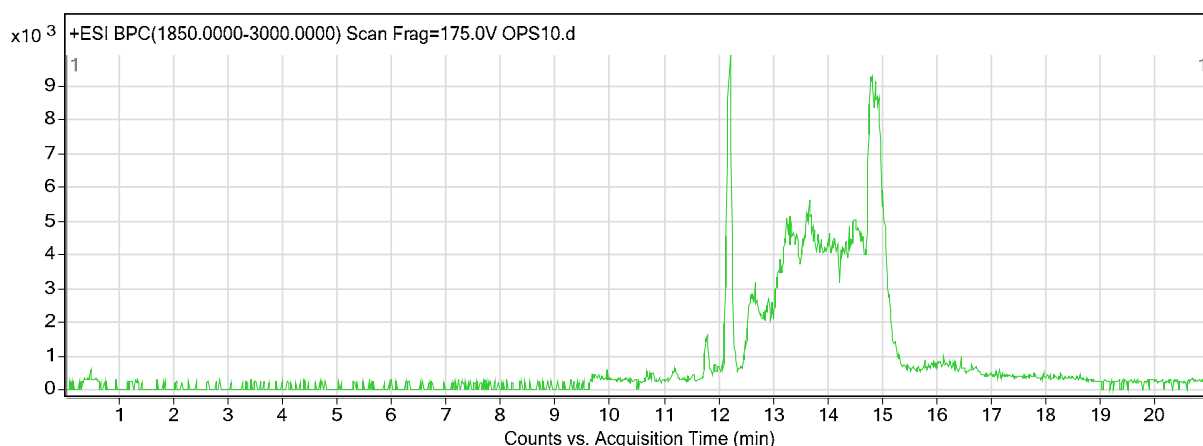


Figure 4.36. Total ion count chromatogram of *B. abortus* S99 OPS eluting from GC column

Total ion count chromatogram of *B. abortus* S99 OPS eluting from GC column with the increase between 12 – 15 minutes being due to the detection of OPS ions.

The mass spectrum from 13.67 minutes is shown below. This shows a nearly symmetrically distribution of ions centred on a peak of 2089 m/z. This relates to an OPS molecule with 43 D-Rha4NFO units. The 2089 m/z ion appears to be part of a quadruply charged polymer series as there are significant peaks that are of 43.26 m/z difference above and below. Other ion OPS related ion series are also present.

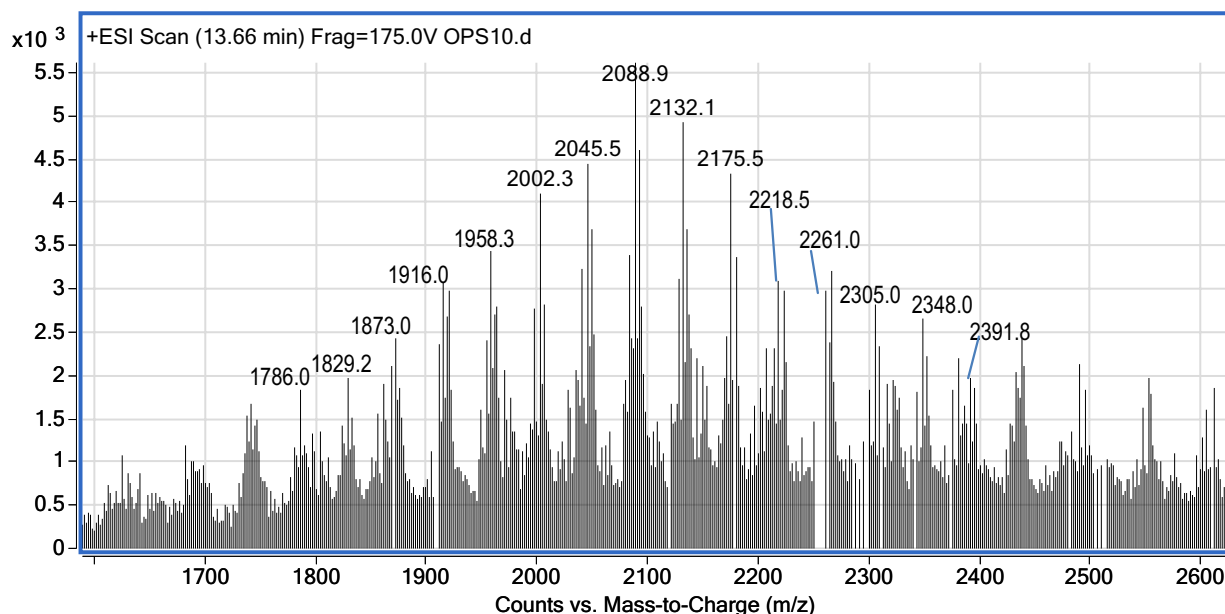


Figure 4.37. ESI-QqQ mass spectrum of *B. abortus* S99 OPS eluting from GC column at 13.6 mins

Spectrum taken at 13.66 min from the GC column showing ion count (y-axis) against m/z (x-axis). The prominent peaks either side of the central 2088.9 m/z ion are mainly repeating quadruply charged ions of the *Brucella* S99 OPS as indicated by their repeating m/z difference of 43.26. A series of such peaks has been annotated.

The spectrum shown in figure 4.38 below was taken at 14.8 mins and shows ions from a single, doubly and quadruply charged series. Ion 2269 m/z for example is part of a series of singly charged ions. The quadruply charged ion polymer series shows ions related to a polymer containing 55 D-Rha4NFO. These results show that the GC column effectively binds and then yields the OPS molecules for detection by the ESI-MS.

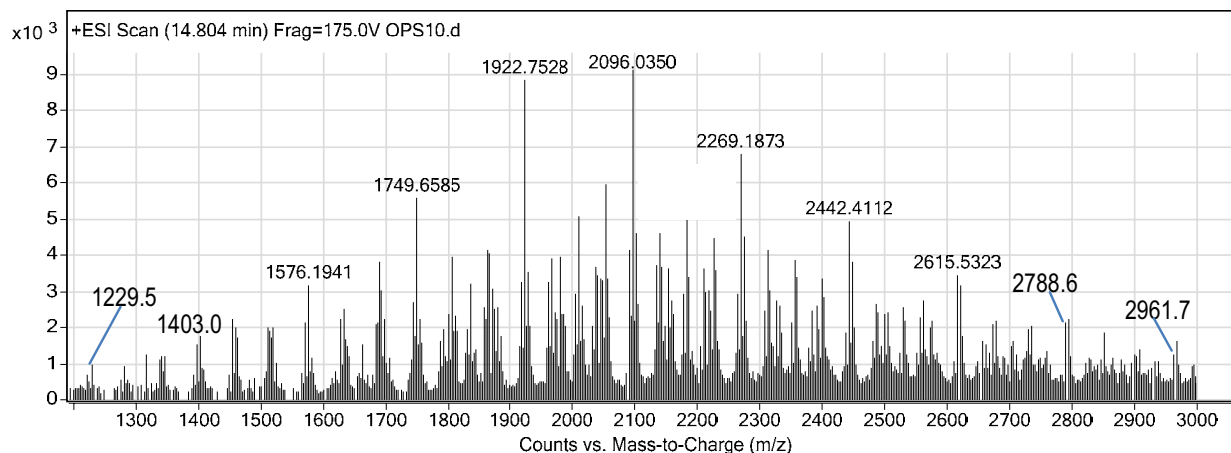


Figure 4.38. ESI-QqQ mass spectrum of *B. abortus* S99 OPS eluting from GC column at 14.8 mins
Spectrum taken at 14.77 min from the GC column showing ion count (y-axis) against m/z (x-axis). The spectrum shows prominent peaks attributable to a singular, doubly or quadruply charged series of OPS ions. Peaks attributable to a singly charged series, 173 m/z apart, have been labelled.

4.9.4. Size and ion-exchange chromatography of hydrolysed *Brucella* and *Y. enterocolitica* O:9 OPS

After partial HCl hydrolysis the *B. abortus* S99 OPS still contained substantial quantities of residual polysaccharides (greater than 12-mer in this context), deformylated products, monosaccharides and the products from degraded monosaccharides. It was particularly important to remove the longer polysaccharides otherwise subsequent antibody immunoselection processes could be biased towards the selection of these larger polysaccharides by virtue of their presentation of multiple epitopes to the bivalent antibodies. This would conflict with the aim of the study which is to identify the binding profiles of single epitope antigens which by definition only bind to a single antibody paratope.

The oligosaccharides were separated from these undesirable components by SEC-HPLC using the Tosoh TSK-GEL PWXL-CP G3000 column described previously. Owing to the positive charged surface of the SEC matrix this also separated the deformylated products based on their charge as well as the mono, oligo and polysaccharides based on their size. The eluted fractions were monitored at 210 nm absorbance as they eluted from the column and the chromatogram is shown in figure 4.39. This clearly shows the early elution of a large quantity of material between 3 and 6 minutes followed by a return to baseline. Given the positively charged nature of the SEC matrix and

the elution profile of the unhydrolysed OPS, as shown in figure 4.26, this is most likely to be partially deformylated material. The two progressively far smaller 'peaks' at approximately 7 to 9 and 9.5 to 10.5 may contain material of interest to subsequent studies.

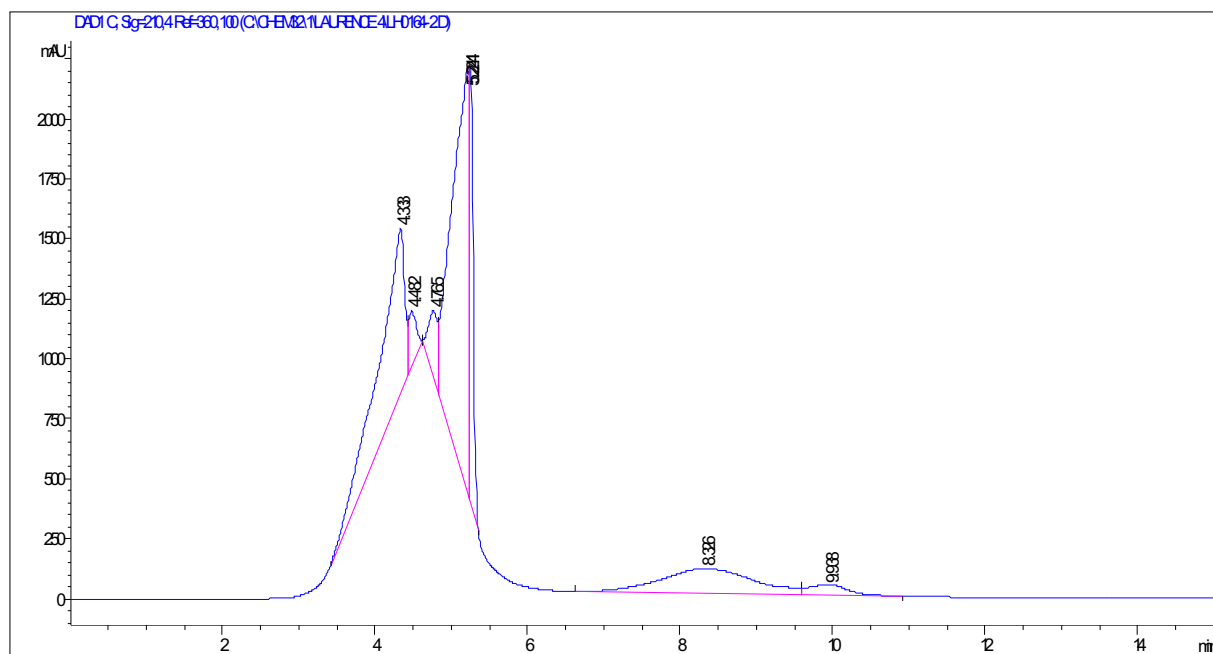


Figure 4.39. Size exclusion chromatograph (UV absorbance) of partially hydrolysed *B. abortus* S99 OPS

Size exclusion chromatograph (UV 210 nm absorbance), showing absorbance (y-axis) against time in mins (x-axis) of *B. abortus* S99 OPS partially hydrolysed by incubation in 10 M HCl at 60°C for 3 mins 45 secs eluting from a Tosoh TSK-GEL PWXL-CP G3000 HPLC size exclusion column. The absorbance between 3 and 4 minutes is due to fully or partially deformylated OPS products. The absorbance between 7 and 9 minutes is due to relatively intact fully formylated OPS. The absorbance between 9.5 and 10.5 minutes is due to D-Rha4NFo homo-oligosaccharides.

4.9.5. GC Column-ESI-QqToF analysis of hydrolysed and fractionated *B. abortus* S99 OPS

The GC column chip integrated with ESI-MS was used to evaluate fractions collected during the described SEC separation of the hydrolysed OPS. The fractions were evaluated by total ion count and specified m/z scans to identify the precise fractions which contained the most purified D-Rha4NFo homo-oligosaccharides. Figure 4.40 shows the total ion scan from the fraction collected from the size exclusion column at 9.5 to 10.5 mins (refer to figure 4.39). This shows regular sharp peaks and through analysis of specified protonated ion chromatograms from this column, examples shown in figures 4.41 to 4.44, it can be seen that the peaks in the total ion count correspond to

oligosaccharides that increase in length with elution time. However, there is some overlap and each of the specified ions have multiple elution peaks.

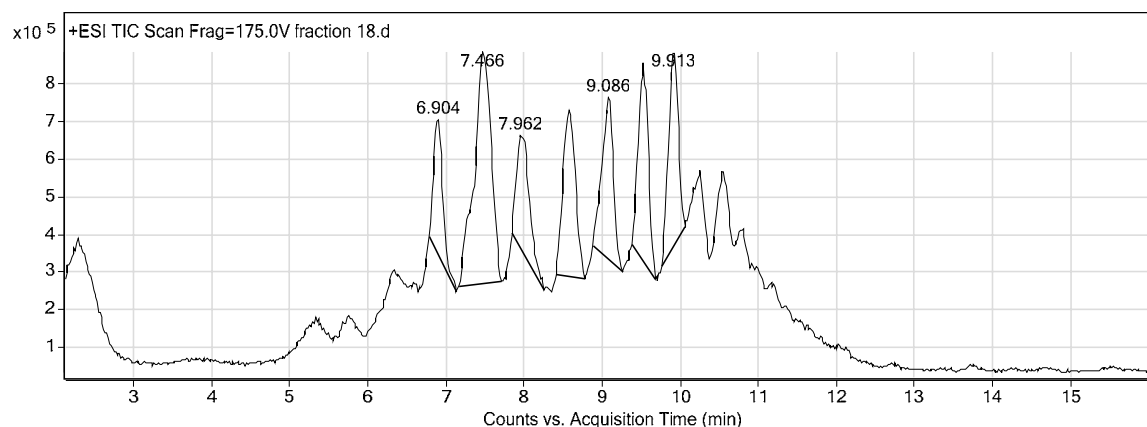


Figure 4.40. Total ion count GC column chromatogram (QqToF) for *B. abortus* S99 oligosaccharides
Total ion count chromatogram for GC column separated *B. abortus* S99 D-RHa4NFo homo-oligosaccharides purified by exclusion chromatography. The highlighted oligomers are: triperosamine (7 mins) tetraperosamine (7.5), pentaperosamine (8.0), hexaperosamine (8.5), heptaperosamine (9.1), octaperosamine (9.6), nonaperosamine (9.9 min). Up to dodecaoligosaccharides can also be detected in this sample using selected ion count.

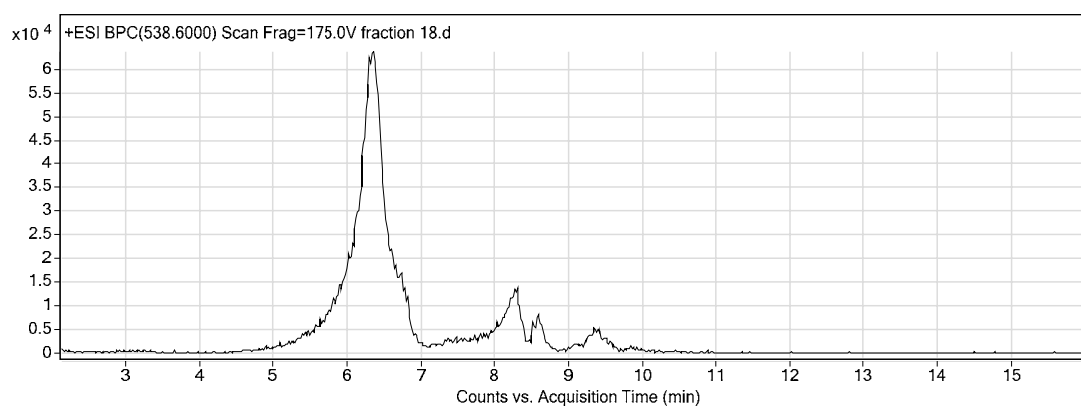


Figure 4.41. Specified ion GC column chromatogram (QqToF) for *B. abortus* S99 tri-D-Rha4NFo

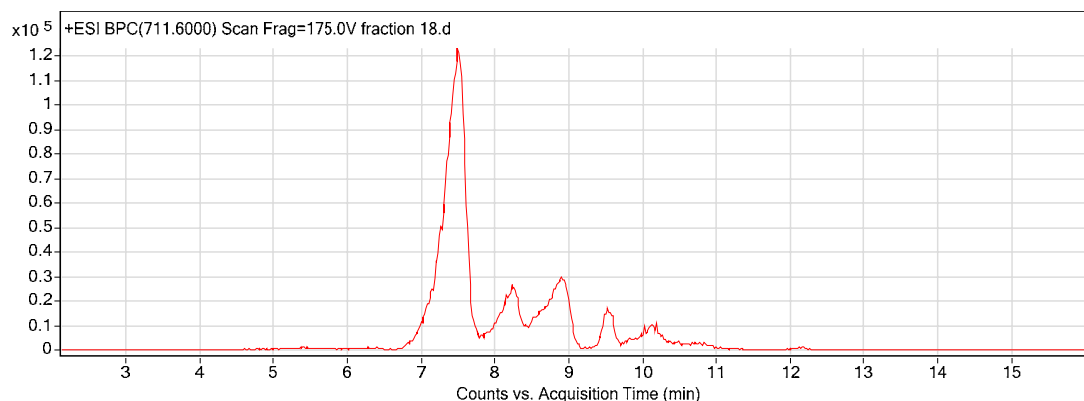


Figure 4.42. Specified ion GC column chromatogram (QqToF) for *B. abortus* S99 tetra-D-Rha4NFO

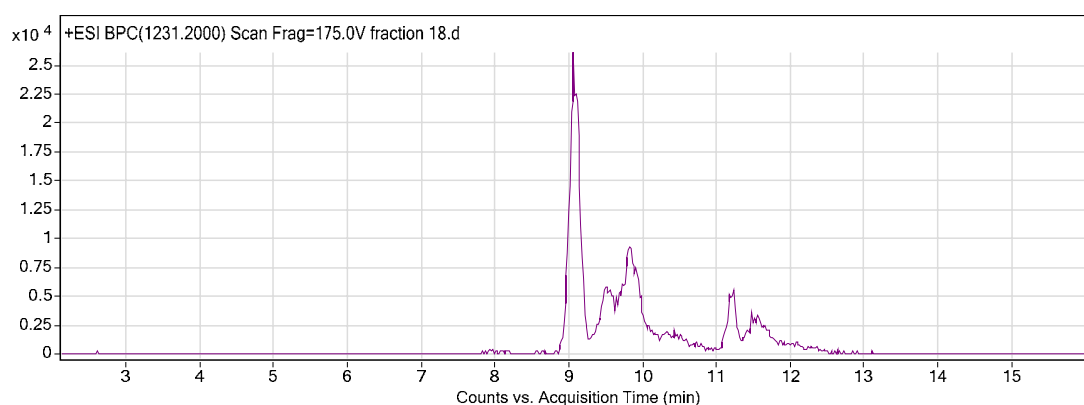


Figure 4.43. Specified ion GC column chromatogram (QqToF) for *B. abortus* S99 hepta-D-Rha4NFO

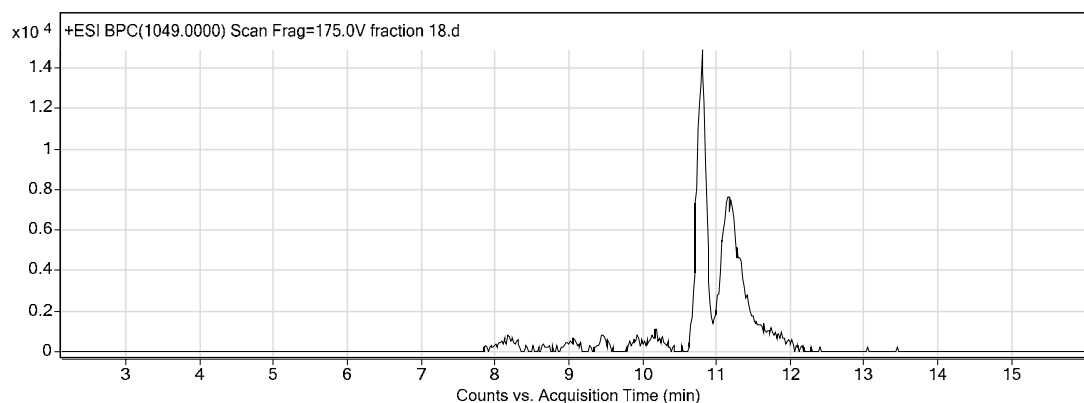


Figure 4.44. Specified ion GC column chromatogram (QqToF) for *B. abortus* S99 dodeca-D-Rha4NFO

The ions detected in this fraction were all fully formylated and the protonated species were relatively high in abundance and they also all corresponded to D-Rha4NFO homopolymers. This is in agreement with the analysis from MALDI-ToF. Analysis of the other fractions from the SEC (elution profile shown in figure 4.39) confirmed that all of the material eluting between 3 and 6 minutes was at least partially deformylated. These molecules are repelled from the positively charged surface of the

SEC and so elute earlier. The molecules eluting between 7 and 9 minutes are larger fully formylated polysaccharides.

The oligosaccharides eluted from the SEC between 9.5 and 10.5 minutes are the perfect material for the next phase of the project as they are fully formylated, they are homopolymers and they are short enough to avoid complications that may subsequently arise due to the availability of multiple antibody binding sites. However, perfect as they may be, they reflect only a very small proportion of the starting material despite attempts to optimise the hydrolysis to minimise de-formylation compared to depolymerisation. This is good evidence to demonstrate that acid hydrolysis is not the optimal approach for this polymer – but it is a far safer option than using hydrogen fluoride, or even trifluoromethanesulfonic acid. Nonetheless, several mgs each of OPS from *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 were hydrolysed by brief incubation for 3 minutes 45 seconds in warm HCl (60°C) followed by dilution with cold distilled water and three rounds of freeze drying and reconstitution. The additional rounds of freeze drying were required to eliminate all the HCl and halt the hydrolysis process.

4.9.6. GC Column-ESI-QqToF analysis of hydrolysed and fractionated *Brucella* and *Y. enterocolitica* O:9 OPS

The three SEC purified OPS D-Rha4NFO homo-oligosaccharide preparations from *B. abortus* S99, *B. melitensis* 16M, *Y. enterocolitica* were all analysed by GC column-ESI-QqQ. The total ion count chromatographs are shown in figures 4.45, 4.45 and 4.46 respectively. Evaluation of the mass spectra at the elution time for each peak confirms that those eluting from 7 to 12 minutes represent oligosaccharides of increasing molecular weight (from 2 to 12 mer) over time although there is some overlap (see below). Mass analysis of the peaks eluting from the column after 14 minutes demonstrate that these ions are not related to the OPS.

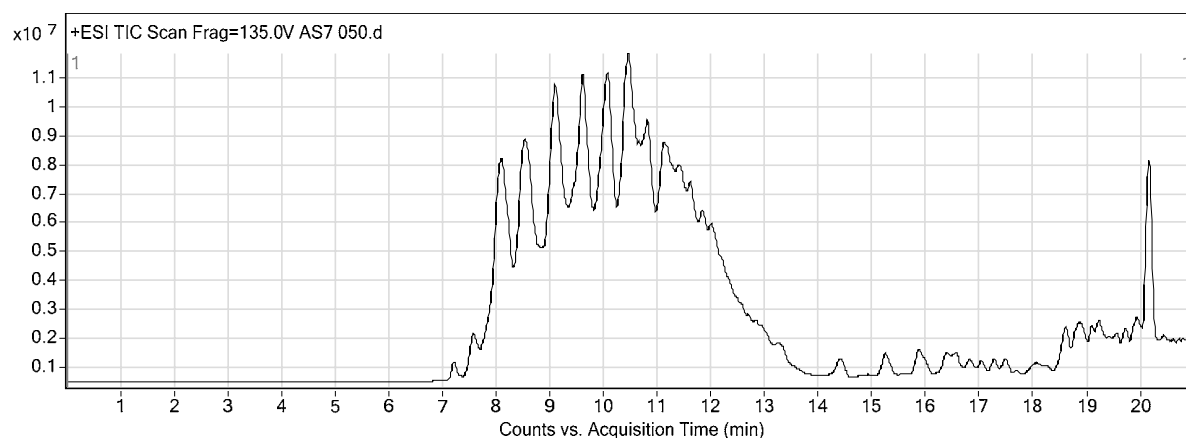


Figure 4.45. Total ion count GC column chromatogram (QqQ) for *B. abortus* S99 oligosaccharides
 Total ion count chromatogram for GC column separated *B. abortus* S99 D-RHa4NFO homo-oligosaccharides purified by exclusion chromatography. The regular repeating peaks from 7 to 11 minutes represent 2 to 10 mer oligosaccharides.

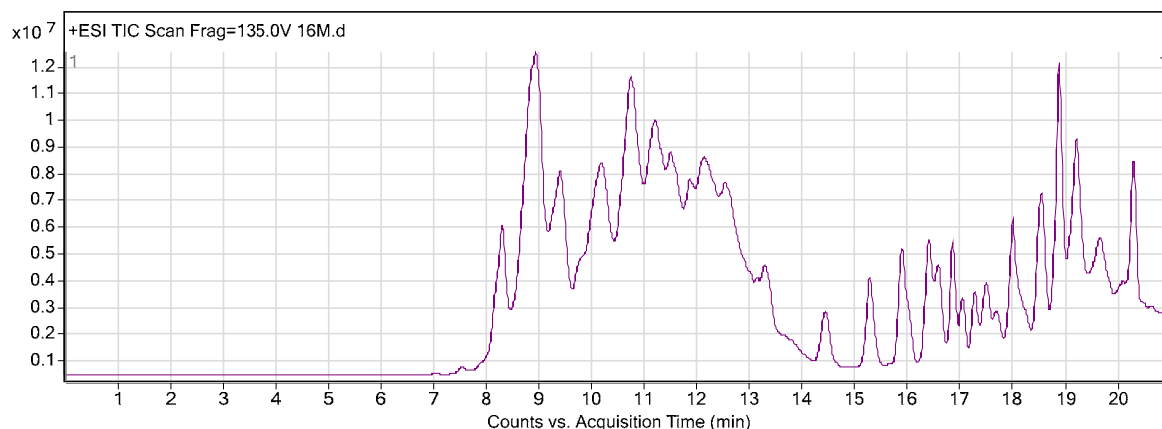


Figure 4.46. Total ion count GC column chromatogram (QqQ) for *B. melitensis* 16M oligosaccharides
 Total ion count chromatogram for GC column separated *B. melitensis* 16M D-RHa4NFO homo-oligosaccharides purified by exclusion chromatography. The regular repeating peaks from 8 to 11 minutes represent 2 to 12 mer oligosaccharides.

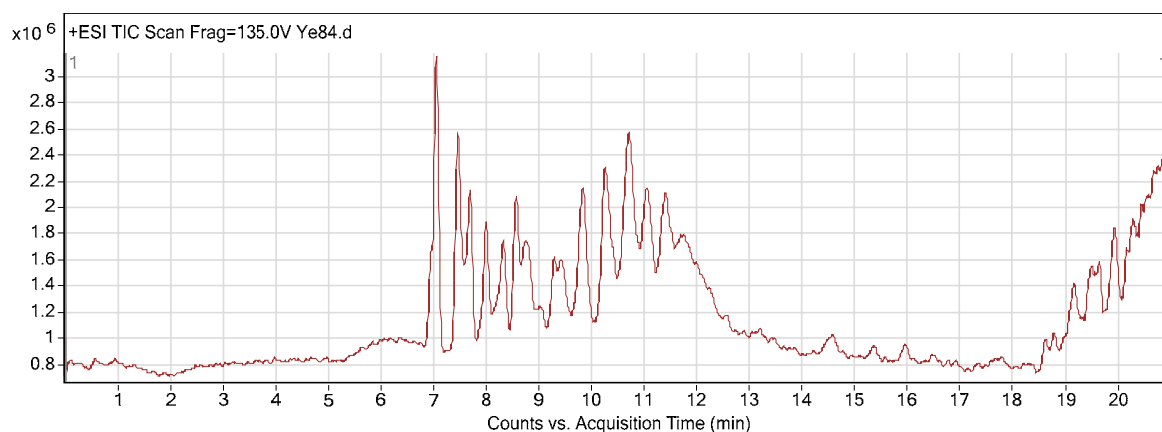


Figure 4.47. Total ion count GC column chromatogram (QqQ) for *Y. enterocolitica* O:9 oligosaccharides

Total ion count chromatogram for GC column separated *Y. enterocolitica* O:9 D-RHa4Nfo homo-oligosaccharides purified by exclusion chromatography. The regular repeating peaks from 7 to 12 minutes represent 2 to 12 mer oligosaccharides.

The results from a selected ion scan for the tetrasaccharide are very interesting, informative and most relevant to subsequent experimentation. Chromatographs for the three *B. abortus* S99, *B. melitensis* 16M, *Y. enterocolitica* O:9 derived tetrasaccharides are shown in figures 4.48, 4.49 and 4.50. These ion scans are more complex than may have been anticipated. There are five significant elution peaks for the tetrasaccharide from *B. abortus* S99 (figure 4.48), two for the *B. melitensis* 16M tetrasaccharide (figure 4.49) and six for the *Y. enterocolitica* O:9 (figure 4.50). This seems paradoxical given that of all the structures for the tetrasaccharide, those from *Y. enterocolitica* O:9 composed completely of $\alpha(1\rightarrow2)$ links should be the simplest and exist in, at most, two isomers (representing alpha and beta forms at the anomeric carbon of the reducing sugar). The tetrasaccharide from *B. melitensis* 16M should be the most complex due to the frequency of alpha-1,3 links and the potential this provides for four different isomers (or eight if including anomeric isomers of each form), yet this has the simplest profile with only two significant peaks. The tetrasaccharide from *B. abortus* S99 should fall somewhere between the two.

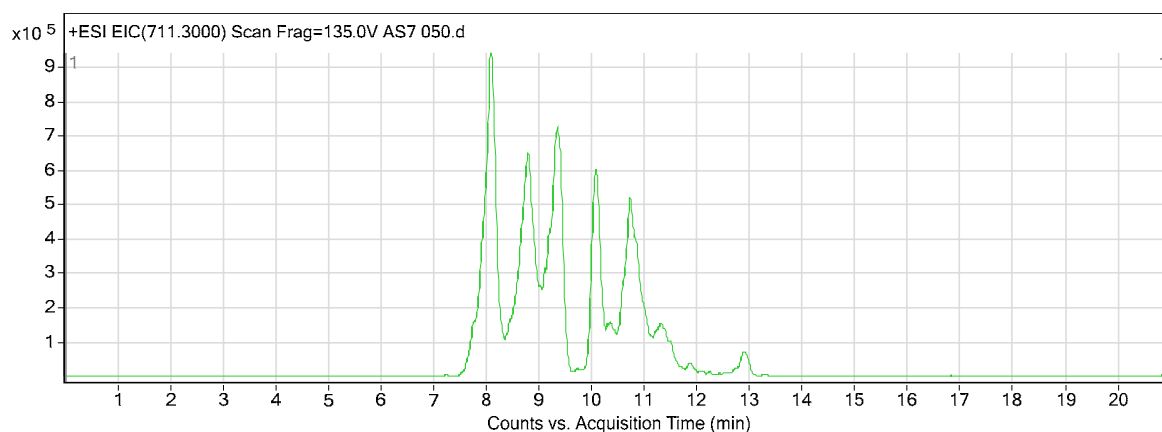


Figure 4.48. Specified ion GC column chromatogram (QqQ) for *B. abortus* S99 tetra-D-Rha4NFO
 Specified ion GC column chromatogram for the protonated tetrasaccharide ($m/z = 711.3$) from the *B. abortus* S99 purified D-Rha4NFO homo-oligosaccharides. Five significant peaks are visible at 8:05, 8:50, 9:20, 10:05 and 10:40 mins:secs.

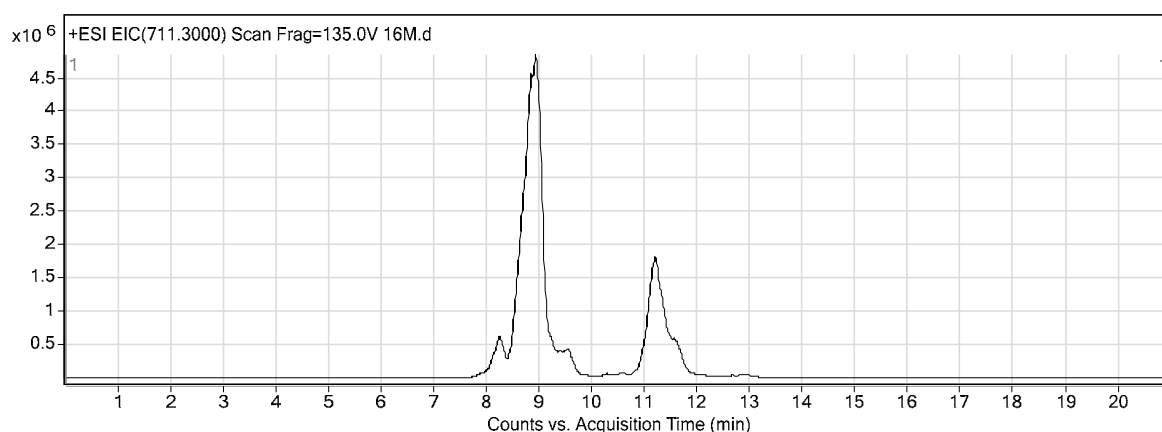


Figure 4.49. Specified ion GC column chromatogram (QqQ) for *B. melitensis* 16M tetra-D-Rha4NFO
 Specified ion GC column chromatogram for the protonated tetrasaccharide ($m/z = 711.3$) from the *B. melitensis* 16M purified D-Rha4NFO homo-oligosaccharides. Three significant peaks are visible at 8:05, 8:50, and 11:10 mins:secs.

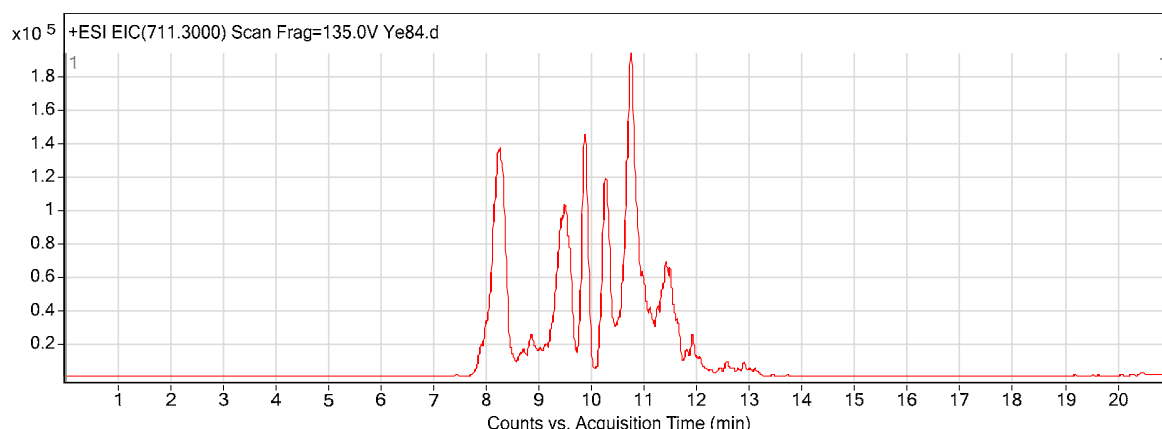


Figure 4.50. Specified ion GC column chromatogram (QqQ) for *Y. enterocolitica* O:9 tetra-D-Rha4NFO
 Specified ion GC column chromatogram for the protonated tetrasaccharide ($m/z = 711.3$) from the *Y. enterocolitica* O:9 purified D-Rha4NFO homo-oligosaccharides. Six significant peaks are visible at 8:05, 9:20, 9:55, 10:10 and 10:40, and 11:30 mins:secs.

One possible reason for this is that the oligosaccharides may form non-covalent bonds with each other and travel through the column in aggregates as well as single molecules. A consideration of their structure suggests that the secondary amino and aldehyde groups within the formyl groups may become interlocked with the same pair of groups on a neighbouring oligosaccharide. This may perform an important biological function within the bacterial cell membrane and may partly explain the existence of the 'Native Hapten'. Oligosaccharides that are kinked by the incorporation of $\alpha(1\rightarrow3)$ links may be less able to form this complementary pairing due to steric hindrance. This may be the reason why the chromatogram for the *B. melitensis* 16M tetrasaccharide is less complex.

As well as the two significant peaks in the *B. melitensis* 16M tetrasaccharide chromatogram there is a smaller peak at 8:10 that has a very similar elution time to the first peak eluted from both the *B. abortus* S99 and *Y. enterocolitica* O:9 tetrasaccharide chromatogram. Furthermore, the *B. abortus* S99 but not the *Y. enterocolitica* O:9 tetrasaccharide chromatogram contains an elution peak at 8:50 that coincides with the timing of the major peak from the *B. melitensis* 16M tetrasaccharide chromatogram. These observations fit the hypothesis that the earlier peak is due to an exclusively $\alpha(1\rightarrow2)$ linked tetrasaccharide whereas the major peak from the *B. melitensis* 16M tetrasaccharide chromatogram is due to a tetrasaccharide containing an $\alpha(1\rightarrow3)$ linkage. There is also a unique peak present in the *Y. enterocolitica* O:9 tetrasaccharide chromatogram eluting at 9:55. This may be due to interactions that only occur between completely $\alpha(1\rightarrow2)$ linked oligosaccharides, although it seems probable that this should occur at least to some degree with the *B. abortus* S99 oligosaccharide preparation.

4.10. Chapter 4: Summary and conclusions

The objective of this part of the project was to generate short homopolymers of D-Rha4NFO. Through the use of MALDI-ToF and modified FACE an effective method for depolymerisation was developed. It was possible to separate the short intact oligosaccharides from the longer and deformed polymers through the use of a size exclusion column with a positively charged surface. HPLC coupled ESI-MS (both QqQ and QToF) proved to be a highly sensitive means to detect specific oligosaccharides and also identified the presence of the core sugars in the OPS antigen preparation. These spectra were highly convoluted and would present a challenge to attempts at *de-novo* analysis. However, the method was sensitive and precise even for high mass polymers. In particular the GC column separated the oligosaccharides based on size but also by conformation through a less well defined mechanism. This method of separation provided some surprising but very useful ion specific chromatograms. This is amply demonstrated by the chromatogram for the D-Rha4NFO tetrasaccharides from the A dominant *B. abortus* S99, the M dominant *B. melitensis* 16M and the exclusively $\alpha(1\rightarrow2)$ linked *Y. enterocolitica* O:9. These chromatograms show a peak that is specific for *Brucella* and more abundant in *B. melitensis* 16M than in *B. abortus* S99. These oligosaccharides will be applied in the next phase of the project, antibody affinity chromatography, and the GC-HPLC-ESI-MS method for analysis will be used to monitor the outcome.

Chapter 5

AFFINITY CHROMATOGRAPHY of OPS HOMO- D-Rha4NFo-OLIGOSACCHARIDES

5.1. INTRODUCTION

The aim of this component of the project was to evaluate the binding of antibodies of particular OPS specificity to the purified homo-D-Rha4NFO-oligosaccharides derived from *B. abortus* S99 OPS that were produced by the means described in chapter 4. The hypothesis is that antibodies of different specificity will select different oligosaccharide types from the pool of those that were generated. In particular, antibodies with specificity for *Brucella* OPS will select oligosaccharides that may only be derived from *Brucella* OPS. The selected oligosaccharides will be compared, by LC-ESI-MS, to the homo-D-Rha4NFO-oligosaccharides that make up the starting pool (*B. abortus* S99 OPS) and to those derived from *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS. The results from the GC column-ESI-MS analysis of these oligosaccharide populations were presented in chapter 4.

In order to test this theory it was necessary to select the desired antibodies. To probe for M-type oligosaccharides the BM40 mAb was selected owing to its high specificity for this antigen. In the absence of mAbs against the A, C ('C specific') and C/Y epitopes a high titre bovine serum sample was used as a source of antibodies. Whilst the purification of the specific polyclonal anti-*Brucella* antibodies from this sample presented challenges, the use of polyclonal antibodies from a primary host created an opportunity to generate data that has more relevance beyond the research laboratory than would be the case if only mAbs were used. The antibodies must be conjugated to an appropriate matrix in order for subsequent separation and elution of the oligosaccharides. Prior to this, the polyclonal antibodies need to be purified from the rest of the serum proteins in order to avoid potentially confounding elements from entering the experiments.

5.2. Affinity purification of anti-*Brucella* OPS antibodies

5.2.1. Conjugation of OPS to affinity chromatography columns

The polyclonal anti-*Brucella* OPS antibodies were purified from a serum sample collected from a cow experimentally infected with *B. abortus* strain 544 (an A dominant strain). The serum was of high anti-*Brucella* titre in all conventional and contemporary immunodiagnostic assays and in plentiful supply. In order to effect the desired purification of antibody, the OPS antigen preparations from *B. abortus* S99, *B. melitensis* 16 and *Y. enterocolitica* O:9 were used. These were conjugated to CarboLink™ (Pierce) columns. These are centrifugal spin columns whereby chromatography occurs in a stepwise

fashion through the addition of reagents to the column followed by centrifugation to remove liquid and unbound components. The conjugation chemistry is the same as that employed by the Carbo-BIND™ ELISA plates (Corning) that were used for the covalent attachment of the OPS (as described in chapter 3). The beaded agarose resin within the CarboLink™ columns is coated with hydrazide groups on the end of spacer arms. Under the appropriate conditions these groups spontaneously react with aldehyde groups within oxidised carbohydrates to create stable hydrazone bonds. Although some re-optimisation was required, the process of coupling the OPS to the CarboLink™ columns was essentially the same as that for coupling the OPS to the Carbo-BIND™ ELISA plates. This was mild oxidation in cold sodium metaperiodate, followed by an additional desalting step to remove excess sodium metaperiodate and then incubation of the oxidised OPS within the column in sodium acetate buffer. Three columns were prepared; one each for *B. abortus* S99, *B. melitensis* 16 and *Y. enterocolitica* O:9 OPS. For each of these columns 2 mg of OPS antigen was used.

5.2.1.1. Evaluation of OPS antigen loss from OPS conjugated affinity chromatography columns

The successful conjugation of OPS to the column can be partially demonstrated by the data in figure 5.1 that shows that, on the basis of what is lost from the column during washing steps post conjugation, most of the antigen was retained. The standard cELISA test (Perrett et al., 2010) was applied to the wash fractions to detect fluid phase OPS. The cELISA sets up a reaction between an HRP labelled BM40 mAb and solid phase *B. melitensis* 16M sLPS. The binding of the BM40-HRP is detected by the use of an HRP sensitive colourimetric substrate. When competing reagents are added, which may be either anti-*B. melitensis* OPS reactive antibodies (which, as shown in chapter 3, need not necessarily arise from infection with *Brucella*) or BM40 reactive antigen (which must contain M epitopes as also shown in chapter 3) they will inhibit this reaction as will be measured by a reduction in colour change. The scale on the y-axis in figure 5.1 is the percentage inhibition of this reaction whereby the higher the value, the more the inhibition and the greater the measure of the competing analyte. In this case this is free, fluid phase, OPS.

The cELISA data for the wash fractions, shown as broken lines in figure 5.1, demonstrates that in the first wash (far left hand side) after conjugation there is a substantial amount of OPS. For the *B. abortus* and *B. melitensis* this is approximately 200 µg/ml and 50 µg/ml respectively. This quantity can be estimated from the cELISA data by using the dose response curves shown as solid lines on

the graph. As shown in chapter 3, the cELISA is more sensitive to *B. melitensis* 16M than *B. abortus* S99 OPS. However the concentration of OPS in the wash fractions rapidly falls with each wash. After the fifth wash there was no further significant detection of OPS. Using these estimates, approximately 1.5 mg of OPS bound to the *B. abortus* column and over 1.8 mg bound to the *B. melitensis* column.

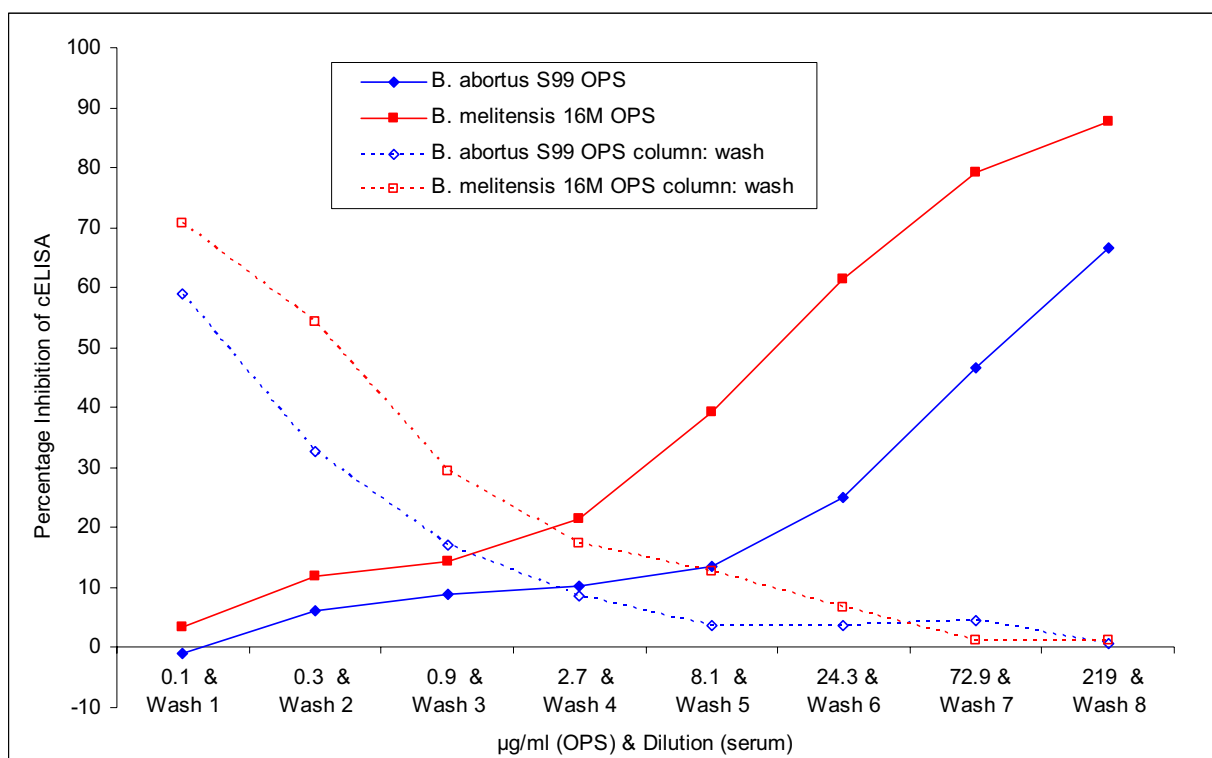


Figure 5.1. Evaluation of OPS loss from CarboLink™ columns after conjugation

The line graph in figure 5.1 shows the detection of fluid phase OPS antigen by measurement of the inhibition of BM40-HRP mAb binding to solid phase *B. melitensis* 16M sLPS in cELISA. The scale on the y-axis shows percentage inhibition of the cELISA whereby higher values indicate higher concentrations of free fluid phase antigen. The solid lines show a dose response curve for fluid phase OPS in cELISA. The broken lines shown the cELISA results for the wash fractions collected after conjugation of OPS to the CarboLink™ columns. The blue lines show results for *B. abortus* S99 OPS and the red lines show results for *B. melitensis* 16M OPS.

5.2.2. Development of affinity chromatography method for anti-*Brucella* OPS antibodies

5.2.2.1. Evaluation of retention of serum proteins by OPS conjugated columns

A process of affinity selection using the OPS conjugated columns was then developed. This involved three basic steps. Addition of sample, washing to remove non-specifically bound material followed by elution of the specifically bound material. The effectiveness of each of these processes was

evaluated by Bradford (Coomassie) protein assay and immunoassay - competitive and indirect. The method of use for the column follows this sequence: addition of sample or reagent, mixing and incubation, centrifugation and collection of eluate. Each one of these steps may be reiterated several times. After use the columns may be stored for reuse if suitably cleansed of the previous sample and buffers.

The results of the protein assay for a prototype, exploratory affinity chromatography process are shown in figure 5.2. This shows the total protein content for each of the fractions shown. The source positive serum had a protein concentration of 64.3 mg/ml, 2 mls of which were added to the *B. abortus* S99 OPS conjugated column in total. Bovine serum typically contains 60 – 80 mg/ml of protein (Alberghina et al., 2011). The sample addition was done in 5 steps, in each one 2 mls of 1/5 diluted (in a bovine casein buffer) serum was added. The protein assay results for the flow through (the liquid collected after centrifugation) after each one of these steps is shown sequentially in figure 5.2 annotated as 'SFT' 1-5. The levels of protein within flow through were high right from the first fraction and although the protein increased to a maximum of approximately 20% the data still suggests most of the protein passes straight through the column even after the first addition. The sum protein measured in the flow through was slightly above the protein content within the sample added due to the use of the casein buffer which contained 1 mg/ml of protein at working concentration. The column was then washed with 7 rounds of 2mls of PBS. The protein content of the wash fractions (the PBS collected after centrifugation) is also shown. After the 4th wash the protein content was very low – less than 20 µg/ml and falling.

The addition to the column of 0.1 M glycine with pH adjusted with HCl lead to a small but perceptible elution of protein. Four elution buffers, of decreasing pH were sequentially applied to the column. Each buffer was added to the column in 2 ml volumes and the process of addition of eluting buffer, mixing and centrifugation of the column to collect eluate was iterated a total of 6 times per pH tested. The 12 ml total eluate volume for each individual elution buffer was pooled and concentrated and the protein content measured. The total amount of protein eluted within each of the four 12 ml volumes corresponding to each elution buffer is shown in figure 5.2. The total amount of protein released from the column as a result of the addition of eluting buffers was approximately 1.25 mg. This represents approximately 1% of the total protein content. These results show that approximately 99% of the protein has been removed from the column prior to the use of the elution buffers. As the specific anti-*Brucella* OPS antibodies would only make up a small fraction of the total serum protein content the

amount of protein collected, assuming that the affinity selection has been effective, is surprisingly high. This assumption required further analysis and scrutiny.

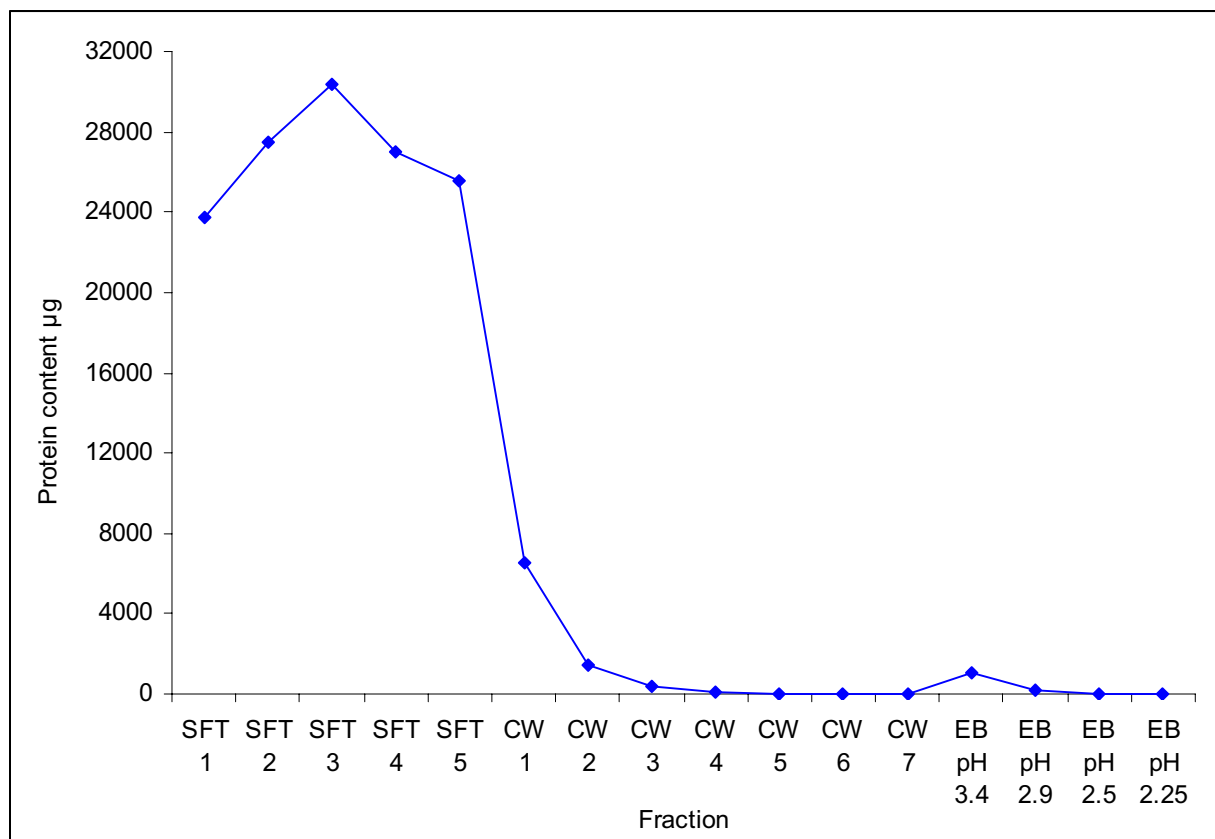


Figure 5.2. Protein assay performed on fractions from OPS affinity selection of serum antibodies

The figure shows the results of the Bradford protein assay, in µg, on the y-axis against the selected fraction (x-axis). The fractions are shown in the order in which they were collected. 'SFT' is the serum flow through that occurs after incubation of the column with serum. 'CW1' to 'CW7' represents seven sequential fractions following wash with PBS. 'EB pH 3.4', 2.9, 2.5 and 2.25 represents the fractions collected after incubation with elution buffer at pH 3.4, 2.9, 2.5 and 2.25. There were 6 individual EB fractions for each pH that were neutralised, pooled, concentrated and then quantified to present an average protein concentration.

5.2.2.2. Evaluation of retention of anti-*Brucella* OPS antibodies by OPS conjugated columns using cELISA

The fractions described above that were evaluated for protein content were also evaluated by the cELISA described above that was used to detect the fluid phase OPS (figure 5.1). An equivalent set of fractions to that derived from the addition of positive serum to the *B. abortus* S99 OPS column were generated by the application of the same process to a *B. melitensis* 16M OPS conjugated column. Fractions from both columns were evaluated by cELISA. The results are shown in figure 5.3

in chronological, order along the x-axis, that follows the sequence of processes applied. Also shown, on the left hand side of the graph, is a dose response curve for the positive serum sample that was applied to the columns. This curve allows for estimations of antibody titre within the chromatography fractions to be made.

The overall protocol shown in figure 5.3 shows 36 iterations per column of the addition, incubation and centrifugation step. There were 5 serum addition (and subsequent flow through) steps so that the column could be loaded with specific antibodies. There were 7 subsequent wash steps. These were followed by 6 elution steps per buffer pH. In the example in figure 5.2 there were four different elution buffers making 24 elution steps in total. The elution buffers were added sequentially with the strongest last so that antibodies of different avidities can be collected separately. The collected eluates were immediately neutralised with tris-base buffer to prevent undue degradation of the antibodies. The use of a strong final eluate also cleans the column in readiness for the next use. The OPS molecule and the hydrazone bond were both robust enough to withstand repeated exposure to 0.1M glycine pH 2.2 and this was used as a final buffer to strip any remaining antibodies from the column so that it was clean for subsequent use.

Figure 5.3 shows that the first eluate (serum flow through) after the first addition of serum contained little competing antibody. This suggests that virtually all of the anti-OPS antibodies that were added were retained. This starkly contrasts with the results of the protein assay which shows that almost all of the protein flowed through into this fraction. This immediately indicates that the columns are specifically retaining the anti-*Brucella* OPS antibodies. With successive additions of sera the quantity of the antibodies in the 'flow through' eluates increased as fewer and fewer binding sites on the OPS became available. The curve relating to the specific antibody concentration in the serum flow through began to level off after five additions of sera so it was considered that the capacity of the column was being reached. After serum addition, the columns were washed with PBS seven times. Within each wash there were antibodies present and any reduction to baseline levels appeared to be only a distant possibility. Therefore, on the basis that washing excess OPS from the columns was effective after 5 iterations (refer to figure 5.1), 7 washes were deemed suitable for the antibody bound columns.

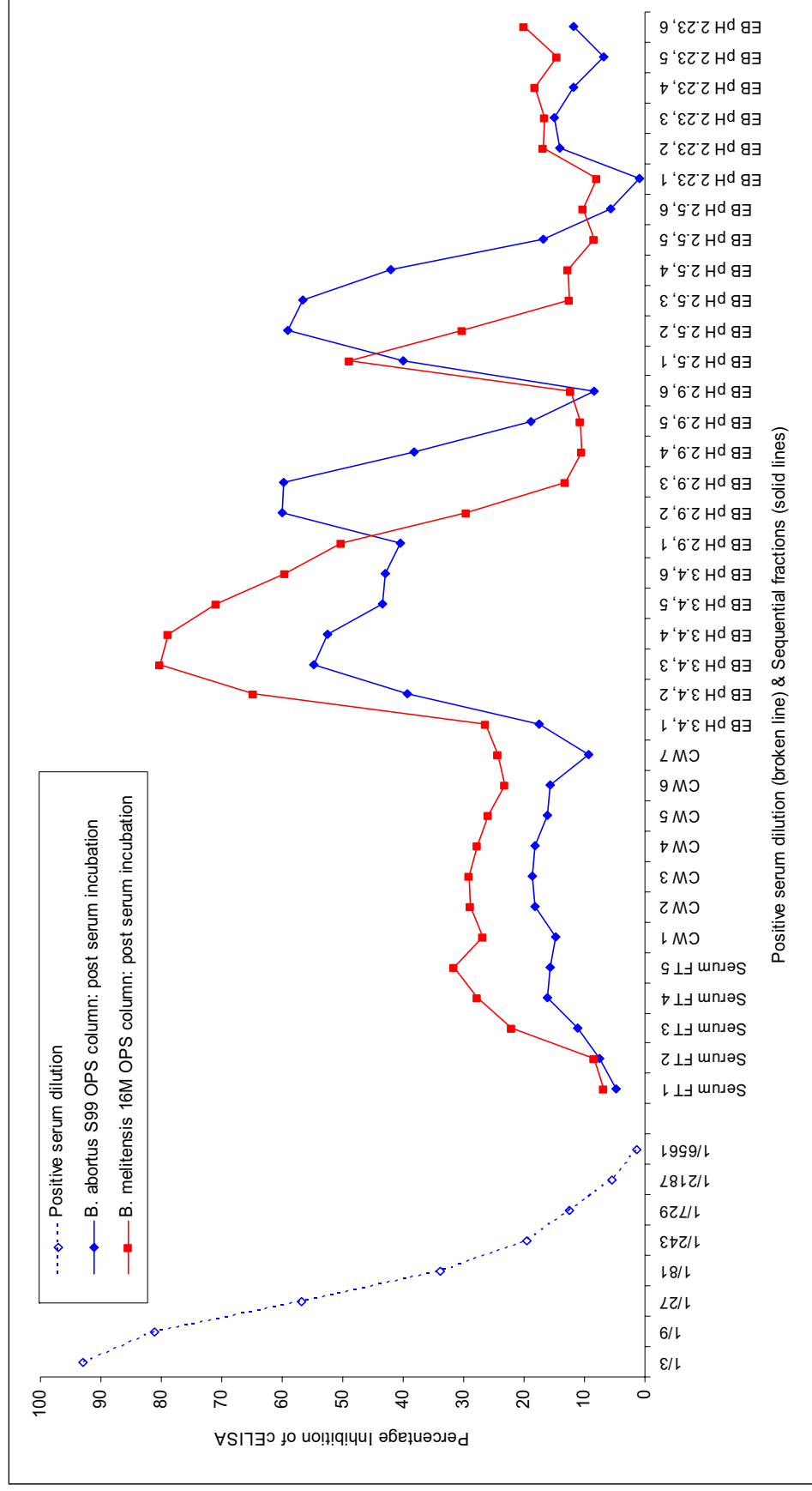


Figure 5.3. cELISA results showing the antibody content washed or eluted from within *Brucella* OPS column fractions

The line graph in figure 5.3 shows the cELISA results (percentage inhibition) of each of the fractions obtained after addition of positive serum to the *B. abortus* S99 OPS (solid blue line) and *B. melitensis* 16M OPS (solid red line) conjugated CarboLink™ columns. Higher values indicate the presence of more antibodies although the relationship is non-linear. The broken blue lines show the dose response curve for serial dilutions of the positive serum. The fractions are shown in chronological sequence from left to right along the x-axis. There are three fraction types: Serum FT 1-5 (the flow through from the column after 5 sequential additions of positive serum), CW 1-7 (the contents of the PBS wash buffer applied sequentially to the column) and EB pH X 1-6 (the eluates obtained after sequential addition of elution buffer). The pH of the elution buffer decreased from 3.4 to 2.23 as shown on the x-axis.

The number of wash iterations was subsequently justified on the basis of the results obtained after the addition of eluting buffer. Although the first 2 mls had little effect (probably due to buffering by residual PBS wash still within the column) the next 4 mls, 2nd and 3rd additions, released large quantities of specific antibodies from both columns. Subsequent additions 4, 5 and 6 of the same elution buffer released more, but not as many, antibodies. Decreasing the pH of the elution buffer from 3.4 to 2.9 created another peak in antibody release in the *B. abortus* S99 but not the *B. melitensis* 16M OPS column. There was another large peak in antibody release from both columns after changing the elution buffer from pH 2.9 to 2.5. A subsequent change to pH 2.23 resulted in a small rise in antibody release suggesting that few antibodies remained on the column. Prior to testing the antibodies obtained within the individual fractions collected for each elution buffer, the eluates were neutralised by the addition of tris-base buffer.

The results, shown in figures 5.2 and 5.3, demonstrated that specific antibodies could be retained on the columns and virtually all non-specific proteins could be removed prior to the pH based sequential release of the, now purified, anti-*Brucella* OPS antibodies. The data demonstrated that there was a lag effect with the addition of new buffers, probably due to the incomplete removal of prior buffer by centrifugation. The data also showed that several iterations of wash and elution buffer addition were required in order for these buffers to exert their full effect.

5.2.2.3. Evaluation of retention of anti-*Brucella* OPS antibodies by OPS conjugated columns using iELISA

The polyclonal antibody fractions derived from the *B. abortus* S99 and *B. melitensis* 16M OPS conjugated columns were also evaluated by iELISA against the homologous and heterologous sLPS antigens. These results are shown in figure 5.4 where the results on the y-axis are expressed as a percentage of the OD of the source serum at a dilution of 1/12,800. The results for the source serum (shown in blue) are shown on the left hand side of the graph in order to assist in the interpretation of the data derived from the column fractions. The data from the fractions from the *B. abortus* S99 column are shown in red and those from the *B. melitensis* 16M column are shown in green. In these instances the fractions obtained after elution with each specific buffer were pooled. They were then concentrated using an Amicon centrifugal filter with a molecular weight cut-off of 30,000 kDa. Then buffer exchanged into PBS using disposable sephadex G-25 columns and then finally, re-

concentrated by centrifugal filtration as just described. This process aided in the storage of the samples and the changes in volume have been factored in to the analysis. The results shown by the solid lines represent data from iELISA using the sLPS from an A dominant strain of *Brucella* (*B. abortus* S99) and the results from the broken lines represent data from an iELISA using the sLPS from an M dominant strain of *Brucella* (*B. melitensis* 16M).

The results shown in figure 5.4 demonstrate that the wash fractions from both columns contain detectable levels of antibody, and this confirms the findings from cELISA shown in figure 5.3. The results also show a large increase in antibody titre after the addition of the elution buffers. The total amount of antibody present in the elutes and in the wash was estimated using the dose response curve, the volumes of the fractions and the dilution with which they were tested. The results of this analysis were that the combined eluates contained between 10 to 40 times more antibody than the combined wash fractions. The estimation for the total percentage of antibody captured from the positive sample used was between 14 – 30% dependent upon exactly which column and sLPS response was used to generate the estimate.

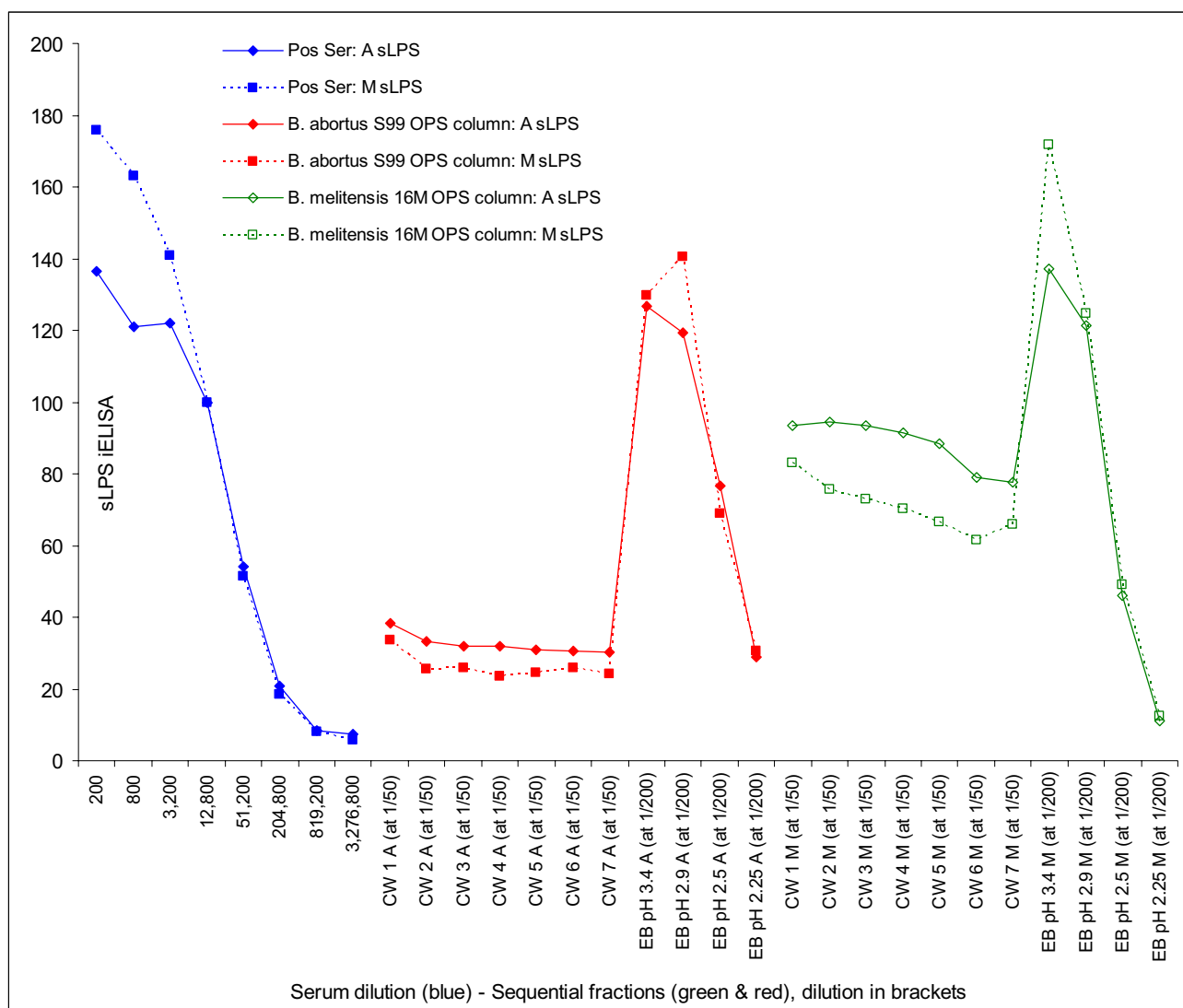


Figure 5.4. sLPS iELISA results for fractions washed and eluted from *Brucella* OPS CarboLink™ columns

The data in figure 5.4 shows data from a positive serum sample from a *B. abortus* infected cow that has been applied to affinity chromatography using OPS. The graph shows the sLPS iELISA results for fractions from this sample washed and eluted from *Brucella* OPS CarboLink™ columns where the results on the y-axis are expressed as a percentage of the OD of the source serum at a dilution of 1/12,800. The dose-response curves for the source serum (shown in blue) are shown on the left hand side of the graph in order to assist in the interpretation of the data derived from the column fractions. The data from the fractions from the *B. abortus* S99 OPS column are shown in red and those from the *B. melitensis* 16M OPS column are shown in green. The results shown by the solid lines represent data from iELISA using the sLPS from an A dominant strain of *Brucella* (*B. abortus* S99) and the results from the broken lines represent data from an iELISA using the sLPS from an M dominant strain of *Brucella* (*B. melitensis* 16M).

The data from the *B. abortus* S99 OPS column (shown in red) shows a relatively low level of antibody response in the wash and that there was not much difference between the relative reactivity between the A and the M sLPS used in iELISA. In the eluates the level of reactivity against the different sLPS

types is also similar. The data from the *B. melitensis* 16M OPS column (shown in green) shows a higher amount of antibody in the wash compared to the equal fractions from the *B. abortus* S99 OPS columns and there is a more pronounced difference between the response to the sLPS types with the response against the M type being lower. In the eluates from the *B. melitensis* 16M OPS column the level of reactivity against the different sLPS types is again similar although the response to the M type OPS is higher. This may be due to the increased retention and subsequent elution of antibodies with a preference for the M epitope, which is more abundant in the *B. melitensis* 16M OPS column. Although these results demonstrated that some OPS specificity selection may be possible with these columns, the antibodies obtained were not more specific to the M epitope than the BM40 mAb.

5.2.3. Production of affinity selected polyclonal anti-*Brucella* OPS antibody populations

The OPS conjugated columns were also used to try and purify from the population of polyclonal anti-*Brucella* OPS antibodies within the positive sera, a sub-population that were specific to *Brucella* (to the 'C specific' epitope). This would be demonstrated by reduced cross reactivity against the *Y. enterocolitica* O:9 OPS antigen relative to the reaction against the *Brucella* OPS. Some of these antibodies may demonstrate some specificity for the 'C specific' epitope. In the absence of a hypothesis for what this structure actually is, conducting the search for it by using specific antibodies as a probe is a reasonable approach. Of the mAbs already evaluated (see chapter 3) mAb 12G12 is reputed to be specific to this 'C specific' epitope. However, the data obtained from this mAb during this project suggests that it is more likely to be demonstrating some specificity for the M epitope rather than against a 'C specific' epitope. As such this mAb was not used for the affinity selection of oligosaccharides.

Therefore the strategy to obtain antibodies with specificity for the 'C specific' epitope was to combine the use of the *B. abortus* S99 OPS column for antibody selection and the *Y. enterocolitica* O:9 column for antibody de-selection (or negative selection). In theory this could separate the anti-*Brucella* OPS antibodies into two populations, both reactive to the *Brucella* OPS but with variable reactivity to the *Y. enterocolitica* O:9 OPS. The sera could either be purified on the *B. abortus* S99 column first and then applied to the *Y. enterocolitica* O:9 column where they could be separated according to their affinity to this antigen. Or they could be applied to and separated on the *Y. enterocolitica* O:9 OPS column first and then applied to the *B. abortus* S99 OPS column for purification. Both approaches were evaluated. The process of antibody affinity selection used was as

outlined above, with sequential additions of positive sera, 7 wash steps and 6 iterations for each elution buffer used.

5.2.3.1. Positive selection of antibodies using *B. abortus* S99 OPS column followed by negative selection using *Y. enterocolitica* O:9 OPS column

In the first instance the positive serum was purified on the *B. abortus* S99 column first and then applied to the *Y. enterocolitica* O:9 column for de-selection. The data shown in figure 5.5 demonstrates the capability of the OPS affinity selection process to create sub-populations of polyclonal antibodies that have different reaction profiles to the source material. The response of the source material against the *B. abortus* S99, *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS is shown on the left hand side of the graph. A range of dilutions for each of the fractions obtained and the source material was tested by iELISA. The results from each dilution series that gave the results closest to each other for the *B. abortus* S99 OPS are shown. This normalisation of the data makes it easier to see how the response to the *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS antigens varies between fractions.

In the source material, the response to these two antigens was close to equal. After affinity chromatography on the *B. abortus* S99 OPS column (fraction 'EB pH 2.9 A') the relative response to *B. melitensis* 16M OPS increased whilst that to *Y. enterocolitica* O:9 OPS decreased. This fraction was applied to the *Y. enterocolitica* O:9 OPS column and the antibodies released in the flow through and wash showed an even greater preference for the *B. melitensis* 16M OPS compared to the *Y. enterocolitica* O:9. This would be as expected as antibodies with stronger preference for *Y. enterocolitica* O:9 would remain attached to the column. This was shown to the case in the eluates from the column. When the buffer used was at pH 4.0 the eluted antibody population had antigen preference similar to that of the material added. Decreasing the pH to 3.4 and then 2.9 to elute antibodies with successively higher binding avidity resulted in the elution of antibodies similar in preference to the source material and with pH 2.9, antibodies with preference for *Y. enterocolitica* O:9 OPS over *B. melitensis* 16M OPS.

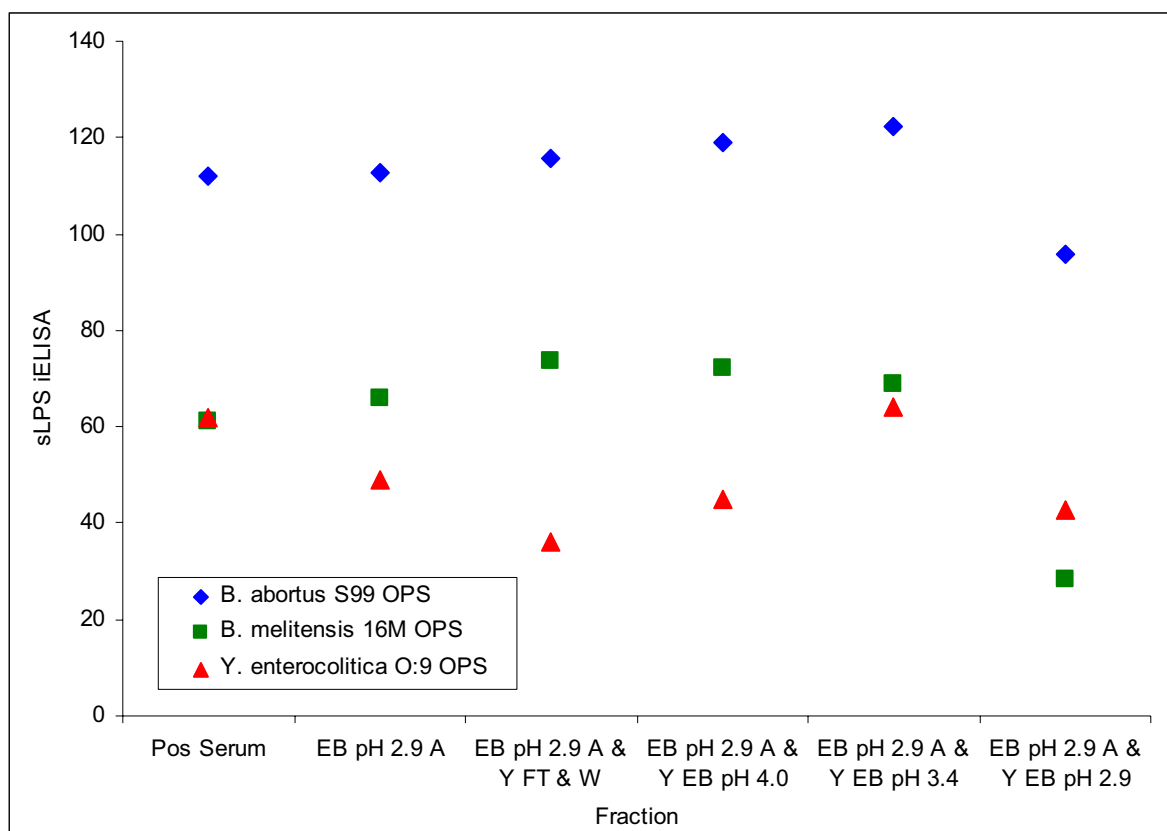


Figure 5.5. OPS iELISA results for antibody fractions obtained from *B. abortus* S99 and *Y. enterocolitica* O:9 OPS conjugated CarboLink™ columns

Figure 5.5 shows data from a positive serum sample from a *B. abortus* infected cow that has been subjected to affinity chromatography using OPS. The graph shows the iELISA results from fractions of the sample that have been eluted from the OPS conjugated CarboLink™ columns. The results from OPS iELISA with *B. abortus* S99 (blue diamonds), *B. melitensis* 16M (green squares) and *Y. enterocolitica* O:9 (red triangles) are shown with the data normalised (approximately) for a similar titre against the *B. abortus* S99 OPS antigen. The different fractions tested are: 'Pos Serum' (the original source positive serum), 'EB pH 2.9 A' (fraction eluted from the *B. abortus* S99 OPS column with pH 2.9 elution buffer), 'EB pH 2.9 A & Y FT & W' (fraction eluted from the *B. abortus* S99 OPS column with pH 2.9 elution buffer that has then been applied to a *Y. enterocolitica* O:9 OPS column and come through in the flow through and wash from this second column), 'EB pH 2.9 A & Y EB pH 4.0' (fraction eluted from the *B. abortus* S99 OPS column with pH 2.9 elution buffer that has then been applied to a *Y. enterocolitica* O:9 OPS column and retained until elution with pH 4.0 elution buffer), 'EB pH 2.9 A & Y EB pH 3.4' and 'EB pH 2.9 A & Y EB pH 2.9' as before but with reduced pH.

The results shown in figure 5.5 can, in the main, be readily rationalised by the current understanding of the relationship between antibody specificity and the OPS antigens. The results most difficult to interpret are from the first affinity selection. It is not clear why selection with a *B. abortus* OPS antigen that possesses a similar A type OPS to that produced by the infective *Brucella* strain that gave rise to the positive serum should result in an antibody binding profile different from the source material. The process will have lead to the enrichment of higher avidity antibodies, both through the process of

competition for binding within the column and through the washing and elution steps. It is possible that antibodies towards the M epitope have, in general, a higher avidity than many of those against the A and C/Y epitopes. The antibodies from this fraction that elute in the flow through and wash when added to the *Y. enterocolitica* O:9 column should be those with comparatively lower preference for this antigen and are de-selected. The ELISA results show exactly that as the difference between the response to the *B. melitensis* 16M OPS and the *Y. enterocolitica* O:9 OPS widened. The antibody response from the samples eluting from this column showed this difference narrowing and finally reversing once the antibodies with higher avidity, eluted with pH 2.9, were removed. This is in keeping with the concept that only the *Y. enterocolitica* O:9 specific antibodies, reactive to the A and C/Y epitopes, but not M were being retained. The reduction of the response of the antibodies in this final fraction against the *Y. enterocolitica* O:9 OPS relative to their response against the *B. abortus* S99 OPS is harder to rationalise.

Owing to the nature of the overlapping epitopes on the OPS of *Brucella*, it is not possible to directly select for antibodies to specific epitopes. Some element of de-selection must be applied. In the example described above, the *Y. enterocolitica* O:9 OPS conjugated column was used to de-select *Brucella* antibodies with specificity to epitopes unique to *Brucella* such as the M epitope and the 'C specific' epitope. This de-selection appears to have worked to a point although residual *Y. enterocolitica* O:9 OPS reactivity is observed in the purified fraction from the *B. abortus* S99 OPS column that was added to but not bound to the *Y. enterocolitica* O:9 OPS column. Should most of the de-selected antibodies have been reactive against the 'C specific' epitope the titre against both *Brucella* antigens would be expected to increase equally. This does not appear to be the case. The dose-response curves (not shown) for dilutions of the source sample against the different OPS antigens shows that at the level of antibody reaction shown in figure 5.5 the dose-response curves for the different antigens are linear and parallel. Therefore differences on the y-axis in figure 5.5 do relate to a linear differences in antibody content. These differences do point more towards the enrichment of antibodies reactive to the M rather than the 'C specific' epitope.

The addition of too many anti-*Y. enterocolitica* O:9 OPS antibodies to the *Y. enterocolitica* O:9 OPS column may have resulted in overloading with the result that some of these antibodies would be outcompeted and not be specifically retained. Consequentially the first de-selected fraction may not be optimal. To test for this the fraction originally eluted from the *B. abortus* S99 OPS column and then released from the *Y. enterocolitica* O:9 OPS column in the flow through and wash was subjected to an additional round of de-selection through the *Y. enterocolitica* O:9 OPS column. The collection and

evaluation of the (now twice) non-retained fraction did not show a decrease in the level of cross reaction to the *Y. enterocolitica* O:9 OPS by iELISA compared to the antibodies in the fraction that was initially added. This demonstrated that, either the antibodies that are present in this fraction have some dual cross reactivity and are not sufficiently stringent, or the de-selection techniques were not sufficiently sophisticated and resolved so as to tease out the more subtle differences in antibody binding. Selection using a continuum of elution parameters, as might be possible using an FPLC or HPLC system, may have improved the resolution between the antibody populations however a suitable matrix to use that would enable OPS conjugation was difficult to find.

5.2.3.2. Negative selection of antibodies using *Y. enterocolitica* O:9 OPS column followed by positive selection of using *B. abortus* S99 OPS column

When the serum is applied to the *B. abortus* S99 column first, all the anti-OPS antibodies – whatever their specificity or preference should in theory be able to bind to the conjugated OPS and will be competing against each other during the serum incubation phase. Antibodies that are lost in the flow through or during washing may therefore represent those from all sub-populations of OPS specificity. Conversely, applying the serum to the *Y. enterocolitica* O:9 column first would result in a process where antibodies with greater specificity for *Brucella* may be at a competitive disadvantage compared to those with an equal preference for binding to *Y. enterocolitica* O:9 OPS. It was therefore considered that the addition of sample to the *Y. enterocolitica* O:9 OPS column first may be a more efficient method for scaling up production of purified polyclonal anti-*Brucella* OPS antibodies for subsequent affinity chromatography for the homo-D-Rha4NFo-oligosaccharides. All the anti-*Brucella* OPS antibodies added to the *Y. enterocolitica* O:9 column could be used. The retained fraction, potentially enriched for antibodies that cross react with *Brucella* and *Y. enterocolitica* O:9 OPS, would be purified and the un-retained, de-selected fraction, could be subsequently purified with a *B. abortus* S99 OPS column.

When this approach was taken (using methods as described above) it was found that the addition of the positive sample to the *Y. enterocolitica* O:9 column resulted in the considerable release of anti-*Brucella* OPS antibodies in the flow through and wash. The addition of elution buffer released considerable quantities of retained anti-*Y. enterocolitica* O:9 antibodies. In this case a single elution buffer was used, 0.1 M Glycine pH 2.5. The addition of the serum flow through and wash to the *Brucella* S99 OPS column allowed for the purification of the anti-*Brucella* OPS antibodies via their

subsequent elution using 0.1 M Glycine pH 4.0 and 2.7. The pH 4.0 fraction provided a good yield of antibodies and these were slightly more selective for *Brucella* OPS than those eluted at pH 2.7.

The fractions derived from the process described were examined by iELISA using *B. abortus* S99 (blue lines), *B. melitensis* 16M (green lines) and *Y. enterocolitica* O:9 (red lines) sLPS antigens, the results of which are shown in figure 5.6. This graph shows the dose response curve for the source serum on the left. The antibodies that were retained by the *Y. enterocolitica* O:9 OPS column and released only after addition of elution buffer ('Y pH 2.5' in figure 5.6) had a comparatively, although only marginally, increased relative reactivity to the *Y. enterocolitica* O:9 sLPS antigen. The antibody population that was unbound by this column but captured by the *B. abortus* S99 OPS column ('Y (SFT+W) x A' in figure 5.6) showed a considerable loss of reactivity to the *Y. enterocolitica* O:9 sLPS antigen.

A range of dilutions for the fractions and source material were tested by iELISA and the results from the dilutions that provided the most informative data are shown. The final data points, on the right hand side of the graph, show selected results from the two analysed fractions, as well as from the source serum. These have been chosen as they have similar results for *B. abortus* S99 OPS iELISA (solid blue diamonds) and this makes it easier to compare the relative responses from the *B. melitensis* OPS iELISA (solid green squares) and the *Y. enterocolitica* O:9 OPS iELISA (solid red triangles). This simple summary shows how the OPS affinity selection process has led to the production of two different and separated antibody populations, one with relatively higher cross reactivity for the *Y. enterocolitica* O:9 OPS antigen and one with relatively reduced cross reactivity for this antigen. This difference in *Y. enterocolitica* O:9 OPS reactivity, for the same given response against the *B. abortus* S99 OPS was approximately fourfold.

In this instance, the reduction in *Y. enterocolitica* O:9 reactivity in the fraction de-selected by the *Y. enterocolitica* O:9 OPS column and purified on the *B. abortus* S99 OPS column was accompanied by a reduction in the reactivity against the *B. melitensis* 16M OPS. It was therefore considered unlikely that the M epitopes were playing a major role in this set of antibody responses. This opens the possibility that these observations were due to the role of the, as yet undefined, 'C specific' epitope. The existence of a residual anti-*Y. enterocolitica* O:9 OPS response from the *Y. enterocolitica* O:9 OPS de-selected antibody population may be due to the different binding conditions within the column and on the iELISA plate. Or the residual activity may be due to the weaker avidity antibodies being outcompeted within the column but able to bind under less selection pressure in iELISA.

However, as before, passing these antibodies back through the *Y. enterocolitica* O:9 OPS column, where they would be under competitive selection pressure, did not change the binding profiles of the antibodies recovered from the flow through and the wash (data not shown). The residual binding may be weak cross reaction to the *Y. enterocolitica* O:9 OPS antigen from antibodies that have greater preference for the *Brucella* OPS antigen.

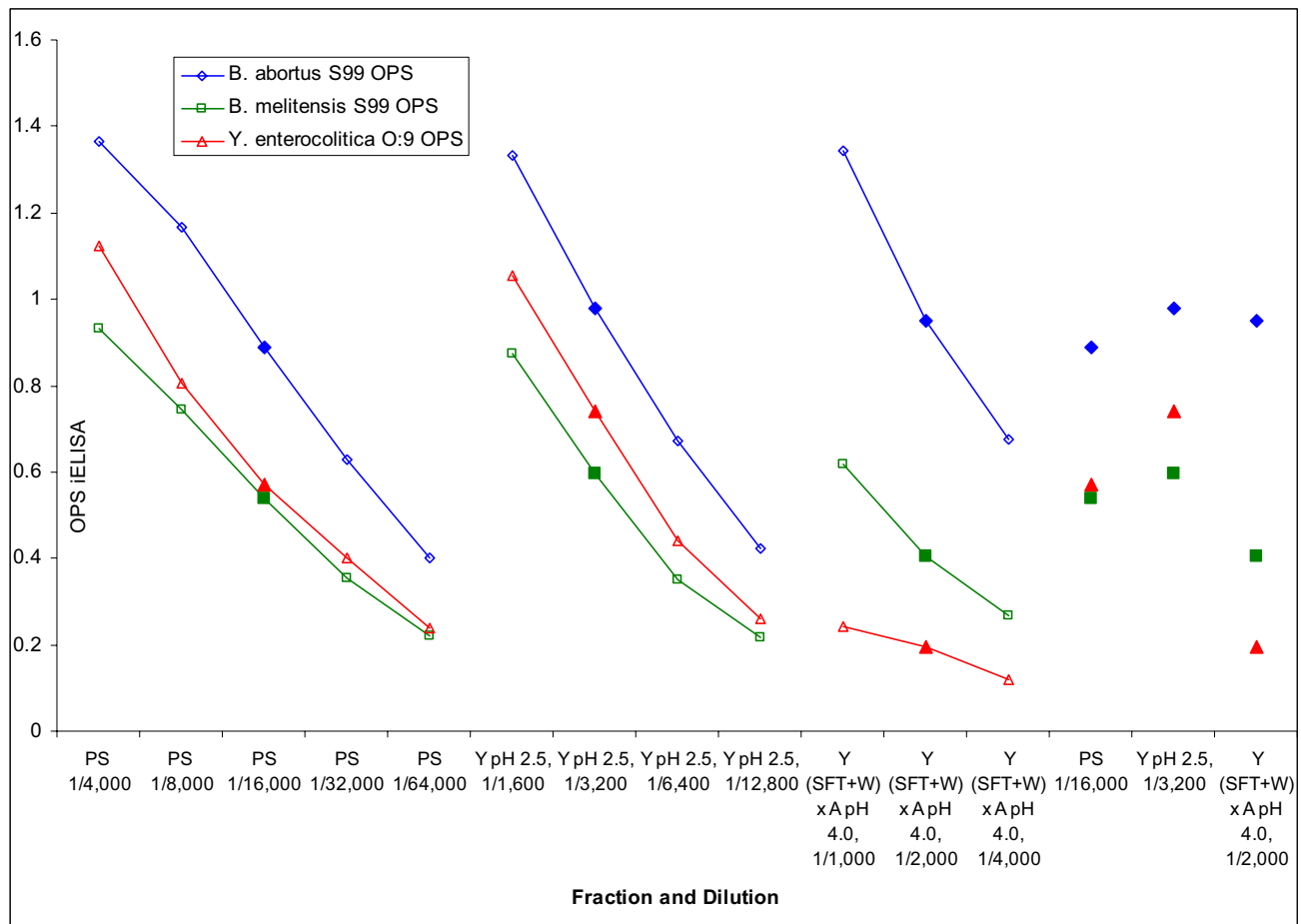


Figure 5.6. OPS iELISA results for antibody fractions obtained *Y. enterocolitica* O:9 and *B. abortus* S99 OPS conjugated CarboLink™ columns

Figure 5.6 shows data from a positive serum sample from a *B. abortus* infected cow that has been subjected to affinity chromatography using OPS. The graph shows the iELISA results from fractions of the sample that have been eluted from the OPS conjugated CarboLink™ columns. The results from OPS iELISA with *B. abortus* S99 (blue diamonds), *B. melitensis* 16M (green squares) and *Y. enterocolitica* O:9 (red triangles) are shown. The different fractions tested are: 'PS' (the original source positive serum), 'Y pH 2.5' (fraction eluted from the *Y. enterocolitica* O:9 OPS column with pH 2.5 elution buffer), 'Y (SFT+W) x A pH 4.0' (fraction deselected from the *Y. enterocolitica* O:9 OPS column and collected in the serum flow through and wash that has then been applied to and retained upon until elution with pH 4.0 elution buffer on a *B. abortus* S99 OPS column). The results with the solid data points have been selected for comparison (far right) as they are normalised (approximately) for a similar titre against the *B. abortus* S99 OPS antigen.

5.2.4. Evaluation of purified anti-*Brucella* OPS antibodies by rLPS iELISA

The specificity of the antibodies within these two separated populations to the OPS rather than any elements of the *Brucella* rLPS, such as the lipid A or core sugars, was demonstrated by iELISA with rLPS extracted from *B. abortus* RB51. The results, shown in figure 5.7, show that all three samples, the source positive serum sample, the *Y. enterocolitica* O:9 OPS column selected antibodies and the *Y. enterocolitica* O:9 OPS column de-selected then *B. abortus* S99 OPS column selected antibodies react vigorously to *B. abortus* S99 sLPS antigen. However, only the source serum sample shows any reaction to the rLPS sample. The affinity selected antibodies show no response at all to this antigen. These results demonstrate the specificity of the purified antibodies to the OPS.

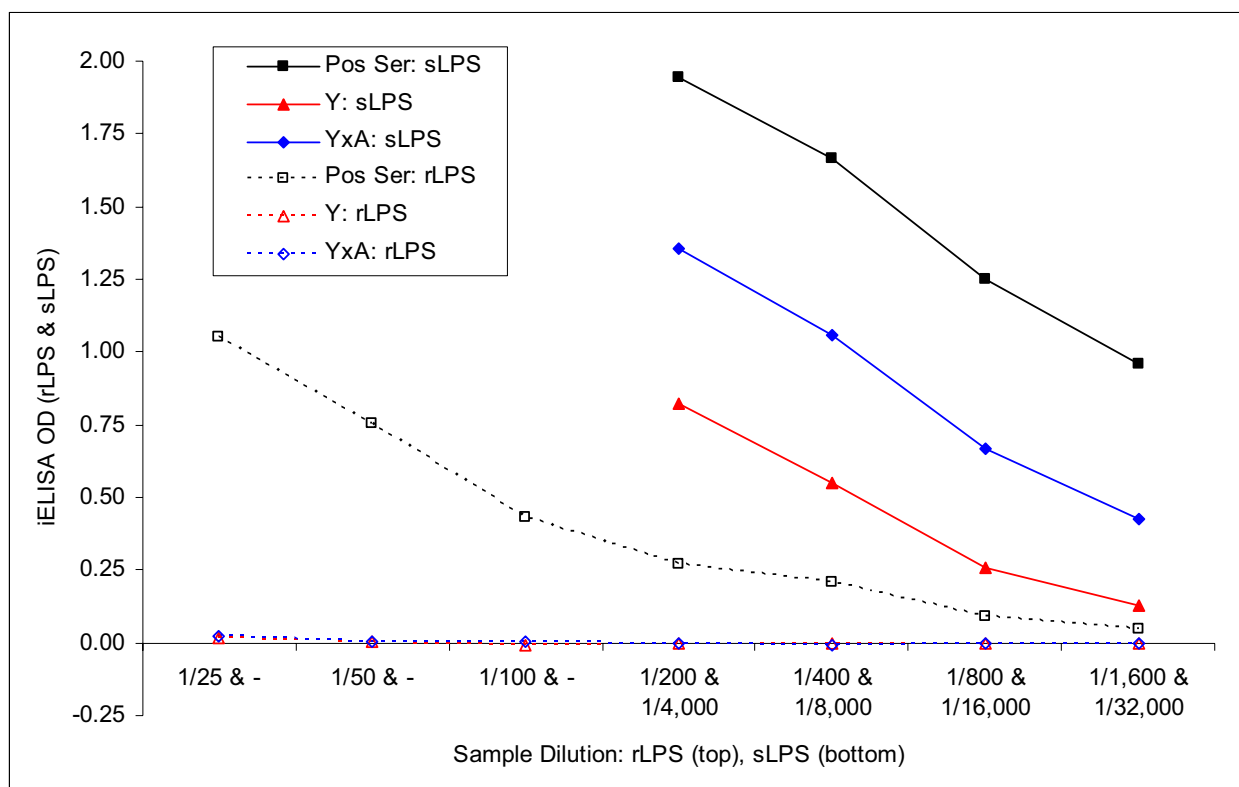


Figure 5.7. rLPS and sLPS iELISA on source positive serum and OPS affinity purified fractions

The graph above shows the results for the rLPS (broken lines) and sLPS (solid lines) iELISA on the source positive serum sample (black lines), the *Y. enterocolitica* O:9 OPS column affinity selected antibodies ('Y', red lines) and the *Y. enterocolitica* O:9 OPS column de-selected then *B. abortus* S99 OPS affinity selected antibodies ('YxA', blue lines). The sample dilutions are shown on the x-axis with the dilution for the rLPS iELISA given first and the dilution for the sLPS iELISA given second.

5.3. Affinity chromatography of *B. abortus* S99 homo-D-Rha4Nfo-oligosaccharides

On the basis of the results described above and the conclusions drawn from them these two purified polyclonal anti-*Brucella* OPS antibody populations (the *Y. enterocolitica* O:9 OPS column selected antibodies ['Y'] and the *Y. enterocolitica* O:9 OPS column de-selected then *B. abortus* S99 OPS column selected antibodies ['YxA']), plus the BM40 mAb, were applied to the affinity selection of the homo-D-Rha4Nfo-oligosaccharides. A sufficient supply of the polyclonal antibodies was obtained through several re-iterations of the production process described above and a total of 2 mls of the positive serum was used. The amount of antibodies obtained was quantified by Bradford (Coomassie) protein assay.

5.3.1. Conjugation of anti-*Brucella* OPS antibodies to AminoLink® Columns

5.3.2. Evaluation of protein loss from AminoLink® Columns subsequent to antibody conjugation

The three antibody sets were coupled to AminoLink® Columns (Pierce) via the binding of primary amines on the antibodies to aldehyde groups on the surface of the agarose beads within the column. To evaluate the success of this conjugation process, the samples and all eluted fractions were evaluated by Bradford (Coomassie) protein assay. A total of 1000 µg of BM40 mab, 525 µg of *Y. enterocolitica* O:9 OPS affinity purified polyclonal antibodies ('Y') and 750 µg of *Y. enterocolitica* O:9 OPS deselected and *B. abortus* S99 OPS affinity purified polyclonal antibodies ('YxA') were added to individual AminoLink® columns. There was no detectable protein in any of the flow through from the columns during their preparation (as described above). This is perhaps not surprising as the capacity of the columns is described to be 1 – 20 mg of protein.

5.3.3. Development of ESI-MS compatible methods for antibody selection of oligosaccharides

The potential for insufficient antigen binding capacity for the anti-*Brucella* OPS antibody conjugated columns was exceedingly small given the small quantities of the oligosaccharide analytes to be selected. In fact the need to maximise the chances of detection of such small quantities of analyte presented more of a concern within the context of this project. To increase the concentration of any eluted analyte the volume of the eluted sample could be reduced. Given the relatively small mass of

the oligosaccharides, centrifugal filtration was not an option, although freeze drying was. The consequent reduction in volume not only increases the concentration of any analyte but also of any other material in solution, for example the PBS, or tris that had previously been used for wash, and neutralisation.

The presence of such salts, especially after concentration, would have a detrimental effect on the ESI-MS equipment required for the analysis of the fractions. Brief experimental evaluation using ELISA confirmed that antibody-antigen interactions did not occur effectively without physiological buffers (data not shown), although the concentration of PBS could be reduced fourfold without significant impediment to the antibody-antigen interaction. PBS was therefore used at this reduced level for the incubation of oligosaccharides within the antibody conjugated columns. The use of 0.1 M glycine for elution would, after freeze drying and reconstitution, lead to a concentration of 5 M glycine in the final sample to be delivered to the ESI-MS. This was also an undesirable situation as the presence of such a highly polar molecule at such high concentrations could have a considerable impact on the separation and ionisation of the oligosaccharides. On a positive, more advantageous note, the reduced need to neutralise the eluates, as the oligosaccharides are not held together by ionic bonds and will not be reused, eliminated the need for such buffers.

To find a means to wash and elute the columns without obtaining PBS, glycine or tris in the analytical sample the use of dilute HCl only was investigated. This was a key partner in the glycine-HCl elution buffers described above and can be eliminated by freeze drying - as described in chapter 4. Initial experimentation using the *B. abortus* S99 OPS conjugated column described above with added positive serum demonstrated that, after washing with water, the addition of 0.0125 M HCl stripped most of the antibodies bound to this column (data not shown). Antibodies that were still bound were removed by addition of 0.025 M HCl. The antibody content was effectively measured by iELISA (after buffer exchange into PBS) indicating that these concentrations of HCl are relatively mild.

A test AminoLink[®] column was prepared with 1 mg of BM40 mAb and 20 µg of *B. melitensis* 16M OPS was added in PBS. After incubation, the column was washed 4 times each with 2 mls of PBS and then washed a further 4 times each with 2 mls of H₂O. This was followed by 4 elutions with 2 mls 0.0125 M HCl and finally 4 elutions with 0.025 M HCl. The water and elution fractions were freeze dried and reconstituted with 0.5 mls of H₂O. These fractions, plus the PBS washes, were evaluated by sLPS cELISA with BM40-HRP and the results are shown (in green), in chronological sequence, in the graph in figure 5.8. The blue curve in figure 5.8 is the dose response curve for a known

concentration of antigen *B. melitensis* 16M OPS antigen. Comparison of the results from the fractions to this dose response curve allows an estimate of the amount of antigen present in the sample to be made.

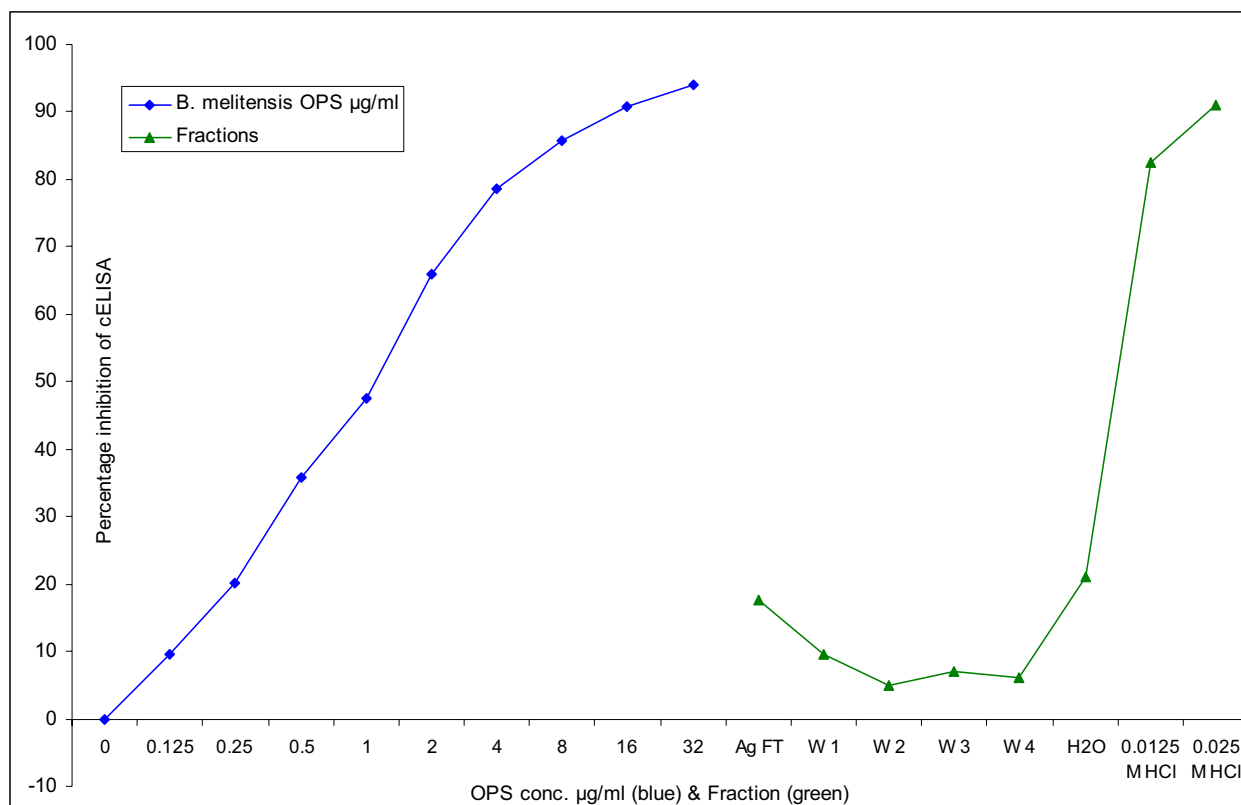


Figure 5.8. Results of antibody (BM40) affinity chromatography of *B. melitensis* 16M OPS

The graph in figure 5.8 shows the cELISA results from fractions derived from the addition of 20 µg of *B. melitensis* OPS to a BM40 mAb conjugated affinity chromatography column. The blue curve on the right is a dose response curve showing the cELISA results (y-axis) for specified concentrations of OPS (x-axis). The green curve shows the cELISA results (in chronological sequence) for flow through ('Ag FT'), for four washes with PBS ('W 1' – 'W 2'), four washes (combined) with H₂O ('H2O'), elution with 0.0125 M HCl ('0.0125 M HCl') and elution with 0.025 M HCl ('0.025 M HCl').

The data in figure 5.8 shows the elution of antigen in the 0.0125 M and 0.025 M HCl fractions. The approximate concentration of *B. melitensis* 16M OPS antigen in the 0.0125 M HCl fraction is between 4 – 8 µg/ml. The percentage inhibition value for the 0.025 M HCl fraction is almost identical to the result for the 16 µg/ml value for the dose response curve. There was an insignificant quantity of antigen present in any of the flow through or wash fractions. Each elution fraction was a 0.5 ml volume (after reconstitution) and therefore a reasonable estimate of total antigen eluted from the column would be in the range of 20 – 24 µg. Only 20 µg of antigen was added to the column, therefore it is fair to conclude (within the scope of the accuracy limitations of

the method) that most of the antigen has been recovered from the column. Further, this antigen remains sufficiently intact structurally to bind as efficiently to the BM40 mAb as before processing.

To be certain that any residual HCl that may have been present in the freeze dried and reconstituted fraction was not adversely effecting the immunoassay, 0.0125 M HCl was added to the assay as a 'sample' and this did not inhibit the antigen-antibody interaction and the addition of pure water as the 'sample' also had no adverse affect (data not shown).

It is quite probable that the antibody conjugate column could have captured and eluted a significantly larger quantity of OPS antigen than that tested in this experiment. However, the analytes to be evaluated are not in abundance. The quantity of D-RhaN4NFO homo-oligosaccharides derived from the *B. abortus* S99 OPS antigen was estimated to be between 50 – 100 µg in total, based upon comparison of ESI-QqToF peak intensity data relative to a chitotriose standard. Therefore the small quantity of antigen used to test the elution conditions was appropriate to the situation. The data gathered demonstrates that: the selected antibody populations have covalently bound to the AminoLink® columns, OPS antigen may be added to and bind to columns in PBS, the PBS may be washed from the columns with H₂O whilst retaining the antigen which may then be eluted from these columns using low concentrations of HCl and that the HCl preparations may be freeze dried and reconstituted to allow for the concentration of eluted analytes.

5.3.4. Antibody affinity chromatography of *B. abortus* S99 derived homo-D-Rha4NFO-oligosaccharides

The quantity of homo-D-Rha4NFO-oligosaccharides derived from *B. abortus* S99 OPS was divided equally for the addition to each of the three antibody conjugated AminoLink® columns prepared: BM40, *Y. enterocolitica* O:9 OPS affinity purified polyclonal antibodies ('Y') and *Y. enterocolitica* O:9 OPS deselected and *B. abortus* S99 affinity purified polyclonal antibodies ('YxA'). After incubation and washing (first with five 2 ml washes with PBS and then with five 2 ml washes with H₂O), the antigen was eluted first with five times 2 mls of 0.00625 M HCl, five times 2 mls of 0.0125 M HCl and then five times 2 mls of 0.025 M HCl. Each 10 ml HCl fraction was freeze dried and reconstituted in 200 µl of H₂O resulting in a 50 times concentration. This was also done for the water wash fractions.

5.4. GC column ESI-MS analysis of antibody affinity selected homo-D-Rha4NFor-oligosaccharides

The reconstituted HCl and water fractions from each column were analysed by GC column-ESI-QqQ. The total ion count of the oligosaccharides evaluated by GC column-ESI-QqQ prior to antibody affinity selection is shown in figure 5.9 (also shown in figure 4.45 in chapter 4). There were few oligosaccharides within the water wash compared to the quantities in the subsequent HCl fractions. The total ion counts for the 0.00625 M HCl fraction, which contained most of the oligosaccharides, are shown for the 'Y', 'YxA' and BM40 conjugated columns in figures 5.10, 5.11 and 5.12 respectively. Comparison of these profiles with the profile prior to affinity separation (figure 5.9) shows that the 'Y' and 'YxA' columns captured all of the oligosaccharides from the tetrasaccharide upwards (as for the pre-column analysis, peaks eluting after 12 mins should be ignored). However the profile for the elution from the BM40 column was very different with only one peak visible (elution time 8:50 mins) that was clearly attributable to an oligosaccharide (the peak 10:50 is part of the series of peaks of non-OPS origin that extends beyond 18:00 mins elution).

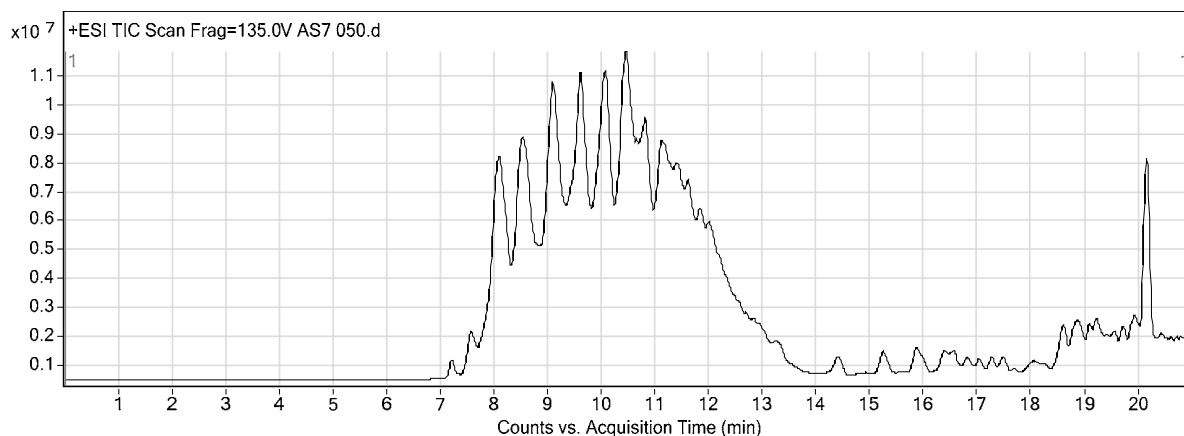


Figure 5.9. Total ion count GC column chromatogram (QqQ) for *B. abortus* S99 oligosaccharides
Total ion count chromatogram for GC column separated *B. abortus* S99 homo-D-Rha4NFor-oligosaccharides purified by exclusion chromatography (also shown in figure 4.45 in chapter 4). The regular repeating peaks from 7 to 11 minutes represent 2 to 10 mer oligosaccharides.

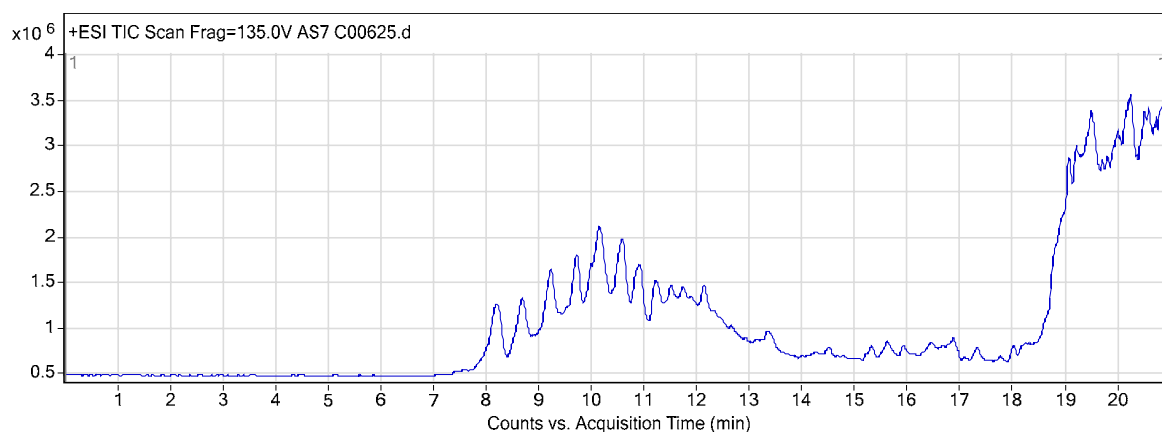


Figure 5.10. Total ion count GC column chromatogram (QqQ) for *B. abortus* S99 oligosaccharides affinity selected with polyclonal antibodies 'Y'

Total ion count chromatogram for GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides that have been eluted from a column bound with *Y. enterocolitica* O:9 OPS affinity purified polyclonal antibodies ('Y'). The regular repeating peaks from 8 to 11 minutes represent 4 to 10 mer oligosaccharides. The peaks between 12 and 19 minutes are not due to structures related to the OPS antigen.

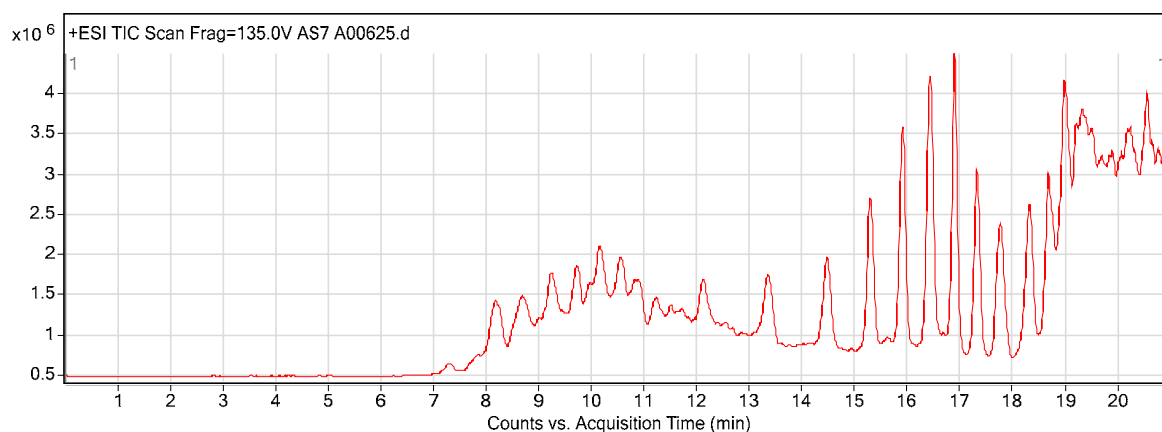


Figure 5.11. Total ion count GC column chromatogram (QqQ) for *B. abortus* S99 oligosaccharides affinity selected with polyclonal antibodies 'YxA'

Total ion count chromatogram for GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides that have been eluted from a column bound with *Y. enterocolitica* O:9 OPS deselected and *B. abortus* S99 affinity purified polyclonal antibodies ('YxA'). The regular repeating peaks from 7 to 11 minutes represent 2 to 10 mer oligosaccharides. The peaks between 12 and 19 minutes are not due to structures related to the OPS antigen.

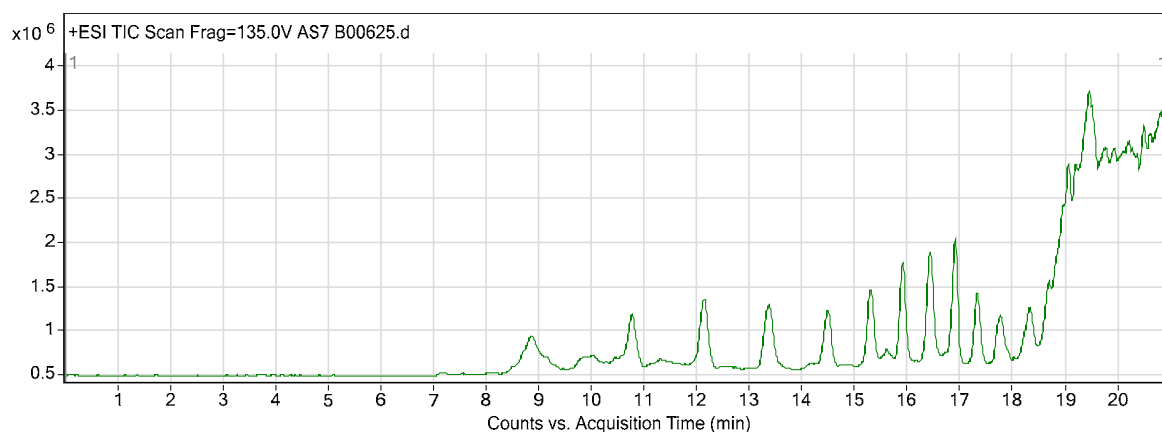


Figure 5.12. Total ion count GC column chromatogram (QqQ) for *B. abortus* S99 oligosaccharides affinity selected with BM40 mAb

Total ion count chromatogram for GC column separated *B. abortus* S99 homo-D-Rha4NFo-oligosaccharides that have been eluted from a column bound with BM40 (anti-M epitope) mAb. The peaks at 8.5 to 10 mins represent oligosaccharides. The peaks between 12 and 19 minutes are not due to structures related to the OPS antigen.

The selected (protonated) tetrasaccharide ion scan chromatograms from the purified OPS oligosaccharide preparations from *B. abortus* S99 subjected to affinity chromatography were compared to the equivalent chromatogram obtained prior to antibody affinity selection as shown in figure 5.13 (also shown in figure 4.48 in chapter 4). The chromatograms for the 'Y' and 'YxA' antibody columns for the 0.00625 M HCl elution fraction are shown in figures 5.14 and 5.15 respectively. These show that the profiles from the two polyclonal antibody columns ('Y' and 'YxA') are essentially identical despite the different ELISA binding profiles of the antibodies (see figure 5.7). The profiles show all of the major tetrasaccharide peaks present in the *B. abortus* S99 oligosaccharide sample prior to addition to the affinity chromatography columns (figure 5.13) albeit that the earliest elution peak has increased in intensity relative to the other peaks visible which may be a reflection of the overall reduction in oligosaccharide concentration.

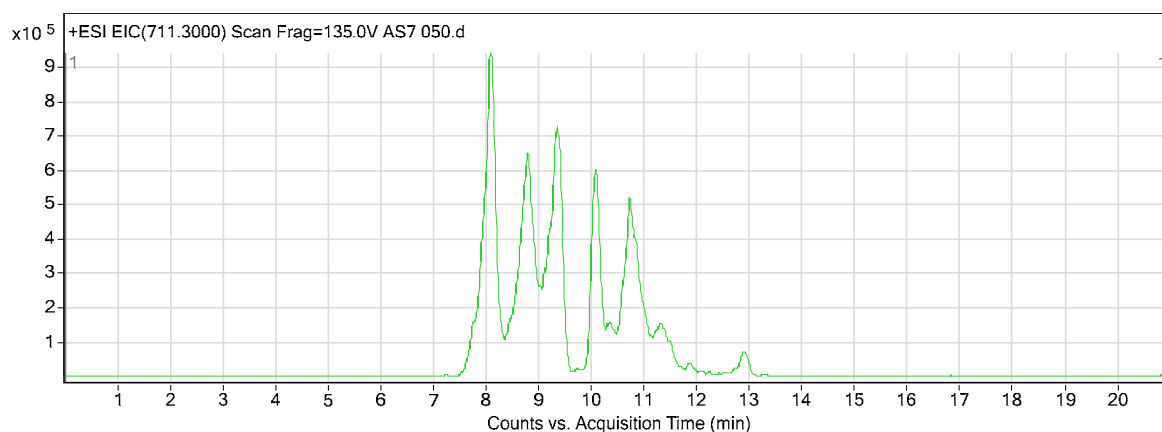


Figure 5.13. Specified ion GC column chromatogram (QqQ) for *B. abortus* S99 tetra-D-Rha4NFO
 Specified ion chromatogram for the protonated tetrasaccharide ($m/z = 711$) on the GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides purified by exclusion chromatography (also shown in figure 4.45 in chapter 4). Five significant peaks are visible at 8:05, 8:50, 9:20, 10:05 and 10:40 mins:secs.

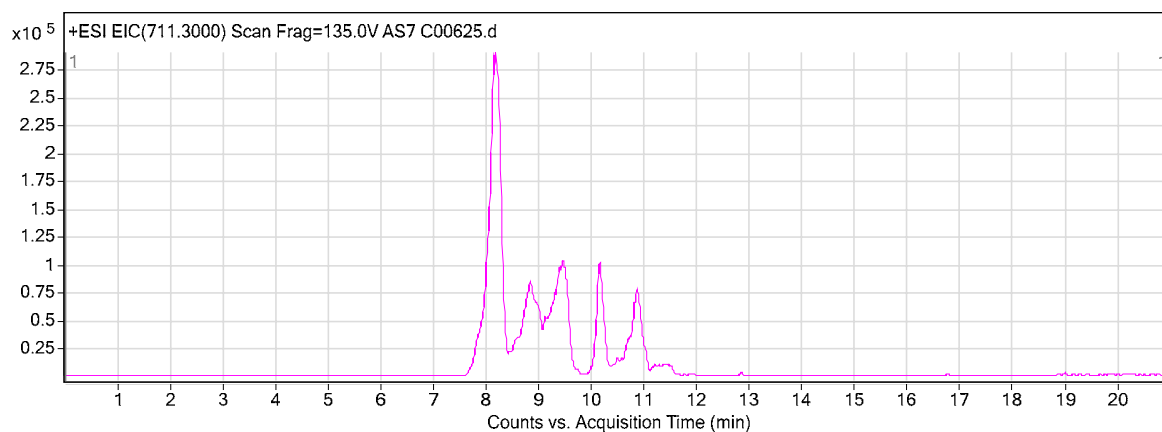


Figure 5.14. Specified ion GC column chromatogram (QqQ) for *B. abortus* S99 tetra-D-Rha4NFO affinity selected with polyclonal antibodies 'Y'
 Specified ion chromatogram for the protonated tetrasaccharide ($m/z = 711$) on the GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides that have been eluted from a column bound with *Y. enterocolitica* O:9 OPS affinity purified polyclonal antibodies ('Y'). Five significant peaks are visible at 8:05, 8:50, 9:20, 10:05 and 10:40 mins:secs.

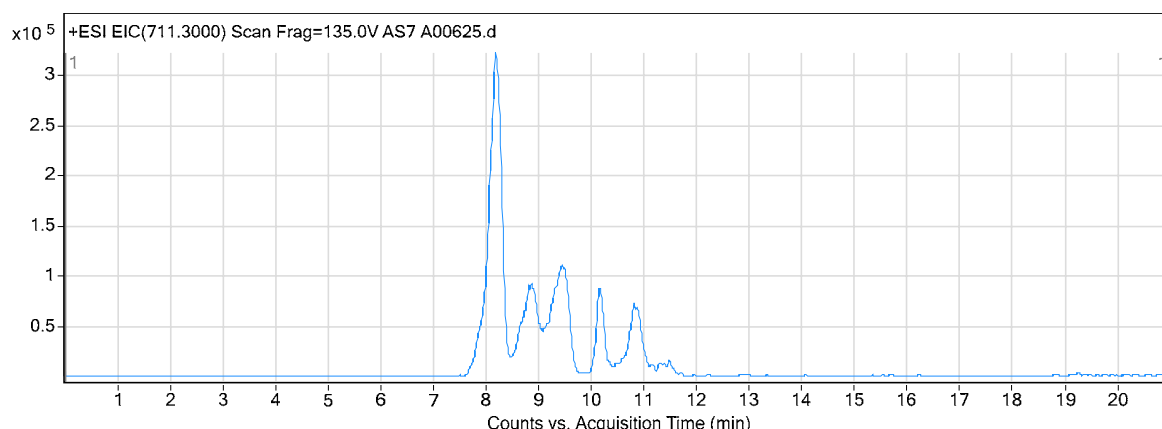


Figure 5.15. Specified ion GC column chromatogram (QqQ) for *B. abortus* S99 tetra-D-Rha4NFO affinity selected with polyclonal antibodies 'YxA'

Specified ion chromatogram for the protonated tetrasaccharide ($m/z = 711$) on the GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides that have been eluted from a column bound with *Y. enterocolitica* O:9 OPS deselected and *B. abortus* S99 affinity purified polyclonal antibodies ('YxA'). Five significant peaks are visible at 8:05, 8:50, 9:20, 10:05 and 10:40 mins:secs.

No peaks or ions unique to *Brucella* were discovered that were able to account for the binding preferences of these polyclonal antibodies. It may be that transition within the columns between binding and elution was insufficiently subtle to detect small differences in antibody avidity or it may be that the $\alpha(1\rightarrow3)$ linkage played no role in this particular antigen mediated affinity separation of polyclonal sera.

The possibility that the differences in relative titre between the two polyclonal antibody populations was due to core sugars was low given that neither population reacted against the rLPS antigen, as shown in figure 5.7. However, some of the difference may be due to the presence of the non-repeating elements of the OPS. These were not contained within the homo-D-Rha4NFO oligosaccharides evaluated by affinity chromatography but would have been present in the OPS conjugated to the affinity chromatography columns and the iELISA plates. Examination of the significance of this non-repeating sequence to antibody binding would be an interesting further line of enquiry. However, one might expect such epitopes to be relatively well sequestered from the host immune system as they lie underneath the repeating elements of the OPS. If antibodies against such epitopes do exist they would probably be of relatively low titre compared to those that react to the repeating D-Rha4NFO element of the OPS, possibly more akin to the titre of the antibodies that react to the core and lipid A. The results from the rLPS iELISA as shown in figure 1.27 in chapter 3 suggests that such antibodies may not be sufficiently abundant within bovine sera to assist in confirmatory diagnosis.

It may also be that fine structural details, as yet unresolved, on the OPS are the key to the differences in the host response to the *Brucella* and *Y. enterocolitica* O:9 OPS antigens. A good example of how small differences can have large implications is provided by the OPS of *Vibrio cholerae* O:1 where a single substitution of an hydroxyl group for a methyl group on the terminal monosaccharide of an otherwise homogeneous polysaccharide accounts for the difference between the Ogawa and Inaba serotype (Ito et al., 1994; Villeneuve et al., 2000). With enough hydrolysed OPS material it may be possible to identify atypical monosaccharides by gas chromatography mass spectrometry (Hitchen et al., 2002).

The selected (protonated) tetrasaccharide ion scan chromatogram from the BM40 mAb bound column did show a very different elution profile (figure 5.16) compared to that of the sample prior to column addition (figure 5.13). In this case the chromatogram showed remarkable similarity to that obtained from the equivalent specified ion chromatogram of the homo-D-Rha4NFO-oligosaccharides derived from *B. melitensis* 16M OPS (shown in figure 5.17 and also figure 4.49 in chapter 4). The major peak in both chromatograms eluted at the same time, 8:50. This is also the peak that is not present in the equivalent specified ion chromatogram of the homo-D-Rha4NFO-oligosaccharides derived from *Y. enterocolitica* O:9 OPS (shown in figure 5.18 and also figure 4.50 in chapter 4). The BM40 mAb has clearly selected a particular tetrasaccharide conformer which is present in the *B. abortus* S99 and *B. melitensis* 16M oligosaccharide preparation but not the *Y. enterocolitica* O:9 preparation. Studies performed earlier (see figure 3.7 in chapter 3) demonstrate how specific this mAb is for oligo- and polysaccharides containing the $\alpha(1\rightarrow3)$ linkage. This is all very strong evidence to show that the particular elution peak at 8:50 is due to a tetrasaccharide containing an $\alpha(1\rightarrow3)$ linkage however it is not possible with this data to state whereabouts specifically in that tetrasaccharide the $\alpha(1\rightarrow3)$ linkage is. The chromatograms in figures 5.14 and 5.15 show that both the polyclonal antibody affinity chromatography columns selected this particular form of the tetrasaccharide as the peak at 8:50 mins:secs is present.

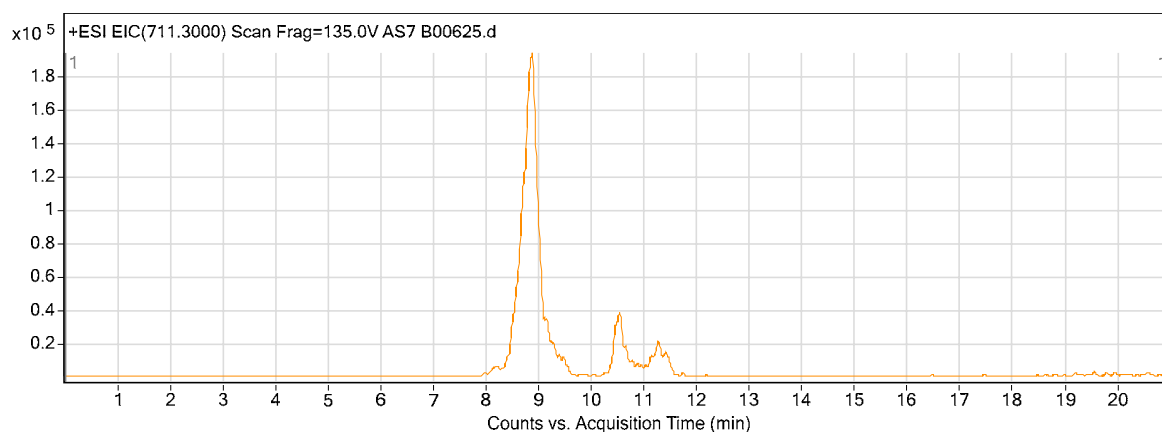


Figure 5.16. Specified ion GC column chromatogram (QqQ) for *B. abortus* S99 tetra-D-Rha4NFO affinity selected with BM40 mAb

Specified ion chromatogram for the protonated tetrasaccharide ($m/z = 711$) on the GC column separated *B. abortus* S99 homo-D-Rha4NFO-oligosaccharides that have been eluted from a column bound with BM40 mAb. Three significant peaks are visible at 8:50, 10:30 and 11:15 mins:secs.

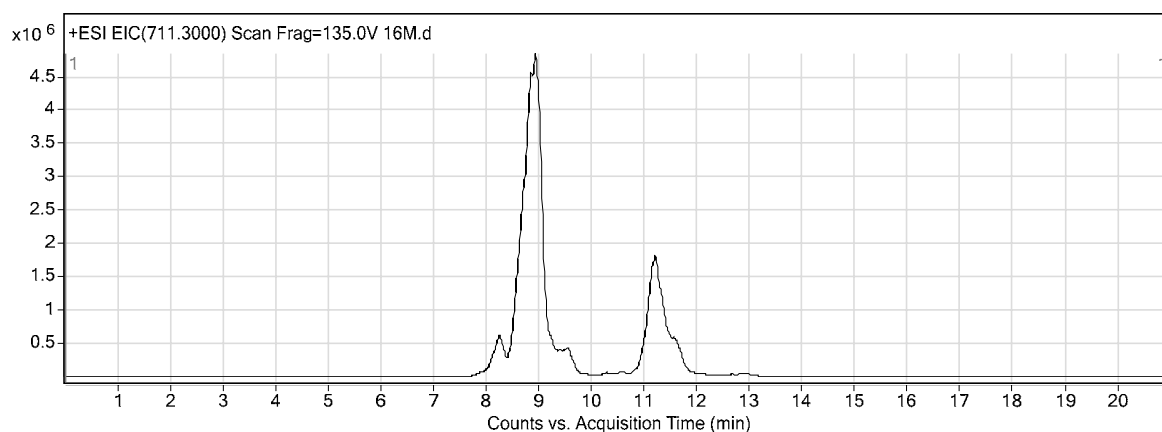


Figure 5.17. Specified ion GC column chromatogram (QqQ) for *B. melitensis* 16M tetra-D-Rha4NFO

Specified ion GC column chromatogram for the protonated tetrasaccharide ($m/z = 711$) from the *B. melitensis* 16M purified homo-D-Rha4NFO-oligosaccharides. Three significant peaks are visible at 8:05, 8:50, and 11:10 mins:secs. Spectrum also shown in figure 4.49 in chapter 4.

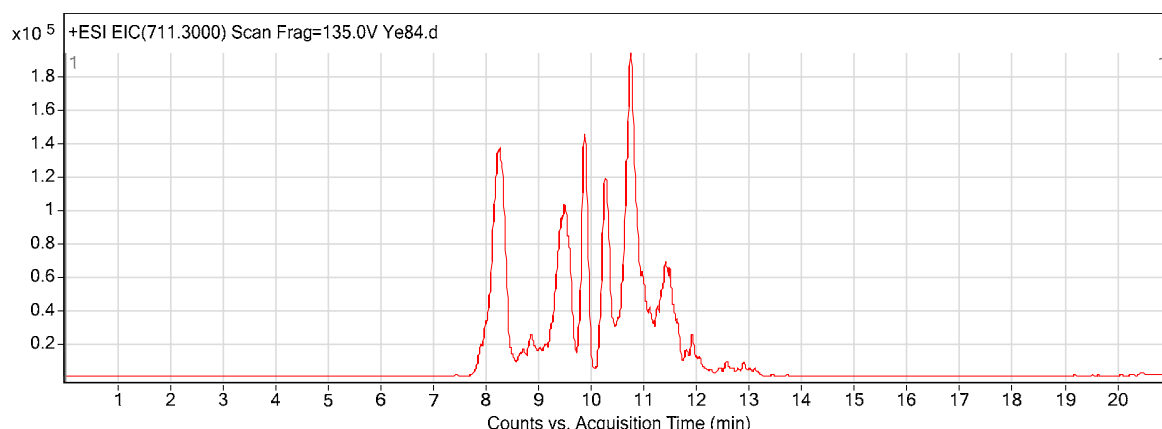


Figure 5.18. Specified ion GC column chromatogram (QqQ) for *Y. enterocolitica* O:9 tetra-D-Rha4NFO

Specified ion GC column chromatogram for the protonated tetrasaccharide ($m/z = 711$) from the *Y. enterocolitica* O:9 purified homo-D-Rha4NFO-oligosaccharides. Six significant peaks are visible at 8:05, 9:20, 9:55, 10:10 and 10:40, and 11:30 mins:secs. Spectrum also shown in figure 4.50 in chapter 4.

5.5. ESI-MS/MS of protonated D-Rha4NFO ion within affinity selected fractions

Collision Induced Dissociation (CID) fragmentation analysis (MS/MS) of the protonated tetrasaccharide ion using the ESI-QqQ was performed to confirm the structure. The spectrum is shown in figure 5.19. Figure 5.20 shows a schematic representation of the homo-D-Rha4NFO-tetrasaccharide with probable fragmentations that would occur during MS/MS (indicated by broken lines). The reducing end of the tetrasaccharide is towards the right, oxygens within the glycosidic bond and the pyranose ring are represented by circles and the positions of carbons 1 and 6 are shown as is the position of the N-formyl group attached to carbon 4. The schematic shows a tetrasaccharide with a central 1→3 flanked by two 1→2 links although the precise location of this linkage is unknown. The position of the 1→3 linkage within the tetrasaccharide does not affect the m/z value of ions produced as a consequence of fragmentation of the glycosidic linkage. The figure has been represented according to the scheme of Domon and Costello (Domon and Costello, 1988) with the m/z values for the predicted ions (positive) shown at the ends of the arrows. These arrows indicate which of two fragments produced corresponds to the m/z value shown.

The MS/MS spectrum of the tetrasaccharide (figure 5.19) shows the parent (protonated) ion ($m/z = 710.8$). It also shows ions derived from the tetrasaccharide C_3 and Y_3 fragments ($m/z = 537.7$), ions derived from the B_3 and Z_3 fragments ($m/z = 519.7$), ions derived from the disaccharide C_2

and Y_2 fragments ($m/z = 364.6$), ions related to the B_2 and Z_2 disaccharide fragments ($m/z = 346.6$) and ions related to the C_1 , Y_1 ($m/z = 190$), B_1 and Z_1 monosaccharide fragments ($m/z = 174$).

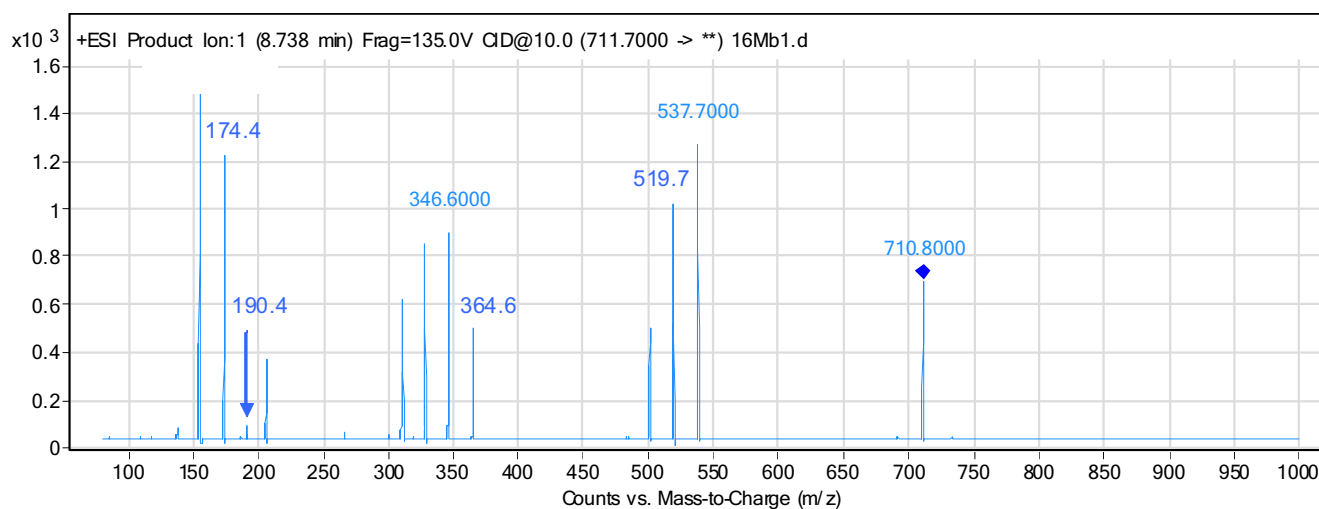


Figure 5.19. MS/MS fragmentation of *B. abortus* S99 OPS derived homo-D-Rha4NFO-tetrasaccharide
MS/MS CID fragmentation of homo-D-Rha4NFO-tetrasaccharide derived from the partial acid hydrolysis of *B. abortus* S99 OPS. The peak at m/z 710.8 shows the parent tetrasaccharide ion. The peaks at 537.7 and 519.7 demonstrate the presence of the trisaccharide fragment (either with or without the oxygen from the glycosidic bond), the peaks at 364.6 and 346.6 demonstrate the presence of the disaccharide and the presence of the monosaccharide is demonstrated by the presence of the 190.4 and 174.4 m/z ions.

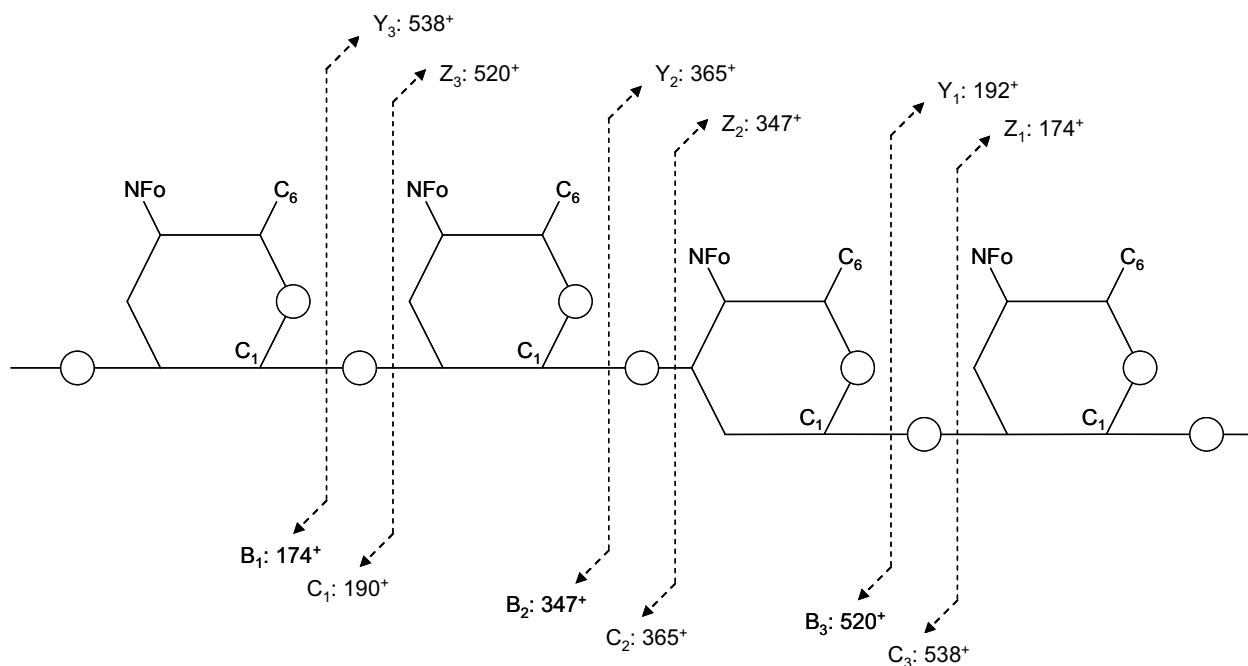


Figure 5.20. Schematic of BM40 mAb selected tetrasaccharide showing MS/MS fragmentation ions

The schematic representation above shows a D-Rha4NFo tetrasaccharide ($m/z = 711$) containing two $\alpha(1\rightarrow2)$ and one $\alpha(1\rightarrow3)$ links (the exact position of the $\alpha(1\rightarrow3)$ linkage in the parent ion is unresolved). The saccharides are represented as hexagons with the position of carbon 1 and carbon 6 shown. The position of the N-formyl group is shown attached to carbon 4. The oxygen in the glycosidic bond (and the pyranose ring) is shown as a circle. The broken lines represent probable fragmentations during CID MS/MS. The predicted m/z values (in positive MS mode) for each of these fragments (the ionised fragment is indicated by the direction of the arrow) is shown at the end of the arrow. The figure has been represented according to the fragmentation scheme of Domon and Costello (Domon and Costello, 1988).

By increasing the CID energy it is sometimes possible to obtain ions relating to cross ring cleavages that may, depending on exactly where the cleavage occurs, identify different glycosidic linkage types (Ashline et al., 2007; Harvey et al., 1997; Mechref et al., 1998; Morelle and Michalski, 2005; Morelle et al., 2004; Robbe et al., 2004; Wuhrer and Deelder, 2006). For linkage information to be gained in this instance, cross ring fragmentation would have to occur between carbon 2 and carbon 3 as these are the carbons that are variably part of the glycosidic links. High energy CID MS/MS was applied to the tetrasaccharide from *Y. enterocolitica* O:9 that was eluted from the GC column in the first significant peak at 8:05 (figure 4.50 from chapter 4) and the *B. melitensis* 16M tetrasaccharide eluted in its first significant peak at 8:50 (figure 4.49 from chapter 4). The first expected to be exclusively $\alpha(1\rightarrow2)$ linked and the second to have at least one $\alpha(1\rightarrow3)$ linkage. However the high CID MS/MS spectra (not shown) showed no differences that could be attributable to the presence of an $\alpha(1\rightarrow3)$ linkage.

For all three of the antibody affinity columns the majority of the oligosaccharides were eluted in the 0.00625 M HCl fraction. In the case of the polyclonal antibody columns there were only trace amounts of the oligosaccharide in the 0.0125 and 0.025 M HCl fractions. The 0.0125 M HCl elution from the BM40 column did contain more oligosaccharides than found for the other columns, especially the larger oligosaccharides. This probably reflects the high avidity of the BM40 mAb and the ability of larger oligosaccharides to bind to more than one antibody paratope or that binding to a single paratope may be strengthened by the incorporation of more saccharide units (Vulliez-Le Normand et al., 2008). In this context it is notable that the affinity chromatography of the *B. melitensis* 16M OPS demonstrated (figure 5.8) that more antigen was released by 0.025 M HCl than was released by preceding elution with HCl at 0.0125 M.

5.6. Chapter 5: Summary and conclusions

The data relating to the retention of the longer oligosaccharides and polysaccharides is interesting and verifies that affinity chromatography was working as may be expected based upon the presence of multiple epitopes and bivalent antibodies. However the results relating to the shorter oligosaccharides are of more interest. The shorter the better as such oligosaccharides are more likely to present only one type of epitope. Therefore the fact that the polyclonal antibodies derived from cattle are able to effectively bind to tetrasaccharides is quite significant as it means that short oligosaccharides may be recognised by other polyclonal sera from livestock and thus have at least some potential to make useful diagnostic antigens.

One feature known to be found on *Brucella* OPS and not, to date, found elsewhere is the $\alpha(1\rightarrow3)$ linkage between the D-Rha4NFo monosaccharides. The evidence presented in chapter 3 suggests that this feature has a significant affect on antibody binding, even antibodies raised by infection with A type OPS dominant *Brucella* strains. The evidence presented in this chapter shows that bovine polyclonal antibodies raised against infection with an A dominant *Brucella* strain bind to a homo-D-Rha4NFo-tetrasaccharide that appears unique to *Brucella* and is bound very selectively by the M epitope specific mAb BM40. In contrast to the strong evidence for the role of the M epitope and $\alpha(1\rightarrow3)$ linkage in *Brucella* specific antibody interactions, there is no hard evidence for the role of a 'C specific' epitope and still no clear evidence for what the structure for this epitope (if it exists) may be.

On the basis of the evidence obtained, the project will continue by pursuing the development of a diagnostic tetrasaccharide antigen which is based on elements that comprise the M epitope.

Chapter 6

DEVELOPMENT and EVALUATION of HOMO-D-Rha4NFo-OLIGOSACCHARIDE iELISAs for ANTIBODY DETECTION

6.1. INTRODUCTION

The conclusion from the earlier work is that D-Rha4NFo tetrasaccharides derived from the *Brucella* OPS are large enough to form viable antibody epitopes that bind to polyclonal antibodies within cattle serum that have been raised against infection by a strain of *B. abortus* (strain 544, biovar 1) with A dominant OPS. This includes a particular tetrasaccharide form that binds specifically to the *Brucella* specific anti-M mAb BM40 and is not present in the OPS of *Y. enterocolitica* O:9. From the pre-existing structural knowledge, the only known difference that can account for this is the presence of an $\alpha(1\rightarrow3)$ linkage in the tetrasaccharide. This conclusion also fits with the serological studies described in chapter 3. These studies, whilst providing evidence for the role of the $\alpha(1\rightarrow3)$ linkage in serology also exemplified the difficulties that the presence of multiple overlapping epitopes present to the unambiguous assessment of the significance of the individual epitope types. The solution to this is to evaluate purified and discrete epitope antigens.

The ability of the BM40 mAb to bind a tetrasaccharide is perhaps not surprising as anti-*Brucella* mAbs have previously been shown to bind oligosaccharides of this length (Bundle et al., 1989a). However, the same study also demonstrated that of the mAbs derived from inoculation with *Brucella*, only half (3 from 6) showed evidence that they could bind pentasaccharides and only one demonstrated evidence that it could bind a tetrasaccharide (no tetrasaccharides were included in the analysis of these mAbs, although penta- and trisaccharides were). The limited permutations of structure evaluated at the time did not prove that the mAbs could not have bound a tetrasaccharide had the appropriate structure been tested. One of the features that may have limited the binding of these mAbs was that the $\alpha(1\rightarrow3)$ links within the synthetic structures evaluated were either at the terminal or reducing end. Structures with central $\alpha(1\rightarrow3)$ links, i.e. flanked on either side by one or more $\alpha(1\rightarrow2)$ links were not evaluated. Such sequences of linkages would be present within the native OPS. The evidence from this study also demonstrated that mAbs specific to the C/Y epitope (found in equal abundance in all *Brucella* OPS and the OPS from *Y. enterocolitica* O:9) could bind to D-Rha4NFo tetrasaccharides joined by two $\alpha(1\rightarrow2)$ links. In order to minimise the binding of antibodies of such specificity to a tetrasaccharide antigen it would therefore be desirable to avoid any contiguous $\alpha(1\rightarrow2)$ links.

6.2. Commissioned synthesis of D-Rha4NFo tetrasaccharide M (TSM) antigen

On the basis of this evidence it was rationalised that a D-Rha4NFo tetrasaccharide with a central $\alpha(1\rightarrow3)$ link flanked by $\alpha(1\rightarrow2)$ links would possess M epitope characteristics, would minimise binding to antibodies that cross react with *Y. enterocolitica* O:9 OPS, would exploit the significant differences observed in antigen binding profiles by antibodies within false positive and true positive sera and would be big enough to present a viable epitope to polyclonal antibodies from the natural host. The synthesis of this structure was commissioned to a commercial laboratory who delivered the bespoke tetrasaccharide shown in figure 6.1. This molecule has not previously been synthesised. Henceforth it will be referred to in this document as the TSM (Tetrasaccharide Synthetic M [epitope] antigen).

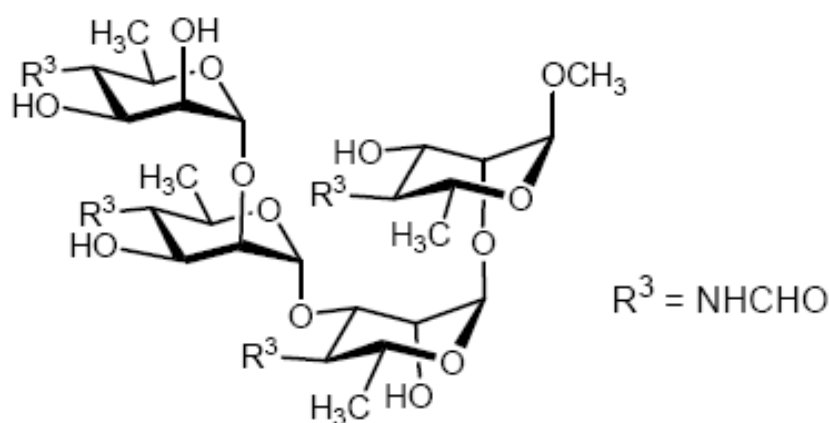


Figure 6.1. The chemical structure of the *Brucella* TSM antigen

The TSM antigen contains D-Rha4NFo monomers linked by consecutive $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow2)$ links. R^3 represents the formamido group. The tetrasaccharide has been 'closed' at the reducing end with an O-methyl group on the anomeric carbon and has a molecular weight of 724.

6.3. Evaluation of synthetic TSM antigen by GC column ESI-MS/MS

On receipt of the TSM antigen from the supplier it was evaluated by GC column ESI-QqToF (CID MS/MS) to evaluate its composition and purity. The total ion count chromatogram from the GC column is shown in figure 6.2. This shows a significant peak eluting at between 11 and 12 minutes. The MS spectrum corresponding to an elution time of 11:33 min:secs is shown in figures 6.3 and in narrower m/z range, 6.4. This shows that the total ion count peak at this time is attributable to ions

of 725 to 747 m/z . In particular these are ions of m/z equal to 725, 742 and 747 (as shown in figure 6.4). These ions are compatible with those of a protonated ($m/z = 725$), ammoniated ($m/z = 742$) and sodiated ($m/z = 747$) form of the TSM. There was no detection of partially or fully deformed variants or tri, di or monosaccharides of D-Rha4NfO. The ESI-MS/MS analysis was also compatible with a pure form of the commissioned TSM structure. The spectrum showing the ions derived from fragmentation of the 725 m/z parent is shown in figure 6.5, A schematic representation of the fragmentation of the TSM antigen, based on the nomenclature of Domon and Costello (Domon and Costello, 1988), showing the predicted ions is shown in figure 6.6. Although the MS and MS/MS data was compatible with the desired structure it did not provide complete confirmation. For example, an exclusively $\alpha(1\rightarrow2)$ linked tetrasaccharide would present the same data. To evaluate some of these details immunoassay was used.

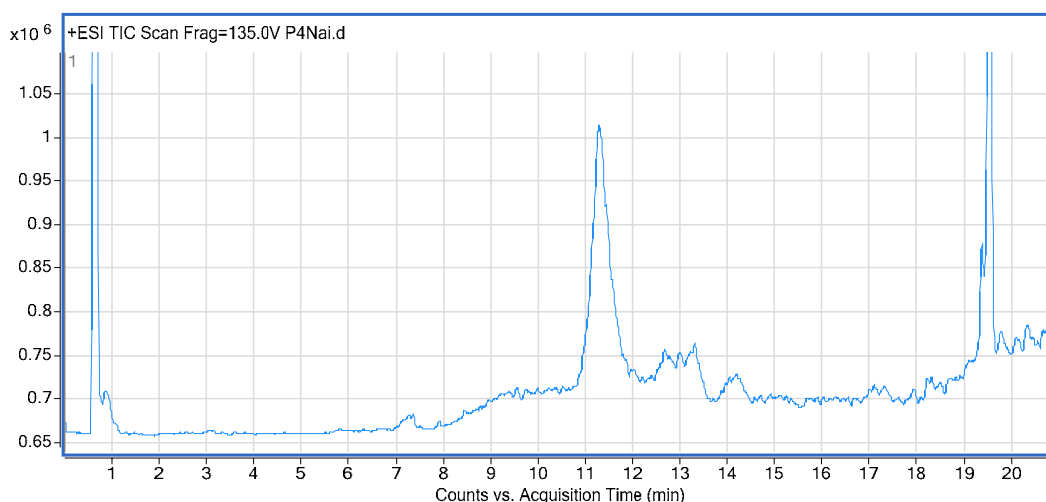


Figure 6.2. TSM antigen total ion count chromatogram from GC column ESI-QqToF

The total ion count chromatogram, with ion frequency on the y-axis against time (mins) on the x-axis, shows a significant peak between 11 to 12 minutes, the mass spectrum for which is shown is figure 6.3.

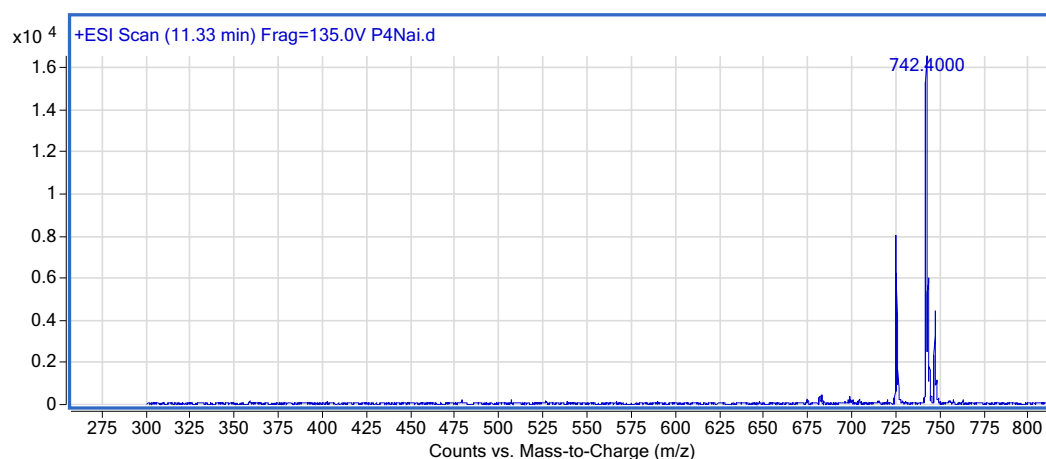


Figure 6.3. ESI-QToF mass spectrum for TSM antigen eluted from GC column (1 of 2)

The mass spectrum, with ion frequency on the y-axis against m/z on the x-axis, for the TSM antigen ($mw = 724$) which shows ions of between 725 and 750 m/z .

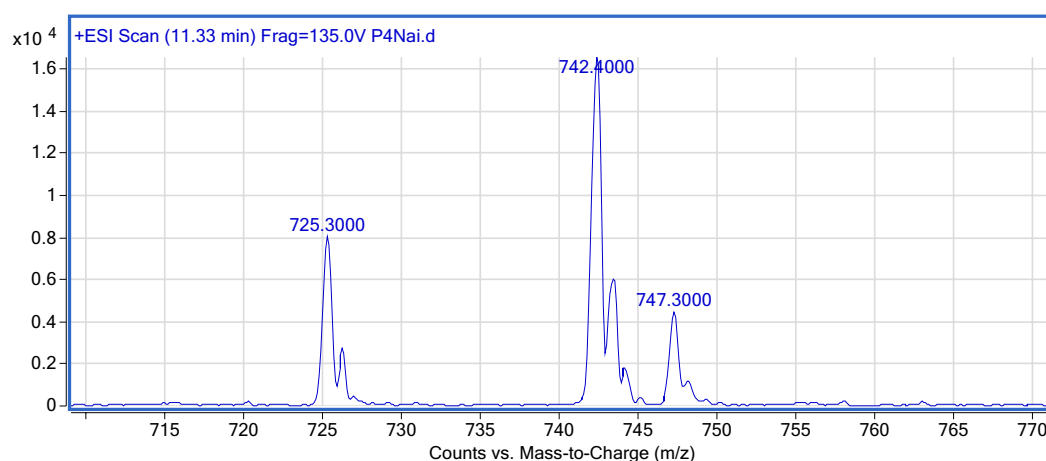


Figure 6.4. ESI-QToF mass spectrum for TSM antigen eluted from GC column (2 of 2)

The mass spectrum, with ion frequency on the y-axis against m/z on the x-axis, for the TSM antigen ($mw = 724$) which shows ions that are compatible with those of a protonated ($m/z = 725$), ammoniated ($m/z = 742$) and sodiated ($m/z = 747$) form of the TSM with additional peaks corresponding to the presence of less abundant elemental isotopes.

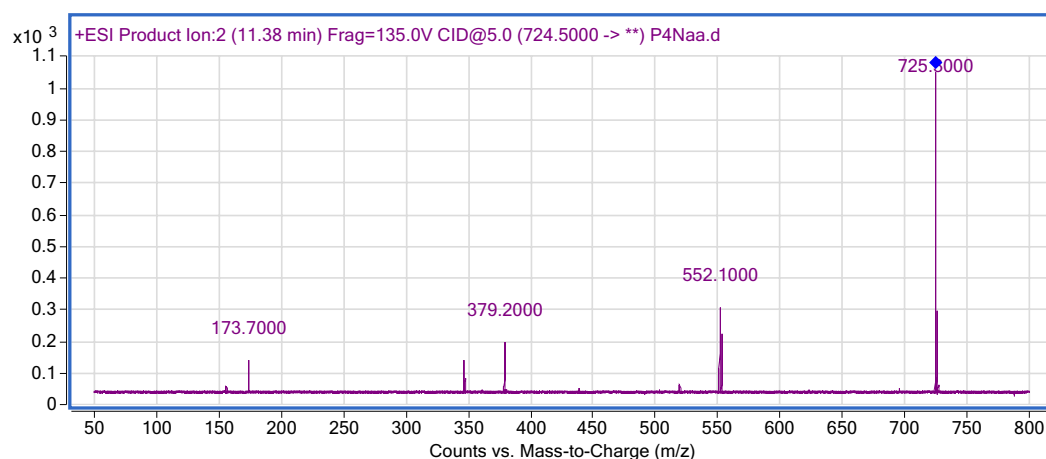


Figure 6.5. MS/MS fragmentation of protonated TSM antigen ion with $m/z = 725$

The CID MS/MS mass spectrum, with ion frequency on the y-axis against m/z on the x-axis, for the ion $m/z = 724$. The observed ion peaks, $m/z = 552$, 379 and 174 are compatible with the Y_3 , Y_2 and B_1 ions (respectively) predicted to arise from the fragmentation of the TSM antigen (see figure 6.6).

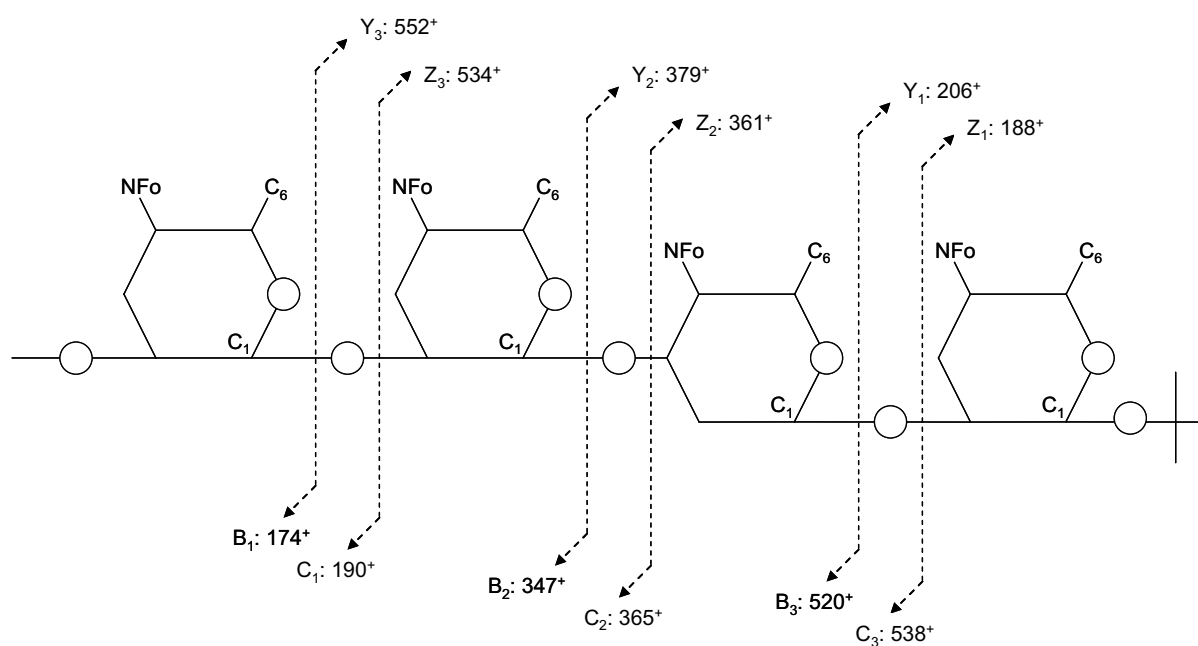


Figure 6.6. Schematic of TSM showing MS/MS fragmentation ions

The schematic representation above shows the TSM antigen, a D-Rha4NFo tetrasaccharide ($m/z = 724$) containing two $\alpha(1 \rightarrow 2)$ and one $\alpha(1 \rightarrow 3)$ links and an O-methyl group at the reducing end. The saccharides are represented as hexagons with the position of carbon 1 and carbon 6 shown. The position of the N-formyl group is shown attached to carbon 4. The oxygen in the glycosidic bond (and the pyranose ring) is shown as a circle. The broken lines represent probable fragmentations during CID MS/MS. The predicted m/z values (in positive MS mode) for each of these fragments (the ionised fragment is indicated by the direction of the arrow) is shown at the end of the arrow. The figure has been represented according to the fragmentation scheme of Domon and Costello (Domon and Costello, 1988).

6.4. Evaluation of synthetic D-Rha4NFO tetrasaccharide by antibody immunoassay

The ESI-MS/MS data provided good evidence that the commissioned laboratory had successfully synthesised the desired structure. Evaluation of the interaction between the TSM and *Brucella* specific antibodies was also performed to provide evidence that the synthetic antigen was of the appropriate structural configuration. The interaction between the TSM and the M-specific mAb BM40 was evaluated by cELISA using the same methods used to evaluate the binding of free OPS, the results of which were shown in chapter 3 (figure 3.10). The results have been incorporated into the data shown previously for the free OPS and these are shown together in figure 6.7 with the results for the TSM antigen shown by the broken purple line. These demonstrate that the TSM antigen was able to inhibit the binding to a far greater extent than the equivalent concentration of *Y. enterocolitica* O:9 OPS and to a similar degree as the *B. abortus* S99 OPS. Taking into account the monovalency of the TSM antigen compared to the multiple binding sites available on 16M OPS, the results provide strong evidence for specific TSM antibody-antibody binding. Given the specificity of the BM40 for the M epitope, the data also provides strong evidence that the TSM antigen conforms to this structure, including the incorporation of an $\alpha(1\rightarrow3)$ link.

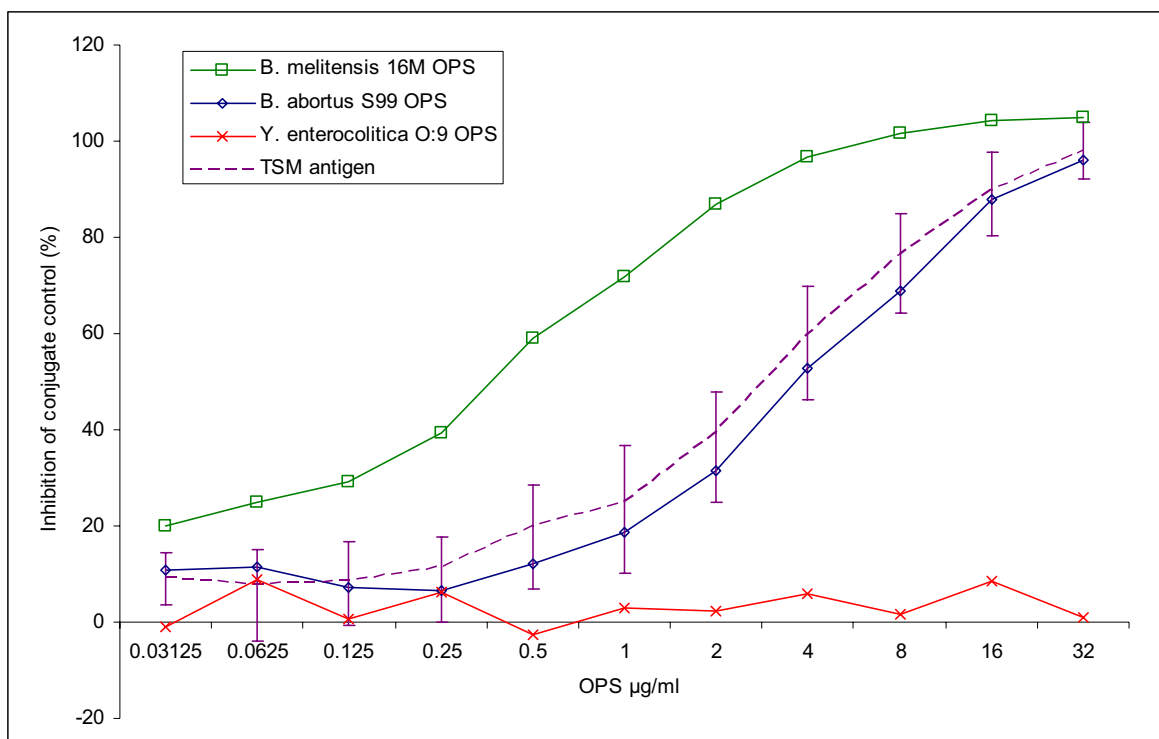


Figure 6.7. BM40 mAb cELISA with TSM antigen, *Brucella* and *Y. enterocolitica* O:9 OPS

The results of a competitive ELISA using *B. melitensis* 16M OPS coated plates and an anti-M epitope mAb (BM40) showing the inhibitory effects on optical density (OD, y-axis) of different quantities of native M dominant (*B. melitensis* 16M, green line), A dominant (*B. abortus* S99, blue line) and A exclusive (*Y. enterocolitica* O:9, red line) OPS. The results for the TSM antigen are shown as a broken purple line with error bars that represent the maximum and minimum data points from triplicate assays with the lines plotted though the mean result.

Further immunoassays were performed to provide evidence that the binding of the TSM was specifically due to the interaction of the antigen with the antibody paratope, rather than due to some non-specific inhibition, and that it was specific to antibodies with preference to the M epitope. Competitive ELISAs using monospecific rabbit anti-A and anti-M antibodies, as used for the serotyping (into A, M or mixed A and M dominance of OPS) of *Brucella* strains (Alton et al., 1994), were developed using ELISA plates coated with *B. abortus* S99 and *B. melitensis* 16M sLPS respectively. This cELISA method was similar in principle to the standard cELISA with HRP conjugated BM40, but in this instance the rabbit anti-*Brucella* monospecific (polyclonal) antibodies were not directly conjugated. However, after competition between the free liquid phase and the solid phase antigen for the binding of the monospecific sera, the antibodies that bound to the solid phase were detected by use of an anti-rabbit antibody conjugate. The results of the monospecific cELISAs for samples containing the TSM, *B. melitensis* 16M, *B. abortus* S99 and *Y. enterocolitica* O:9 OPS are shown in figures 6.8 (anti-M monospecific antibodies) and 6.9 (anti-A monospecific antibodies).

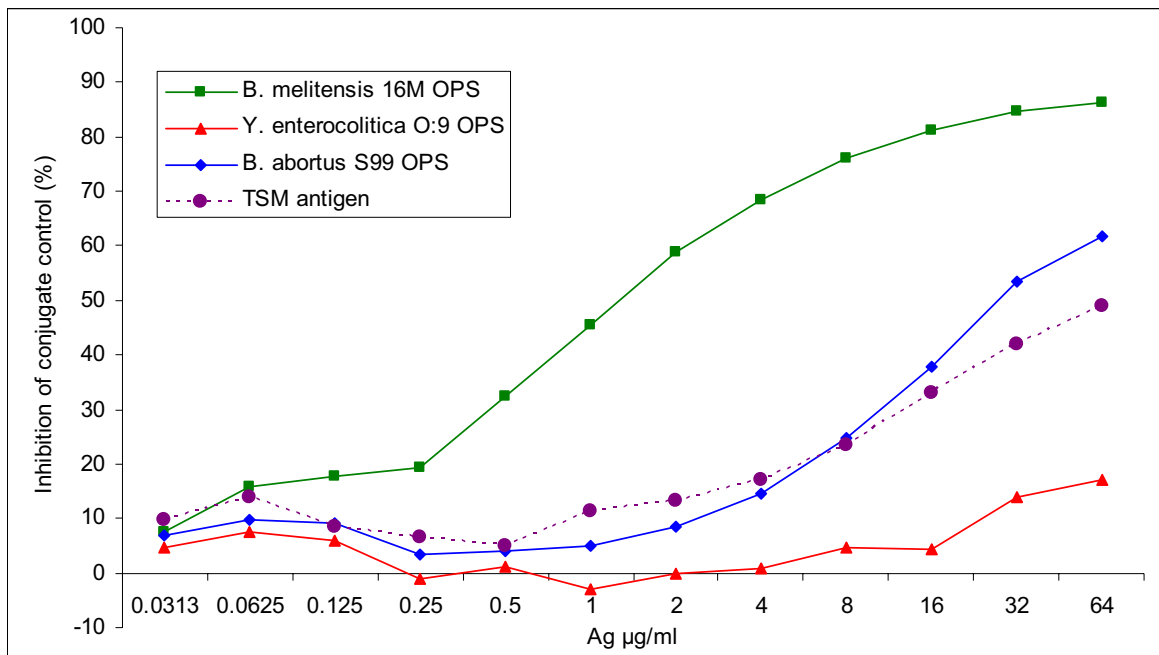


Figure 6.8. Monospecific anti-M cELISA with TSM antigen, *Brucella* and *Y. enterocolitica* O:9 OPS

The results of a competitive ELISA using *B. melitensis* 16M OPS coated plates and anti-M monospecific antisera showing the inhibitory effects on optical density (OD, y-axis) of different quantities of native M dominant (*B. melitensis* 16M, green line), A dominant (*B. abortus* S99, blue line) and A exclusive (*Y. enterocolitica* O:9, red line) OPS. The results for the TSM antigen are shown as broken purple line.

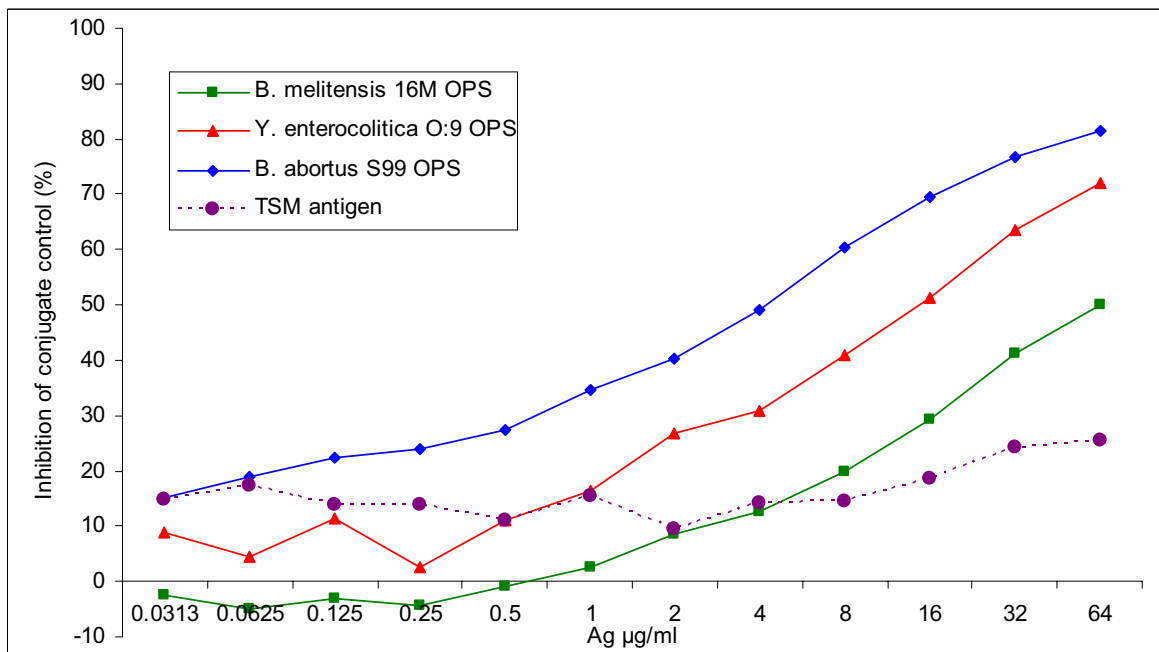


Figure 6.9. Monospecific anti-A cELISA with TSM antigen, *Brucella* and *Y. enterocolitica* O:9 OPS

The results of a competitive ELISA using *B. abortus* S99 OPS coated plates and anti-A monospecific antisera showing the inhibitory effects on optical density (OD, y-axis) of different quantities of native M dominant (*B. melitensis* 16M, green line), A dominant (*B. abortus* S99, blue line) and A exclusive (*Y. enterocolitica* O:9, red line) OPS. The results for the TSM antigen are shown as a broken purple line.

The results from figure 6.8 are similar to those for the cELISA with BM40 shown in the preceding figure. The TSM antigen has similar inhibitory capability as the *B. abortus* S99 OPS. The results shown in figure 6.9 for the anti-A monospecific cELISA show that the TSM antigen has much reduced competitive and thus inhibitory capability. The data for the native antigens demonstrate the specificity of this assay for antigens with minor or no amount of $\alpha(1\rightarrow3)$ links. The *B. abortus* and *Y. enterocolitica* O:9 OPS antigens are both more inhibitory than the *B. melitensis* 16M OPS antigen, which is more inhibitory than the TSM antigen. These results provide compelling evidence that the antibody binding capability of the TSM antigen is due to specific binding to the paratope of antibodies that have a preference to the M epitope of *Brucella* OPS. The results therefore indicate the presence of an $\alpha(1\rightarrow3)$ linkage within the TSM antigen – as designed.

6.5. Application of the TSM antigen to serodiagnosis

On the basis of the antibody specificity of the TSM antigen, the same core structure was commissioned for synthesis with the addition of a linker to enable the attachment of the TSM to a solid phase thus enabling the development of a TSM iELISA. However, despite accepting the commission, the synthesis laboratory was unable to produce a product of sufficient quantity or purity to be acceptable for use. This jeopardised the completion of this project. The TSM antigen as shown in figure 6.1 demonstrated its ability to bind antibody, but what it did not have was an ability to bind a suitable matrix for the construction of a TSM iELISA (or any other suitable platform). Like purified OPS, the TSM is highly hydrophilic and will not passively bind to appropriate immunoassay surfaces. Any chemical modification of the molecule to facilitate conjugation will alter the structure of the antibody binding elements.

6.5.1. Oxidation of the TMS antigen and conjugation to Carbo-BIND™ plates

The approach taken to address the difficulties presented above was to oxidise the TSM antigen under very mild and monitored conditions. In theory this would create aldehyde groups across the vicinal diols in the terminal D-Rha4NFo as shown in figure 6.10. This will break open the terminal D-Rha4NFo leading to creation of a structure that is somewhere between a tetra- and a trisaccharide

(a tri-5!). The aldehyde groups could then be used to bind the oxidised TSM antigen to Carbo-BIND™ plates in the same fashion as described for the covalent conjugation of the oxidised native OPS (refer to chapter 3).

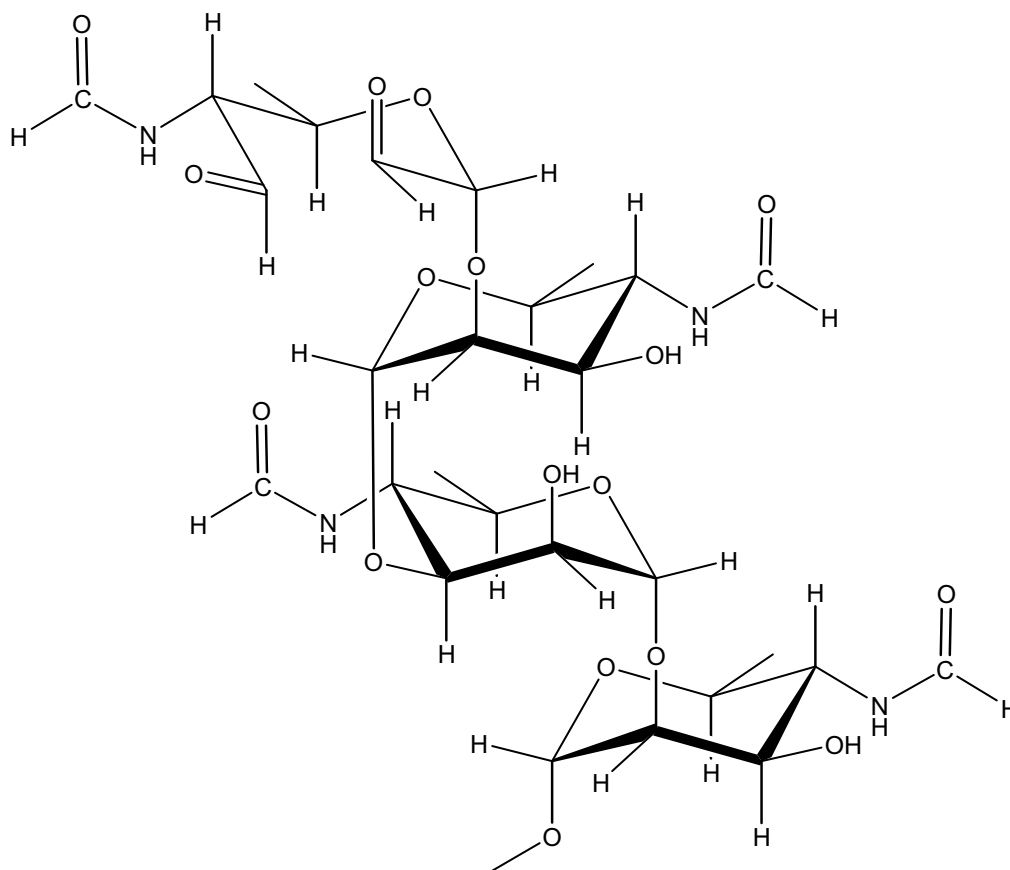


Figure 6.10. The proposed structure for the TSM antigen after mild oxidation

The figure shows the proposed structure of the D-Rha4NFO tetrasaccharide after mild oxidation with sodium metaperiodate. Vicinal diols are the most susceptible to oxidation and the only pair within the molecule are those present in the terminal D-Rha4NFO. The figure shows a pair of aldehydes where the vicinal diols were present pre-oxidation. These aldehydes may be used for conjugation with hydrazide.

Despite developing the oxidation and conjugation process for the OPS antigen and Carbo-BIND™ plates the process of oxidising the TSM antigen presented several additional challenges. There was a much smaller margin for error regarding over oxidation as each molecule to be oxidised only contained one epitope. Any unintended degradation would be more significant than the case were multiple epitopes, and hence some element of redundancy, were present. Separation of excess sodium metaperiodate from the TSM prior to addition to the Carbo-BIND™ plates could not be readily facilitated by size exclusion chromatography due to small size of the TSM compared to the native OPS. This separation was not needed for the OPS antigen as the sodium metaperiodate was

effectively diluted out by the very low working strength concentrations for the OPS – 0.5 µg/ml or less. High levels of residual sodium metaperiodate had a detrimental affect on the antigen binding and presentation process (data not shown). The monovalent nature of the TSM suggests that it may have to be used in higher concentrations than the OPS and the ability to dilute out sodium metaperiodate was consequentially more limited.

A means to measure the concentration of residual sodium metaperiodate after trial oxidation processes was developed using ABTS. This changes colour when oxidised and is typically used in iELISA to quantify the presence of HRP which catalyses the conversion of H_2O_2 into $2\text{H}_2\text{O}$ and oxidised ABTS. It is just as effective at detecting the presence of the oxidising agent sodium metaperiodate in a dose dependent (and enzyme independent) manner. This reaction was used to minimise the potential for over oxidation as the molar concentrations of TSM antigen and sodium metaperiodate could be carefully balanced (data not shown). This detection tool also demonstrated that sodium metaperiodate is efficiently quenched when passing through sephadex columns. Sephadex is essentially a cross linked dextran gel. Although the glucose units do not contain vicinal diols (they are all *trans*) they are in sufficient abundance to overcome any limitations to the efficiency of their reaction with sodium metaperiodate. The gently oxidised TSM could therefore be very efficiently separated from residual sodium metaperiodate by passing it though a disposable sephadex G-10 elution column. As the sodium metaperiodate was quenched rather than retained, the near complete recovery of the TSM antigen can be obtained by adding the total column volume of buffer.

The BM40 cELISA, as used to derive the data in figure 6.7, was used to determine if the oxidised TSM antigen retained its antibody binding properties. The results are shown in figure 6.11, and demonstrate that the oxidised TSM antigen (blue line) had lost none of the BM40 mAb binding capability. The oxidised TSM antigen was dissolved in sodium acetate buffer as a consequence of the oxidation process although this had no significance on antibody reactivity as also shown in figure 6.11. To confirm that this oxidised TSM antigen could bind to hydrazide groups it was incubated with hydrazide-LC-biotin (Pierce) and ESI-MS/MS confirmed that the oxidised TSM antigen had bound to this conjugate (data not shown). Addition of the hydrazone conjugated biotinylated oxidised TSM antigen to cELISA confirmed that the oxidised and conjugated form still bound to the BM40 mAb (data not shown).

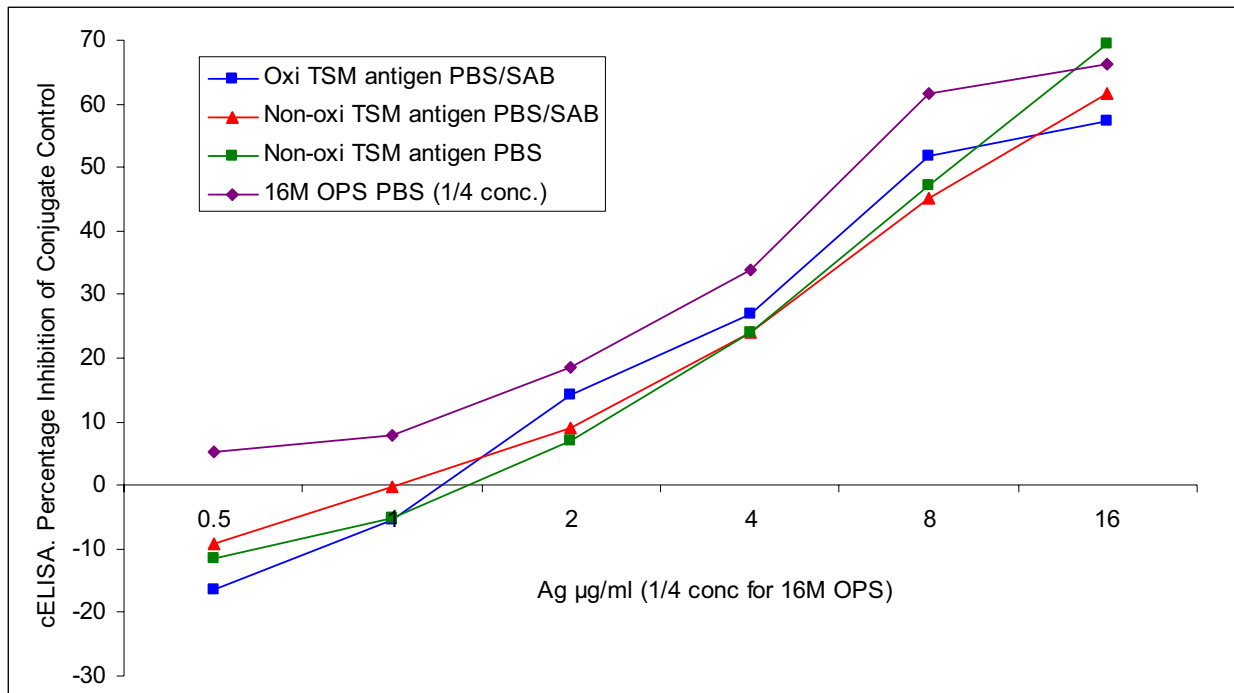


Figure 6.11. BM40 mAb cELISA with oxidised TSM antigen

The results of a competitive ELISA using *B. melitensis* 16M OPS coated plates and an anti-M epitope mAb (BM40) showing the inhibitory effects on optical density (OD, y-axis) of different quantities and oxidation states of TSM antigen. The results for oxidised TSM (in partially diluted oxidation buffer, 'PBS/SAB') are shown as a blue line, the results for the non-oxidised TSM antigen are shown as red (in partially diluted oxidation buffer, 'PBS/SAB') and as green (in PBS). As an external control *B. melitensis* 16M OPS (diluted an additional 4x) was also included with the results shown in purple.

6.5.2. Development and application of TSM antigen iELISA

The oxidised (oxi) TSM antigen was chemically conjugated to Carbo-BIND™ plates to enable the development of an indirect ELISA for the detection of specific bovine antibodies. The ELISA conditions used were essentially the same as those used for the evaluation of cattle sera by OPS iELISA, the results for which are described in chapter 3. The optimal working strength of the oxi-TSM antigen was 10 µg/ml compared to an equivalent working concentration of less than 0.5 µg/ml for the native OPS antigens. This probably reflects the monovalent nature of the TSM antigen, as well as at least some degradation due to the oxidation process and loss of reactivity upon covalent conjugation (even though the cELISA described above data suggests that this was minimal).

The oxi-TSM antigen iELISA was evaluated on the same set of *B. abortus* field infected and FPSR cattle samples as described in chapter 3. These were 46 samples from individual animals confirmed by culture to be infected with *B. abortus* biovar 1 (A dominant). Also 68 samples from individual animals within Great Britain whose sera was collected from 1996 to 1999, more than 10 years since

the declaration of officially brucellosis free national status, that were positive in conventional serology for brucellosis such as *B. abortus* S99 sLPS iELISA, SAT and CFT (Nielsen and Ewalt, 2010) but for which there was no cultural or epidemiological evidence of brucellosis.

For comparison, the results from the *B. melitensis* 16M and *Y. enterocolitica* O:9 OPS iELISAs are presented again here in figure 6.12 (as presented initially in figure 3.25 in chapter 3). The results for the oxi-TSM antigen iELISA on these same sera are presented against the *B. melitensis* 16M OPS iELISA results in figure 6.13. These results clearly demonstrate an improved specificity for true positive samples when the oxi-TSM antigen is used and many of the FPSR samples present very little response. However several of the samples from the *Brucella* infected cattle also present a weak response which would limit the sensitivity of the method if gains in specificity were to be realised. The structural changes due to the necessary oxidation of the TSM antigen are likely to have had some impact on the results.

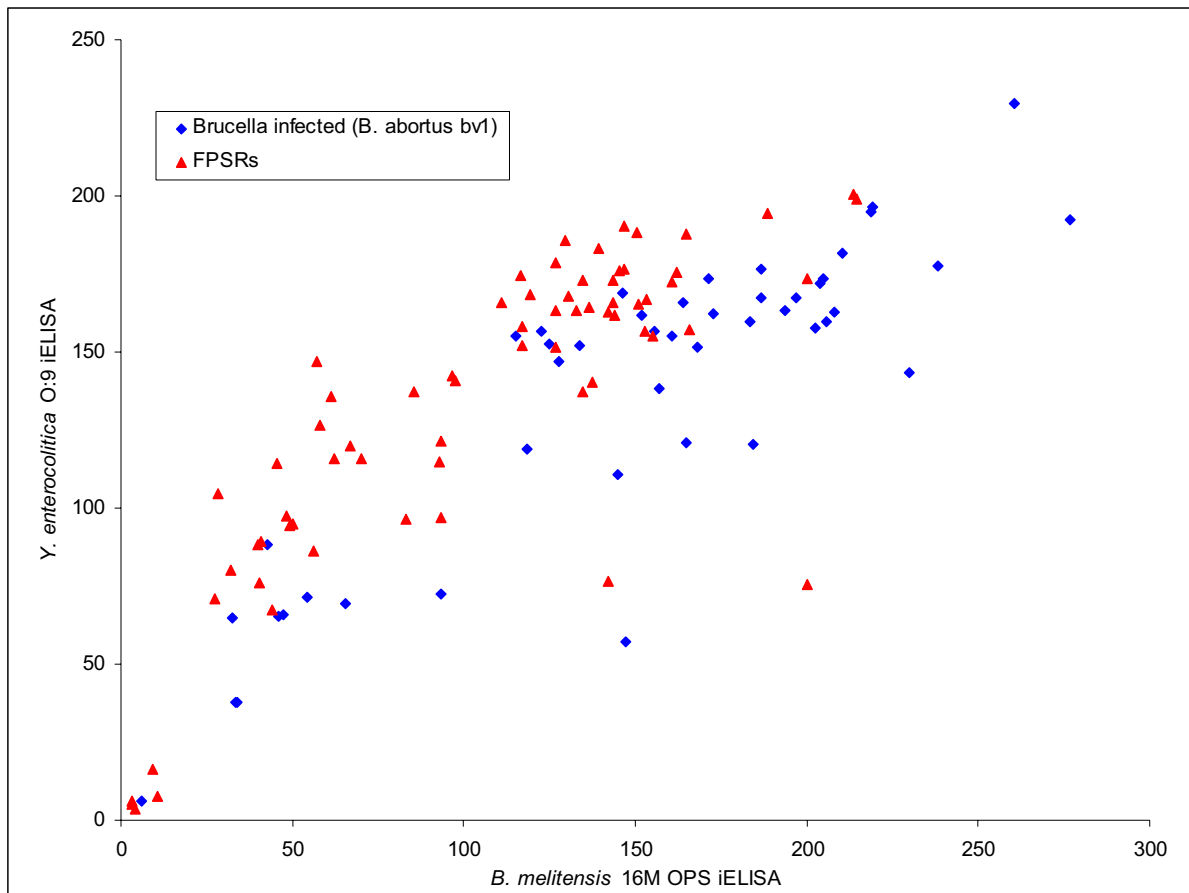


Figure 6.12. *B. melitensis* 16M against *Y. enterocolitica* O:9 OPS iELISA results for cattle sera

The results (expressed as a percentage of a common positive control) for *B. melitensis* 16M OPS iELISA (x-axis) and *Y. enterocolitica* O:9 OPS iELISA (y-axis) ELISAs; the solid blue diamonds represent the results for sera from cattle confirmed as infected with *B. abortus* biovar 1 (n=46) and the red diamonds represent the results for sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

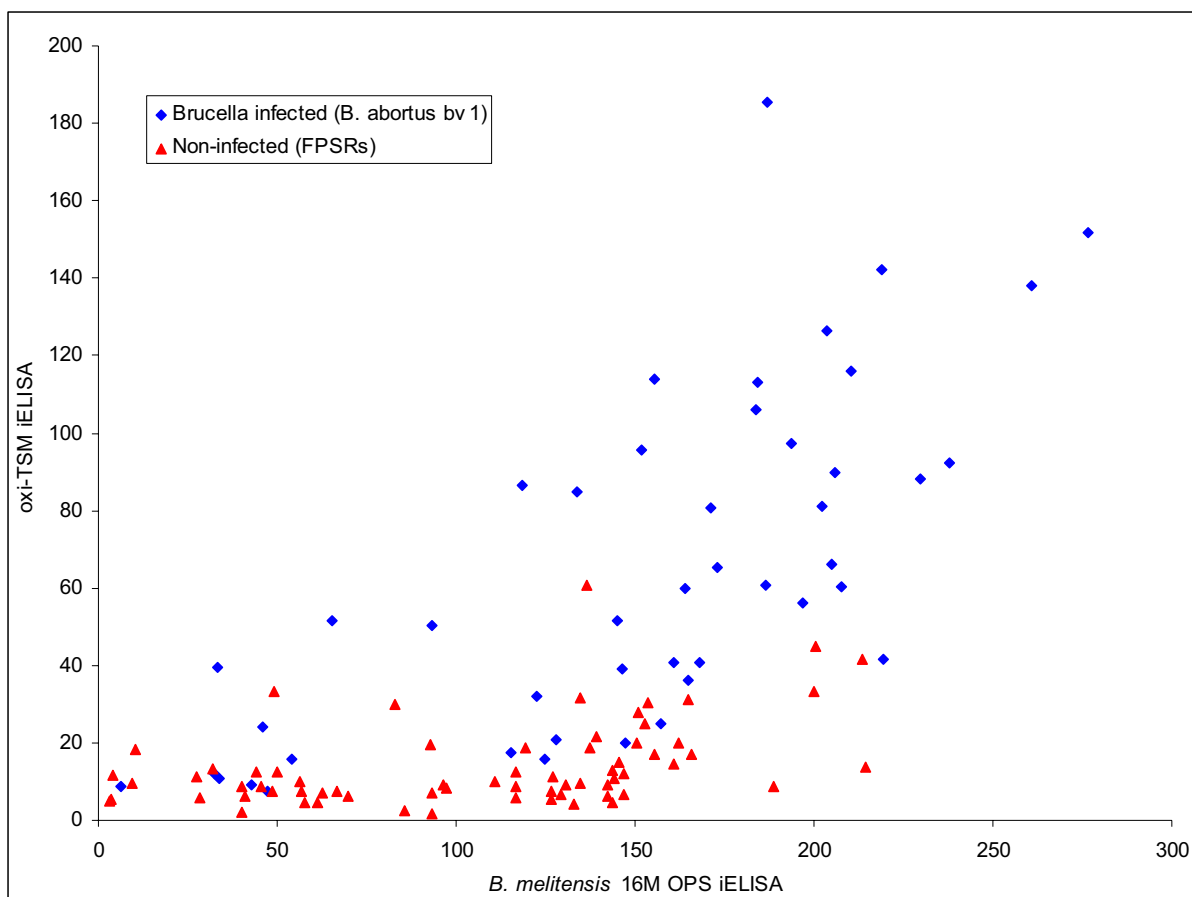


Figure 6.13. *B. melitensis* 16M against oxi-TSM OPS iELISA results for cattle sera

The figure above shows the results (expressed as a percentage of a common positive control) for *B. melitensis* 16M OPS iELISA (x-axis) and oxi-TSM antigen (y-axis) ELISAs; the solid blue diamonds represent the results for sera from cattle confirmed as infected with *B. abortus* biovar 1 (n=46) and the solid red triangles represent the results for sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

6.6. Development and application of penta- (M) and nonoasaccharide (A & M) antigen iELISAs

Before completing the statistical analysis on the data from the oxi-TSM antigen some additional antigens of interest became available. Professor David Bundle (University of Alberta, Department of Chemistry) provided these antigens and requested that they were used to develop iELISAs and evaluated as antigens for the serodiagnosis of ruminant brucellosis. These were BSA conjugated synthetic D-Rha4NFo penta- and nonasaccharides as described recently (Guiard et al., 2013) and as shown in figures 6.14 and 6.17. The published data shows that these antigens had been evaluated by mAbs (YsT9-1 and Bm10) previously shown to be specific for the *Brucella* A and M

OPS antigens respectively (Bundle et al., 1989a). The nonasaccharide antigen bound the anti-A and anti-M specific mAbs with equivalent avidity, whereas the pentasaccharide displays a preference for the M specific mAb, while still binding the A specific mAb but with an approximately 10 fold reduced avidity. The reduction of anti-A antibody binding would be commensurate with the reduction in the number of continuous $\alpha(1\rightarrow2)$ links. The equal binding to each antigen by the anti-M mAb demonstrates that these eliminated $\alpha(1\rightarrow2)$ links have little or no impact on this antibody.

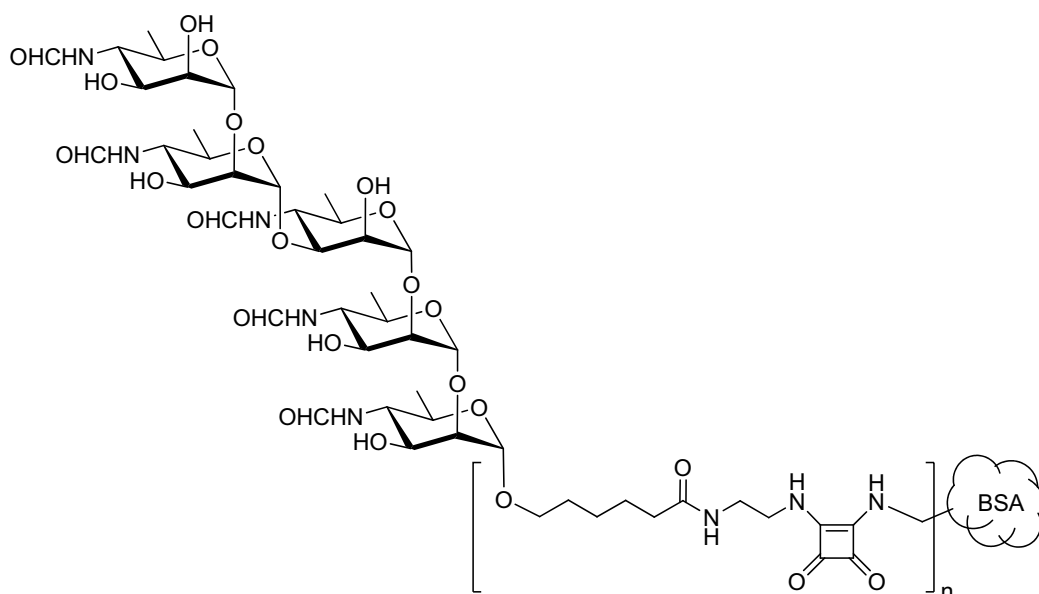


Figure 6.14. Structure of the D-Rha4NFo pentasaccharide-BSA conjugate provided by Professor D. Bundle (image also provided)

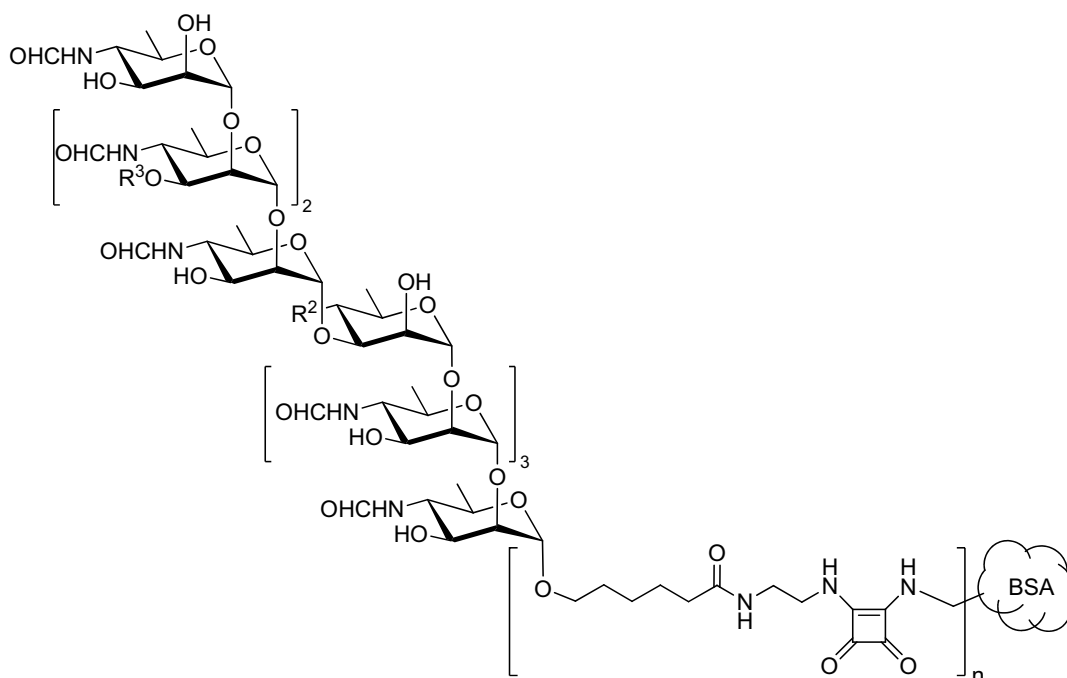


Figure 6.15. Structure of the D-Rha4NFO nonasaccharide-BSA conjugate provided by Professor D. Bundle (image also provided)

The initial invitation was to evaluate these antigens against a conventional panel of sera from *Brucella* infected and non-infected animals. This would test the hypothesis of Professor Bundle which was that these synthetic surrogates make effective alternatives to the use of native antigens for serodiagnosis. Owing to their structure, in particular their length and the incorporation of $\alpha(1\rightarrow3)$ links flanked by $\alpha(1\rightarrow2)$ links they were also of considerable value for testing the hypothesis of this project. With the consent of Professor Bundle and the understanding that the data would be shared and could be presented here, these antigens were applied to the FPSR samples in addition to the original, more conventional, sample set.

The two iELISAs that were developed with these novel oligosaccharide BSA conjugate antigens were similar in most respects to the OPS iELISAs described previously. The major difference was that the conjugation of the oligosaccharides to the BSA facilitated the passive absorption of the antigens to the surface of standard ELISA plates via this benign protein carrier. The pentasaccharide and nonasaccharide iELISA results from the cattle serum sample set (as used above) are shown in figure 6.16. The optimal working strength for these antigens was 2.5 $\mu\text{g/ml}$. This is five times higher than the working strength concentration of the OPS in iELISA. This

difference may, of course, be due to the significant portion of the mass that is the BSA antigen. Given that the BSA constitutes a significant proportion of the antigen it is interesting that the optimal working strength is not higher. This may be because their conjugation to the BSA carrier places the oligosaccharides with sufficient proximity to each other to provide bivalent binding opportunities to specific antibodies. Their working concentration is considerably lower than that for the oxidized TSM antigen (especially given their BSA content) and this may be in part due to their more proximal nature on the surface of the BSA carrier absorbed to the ELISA plate or a reflection of their longer (and more intact) form.

The results from testing the 46 samples from *B. abortus* biovar 1 infected cattle, 68 FPSR samples and 125 randomly selected sera from non-*Brucella* infected cattle by the D-Rha4NFO oligosaccharide-BSA conjugate antigen iELISAs are shown in figure 6.16. Several features of the graph stand out. One is that the randomly selected samples from the non-*Brucella* infected cattle cluster at the origin and can therefore be considered to be, in the main, negative. The FPSR samples show a response against both antigens that is considerably raised compared to the equivalent data from the oxi-TSM iELISA and compared to the randomly selected samples from the non-*Brucella* infected cattle. For the nonasaccharide antigen, almost all the FPSR samples show a reaction that is higher than all of the randomly selected samples. The results for the pentasaccharide antigen show that more of the FPSR samples have very low responses equal to those from the randomly selected samples from the non-*Brucella* infected cattle. For the pentasaccharide antigen, the results from all samples from the *Brucella* infected cattle are higher than those from the randomly selected samples from the non-*Brucella* infected cattle. With one exception this is also the case for the nonasaccharide antigen. What is also striking is that the population of results from the FPSR samples and those from the *Brucella* infected cattle appear to be more distinct compared to the data derived from iELISA with the native OPS (refer to figure 6.12).

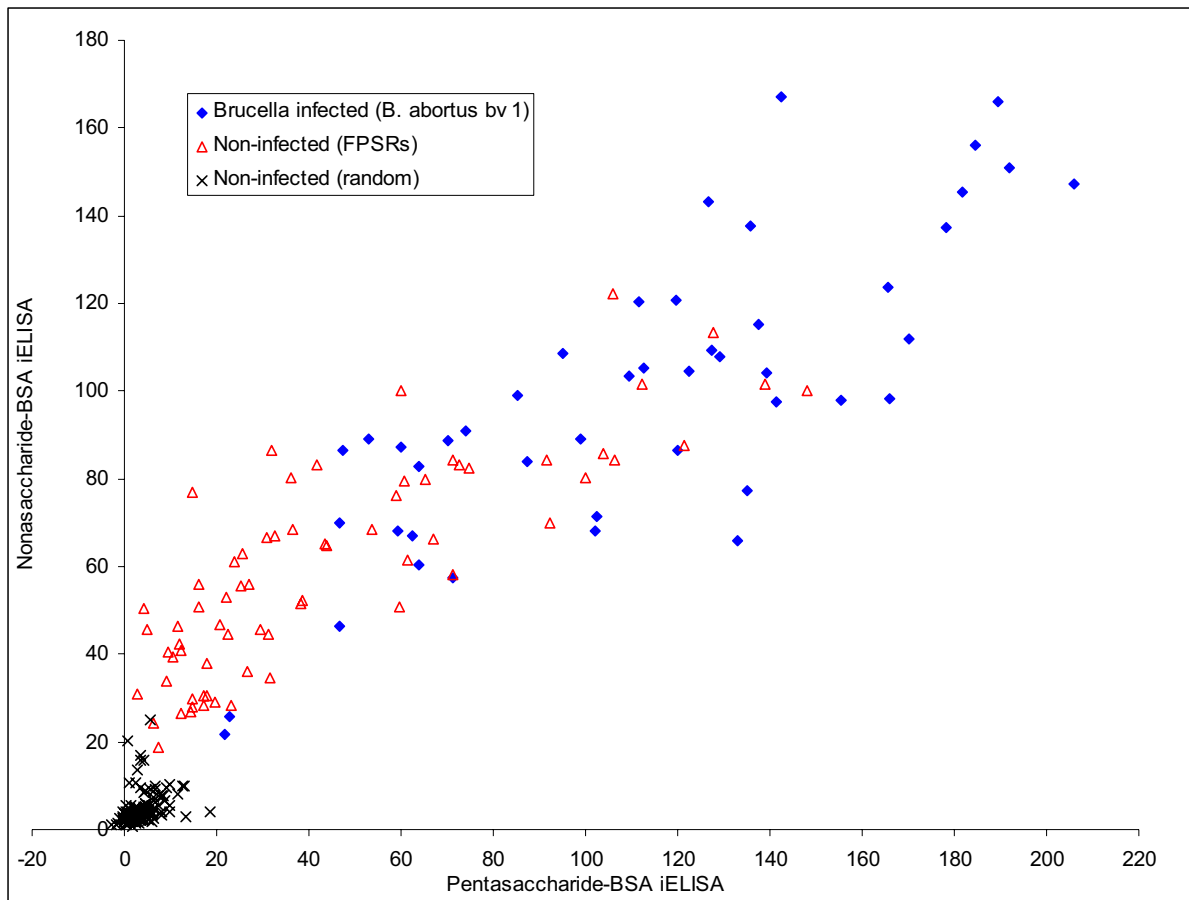


Figure 6.16. Results of D-Rha4NFo penta- and nonasaccharide conjugate antigen iELISAs for cattle sera

The results (expressed as a percentage of a common positive control) for the pentasaccharide-BSA conjugate antigen (as shown in figure 6.14) and the nonasaccharide-BSA conjugate antigen (as shown in figure 6.15) iELISAs; the solid blue diamonds represent the results for sera from cattle confirmed as infected with *B. abortus* biovar 1 (n=46) and the red diamonds represent the results for sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

One other feature of the data in figure 6.16 that is obvious yet less striking but nonetheless highly relevant is that the data from the nonasaccharide and pentasaccharide iELISAs look very similar. Given that the pentasaccharide had tenfold less affinity for the anti-A mAb than the nonasaccharide one might hypothesise that the M like pentasaccharide would be far less effective than the mixed A and M nonasaccharide at detecting antibodies raised by infection with A dominant strains of *Brucella*. This was not the case. It appears that the polyclonal antibodies are either of low stringency (a theory that is not supported by the improved differentiation of the FPSR samples) or that the true positive samples contain many antibodies with specificity for the M epitope. This supports the evidence for this gathered elsewhere in this project. The recent discovery of the location of the M epitope, at the end of the OPS from A and M dominant strains - excluding the afore mentioned exceptions of OPS from *B. suis* biovar 2 (Zaccheus et al., 2013) and *B. inopinata* BO2 (Wattam et

al., 2012) - provides a rationale for this (Kubler-Kielb and Vinogradov). These findings all have major ramifications regarding the universal application of an M epitope antigen for the serodiagnosis of brucellosis.

6.7. Statistical analysis of the diagnostic capability of the synthetic oligosaccharide and native OPS iELISAs

The results from all three of the synthetic antigen iELISA assays were evaluated for their diagnostic capability to minimise the FPSR problems by considering the FPSR samples as the only group within the non-infected population. The results were evaluated to find the optimal Youden Index for each and ROC analysis was used to determine the ROC Curves (figure 6.17) and AUC. The ROC curves for the native OPS iELISAs (initially presented in figure 3.26 in chapter 3) have also been included in figure 6.17 for comparison. The metrics are presented in Table 6.1 below, along with the 95% confidence intervals for the AUC. The table also shows the equivalent data derived from the native OPS iELISAs that was shown in table 3.3 from chapter 3. The results from the testing for significant differences between the AUC data for all of these iELISAs are shown in table 6.2, this incorporates the data previously shown in table 3.4 of chapter 3. In this context, the AUC represents the ability of the assay to correctly classify samples from animals that are *Brucella* infected and those that are not (in this case the FPSR samples).

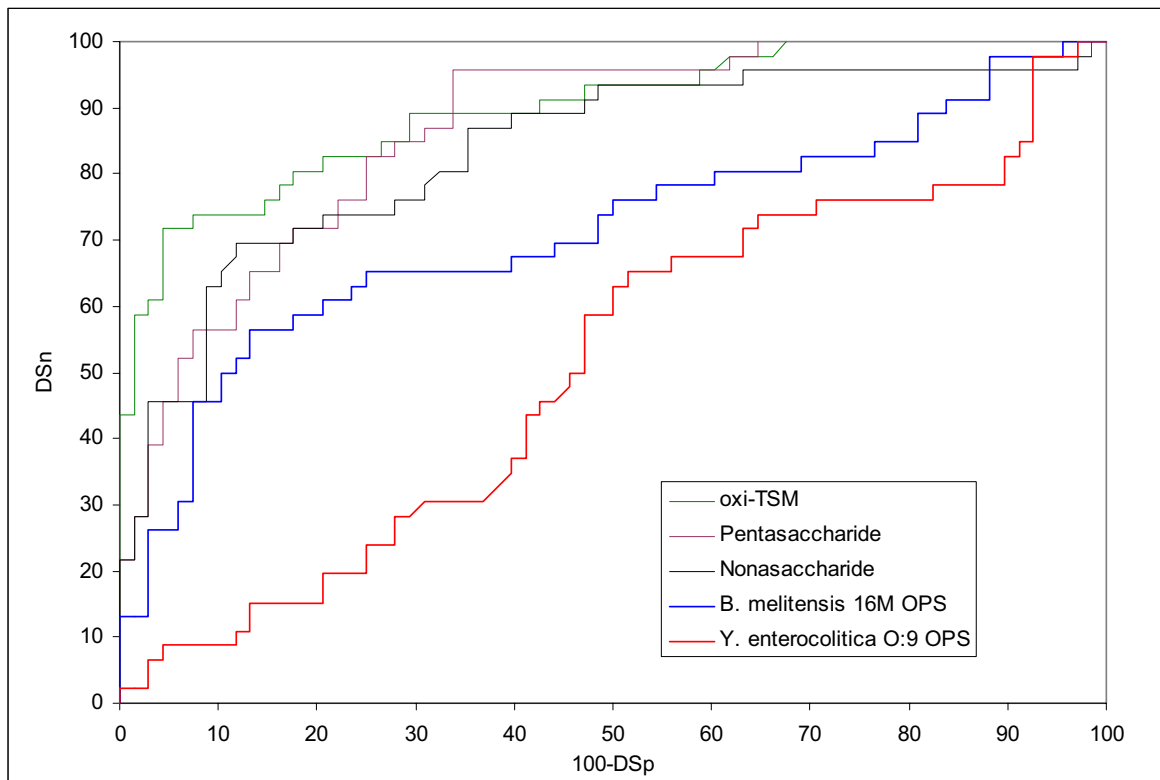


Figure 6.17. ROC curves for native OPS and synthetic antigen iELISAs applied to cattle sera

ROC Curves generated from the results of the *B. melitensis* 16M OPS, *Y. enterocolitica* O:9 OPS, oxidised TSM, BSA-pentasaccharide and BSA-nonasaccharide antigen iELISAs as applied to sera from cattle confirmed as infected with *B. abortus* (n=46) and sera from non-*Brucella* infected cattle that are positive in one or more conventional serodiagnostic assays (n=68).

Assay	Optimal Youden Index (YI=DS _n +DS _p -1)			ROC - Area Under Curve	
	YI Estimate	DS _n %	DS _p %	AUC	95% Confidence Interval
<i>Y. enterocolitica</i> O:9 OPS iELISA	0.1375	65.22	48.53	0.5083	0.3990 - 0.6176
<i>B. melitensis</i> 16M OPS iELISA	0.4328	56.52	86.76	0.7123	0.6096 - 0.8149
Bm16M/YeO:9	0.5415	67.39	86.76	0.8090	0.7264 - 0.8916
Nonasaccharide iELISA	0.5781	69.57	88.24	0.8347	0.7555 - 0.9139
Pentasaccharide iELISA	0.6183	95.65	66.18	0.8686	0.8047 - 0.9325
Tetrasaccharide (TSM) iELISA	0.6733	71.74	95.59	0.8971	0.8379 - 0.9562

Table 6.1. Performance statistics for OPS and Synthetic Oligosaccharide iELISAs as tested on cattle sera

Assay	<i>B. mel</i> 16M	Bm16M/YeO:9	NS	PS	TSM
<i>Y. enterocolitica</i> O:9 OPS iELISA	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
<i>B. melitensis</i> 16M OPS iELISA (<i>B. mel</i> 16M)		0.202	0.0013	0.0005	< 0.0001
Bm16M/YeO:9			0.3015	0.0901	0.0222
Nonasaccharide iELISA (NS)				0.0951	0.0262
Pentasaccharide iELISA (PS)					0.1587

Table 6.2. Comparison of ROC AUC statistics for the OPS and Synthetic Oligosaccharide iELISAs as tested on cattle sera

The optimised YI and the AUC for the oxi-TSM antigen iELISA were superior to any of the other assays and antigens tested. The pattern for the YI and the AUC was the same: TSM > pentasaccharide > nonasaccharide > Bm16M/YeO:9 > *B. melitensis* OPS > *Y. enterocolitica* O:9 OPS iELISAs. The oxi-TSM AUC was significantly greater than all other assays and antigens with the exception of the synthetic pentasaccharide-protein conjugate, where the difference was not statistically significant. Therefore, with this singular exception, the TSM is a significantly superior antigen for the discrimination of FPSR sera and sera derived from truly infected cattle. This is the case even when these cattle are infected with A dominant strains of *Brucella*.

6.8. Chapter 6: Summary and conclusions

The evidence indicates that the presence of the $\alpha(1\rightarrow3)$ link in the TSM antigen and the absence of contiguous $\alpha(1\rightarrow2)$ links is the basis for this superior performance. The next best antigen, the pentasaccharide, contains, in addition to the $\alpha(1\rightarrow2)$ flanked $\alpha(1\rightarrow3)$ linkage, one additional $\alpha(1\rightarrow2)$ link. The contiguous $\alpha(1\rightarrow2)$ links within pentasaccharide may be the reason for the lower, albeit insignificantly, AUC and YI values. The nonasaccharide contains 4 contiguous $\alpha(1\rightarrow2)$ links on one side of the $\alpha(1\rightarrow3)$ link and 3 contiguous $\alpha(1\rightarrow2)$ links on the other, i.e., the α -1,3 link is centrally positioned in the oligosaccharide. This is consequentially likely to be the reason for the relatively (and significantly when compared to the TSM antigen) reduced AUC and YI values for the iELISA with this antigen compared to those with the two other synthetic antigens. It is less apparent why the nonasaccharide iELISA should have a significantly higher AUC value than the *B. melitensis* OPS

iELISA. This may be to do with the regular, or otherwise, order of the two linkage types within the native structure. The multivalent nature of the native OPS antigen may also play a role compared to the monovalent nonasaccharide.

Chapter 7

DISCUSSION

Brucellosis is a zoonosis of global significance. It is endemic in many areas, present in most countries and even in regions that are free of the disease the threat of reintroduction into livestock or wildlife means considerable costs continue to be incurred. These costs include surveillance, monitoring of livestock moving across borders and epidemiological investigations. Diagnosis by clinical observation is impossible and methods for the direct detection of *Brucella* either by culture or identification of specific DNA from material obtained from live animals (with the exception of tissues related to abortions) are currently insufficiently reliable to aid in diagnosis. Furthermore both approaches are considerably more expensive than immunological assays of which the most effective and cheap are serological. Therefore it remains the case that serological techniques underpin the surveillance for brucellosis and resolution of outbreaks as well as facilitating the international trade in livestock.

The serodiagnosis of brucellosis is primarily achieved via the detection of antibodies against the OPS component of the smooth lipopolysaccharide (sLPS) of *Brucella*. This is the immunodominant humoral antigen and serological assays that make use of it, such as all of those described in the OIE Manual for Diagnostic Tests and Vaccines for Terrestrial Animals (hereafter described as the standard serological assays), may have good sensitivity (Nielsen and Ewalt, 2010). The *Brucella* OPS (applied as part of the sLPS or on the surface of whole cells) is, and will remain, an effective serodiagnostic screening antigen due to its dominance of the humoral immune response and the availability of reagents.

Whilst serology is an essential tool it is known that experimental infection with Gram-negative bacteria possessing OPS with sufficient structural similarity to that of *Brucella* can give rise to cross reactive antibodies and generate FPSRs. The effective detection and tracing of future incursions of brucellosis would be significantly enhanced by the ability to eliminate these FPSRs as they incur costly and protracted epidemiological enquires that waste resources and undermine confidence. In genuine outbreaks the transition back to disease freedom would also be assisted by the development of a reliable confirmatory assay. The objective of this project was to develop such an assay so as to significantly and efficiently assist in the resolution of these FPSRs.

Attempts to resolve FPSRs have revolved mainly around efforts to identify and apply diagnostic antigens other than OPS as this dominates the currently available serological portfolio. Whilst many studies have been published extolling the virtues of one antigen or another for the resolution of FPSRs none have made it into assays that have gone beyond the research laboratory into routine

and effective use. It would seem to be the very dominance of the OPS that explains the relative failure of alternative antigens. Given this situation it seemed appropriate to return to this structure, there seeming to be few viable alternatives, and examine whether its full potential is being delivered.

A review of the literature would suggest that there is untapped diagnostic potential within the OPS. Although a linear homopolymer it is not a continuously uniform structure. The combination of evidence presented by the structural studies added to the mAb and monospecific polyclonal binding profiles suggests that it contains, by virtue of the type of glycosidic linkage, a variety of features that are distinctly recognised by antibodies. At least one of these features, the M epitope, exists in nearly all *Brucella* OPS types and has not been identified as part of a molecule in any other organism. Other unique structures, such as the C (specific) epitope may also exist – although there is no structural hypothesis for this epitope.

The existence of these specific epitopes has been known for many years, since the late 1980s at least. What has also been demonstrated, largely by competitive ELISA, is that these epitopes overlap and that antibodies of one specificity may inhibit the binding of antibodies of a different specificity due to steric hindrance. This project aimed to remove this impediment by discovering the exact form of the specific epitopes, to develop and apply them as single epitope types, therefore producing discrete antigens for use in serological assay. The aim was to apply these antigens to true positive and false positive sera to evaluate their ability to resolve FPSRs in cattle.

Much of the information that suggests that untapped diagnostic potential may exist within the OPS is several decades old. Its possible application has therefore been overlooked for a long time. Although time suggests this project hypothesis is not a fruitful topic of research there are some plausible explanations as to why this potential may have been overlooked.

- 1) Within the broad brucellosis community it was obvious to appreciate that the OPS was the origin of the FPSR problem. The structural data relating to the OPS from *Brucella* and *Y. enterocolitica* O:9 demonstrated that they were very nearly identical. From 1984 to 1989 the structures within the public domain for *Y. enterocolitica* O:9 and *Brucella* A type OPS were given as identical. In 1989, the *Brucella* A type OPS was discovered to have a small proportion of α -(1→3) linkages.

- 2) Although epitope mapping of the OPS with mAbs demonstrated that some bound to *Brucella* OPS with high specificity many also bound equally to all OPS types. It was, and still isn't, well understood to what degree the mAb repertoire, specificity and fidelity reflects the properties of a polyclonal antibodies from a population within a natural host that has been naturally infected.
- 3) Should one hypothesise that antibodies with sufficient OPS epitope specificity do exist in naturally infected natural hosts then the ideal means to test this would be with the discrete epitope structures. However, these structures do not appear to exist in isolation in nature and their synthesis is complex and only possible in very specialised laboratories and at considerable cost.
- 4) Antibody absorption methods employed to evaluate the antibody response to specific OPS epitopes are awkward to perform and the outcomes are insufficiently reliable for diagnosis (Kittelberger et al., 1998).
- 5) Were consideration given to testing the properties of a discrete epitope a structure to isolate or synthesise would be required. Based on the mAb binding literature the preferred structure to evaluate would be the 'C specific' epitope as it is unique to *Brucella* and equally distributed across all *Brucella* OPS types. However there is insufficient evidence with which to make a genuinely knowledge based hypothesis as to its structure. It may not exist. Therefore it has not been synthesised and applied.
- 6) The M epitope also appears to be specific to *Brucella* and there is sufficient structural and antibody binding data to enable the generation of a hypothesis as to what the ideal M epitope structure might be. However, the abundance of the M epitope is not evenly distributed across *Brucella* species, biovars and strains. In many highly significant epidemiological biovars the M epitope is present in relatively low quantity. For example: *B. abortus* biovars 1-3, *B. melitensis* biovar 2, *B. suis* biovars 1 and 3. Recently it has been shown that *B. suis* biovar 2 does not possess any M epitopes. A diagnostic assay that can only detect antibodies derived from infection with M dominant strains, or where titre is governed largely by serotype, is of limited use.

- 7) The classical biotyping techniques used to distinguish between M dominant and A dominant *Brucella* strains makes use of sera that has absorbed to become monospecific to these antigens. This absorbed sera agglutinates smooth *Brucella* cells based on the type of the OPS. The value of this method is that the A dominant strains do not agglutinate with the monospecific anti-M sera (and visa versa). This suggests that the low abundance of M epitopes present on A dominant strains is immunologically insignificant.
- 8) The polyclonal antibody response to *Brucella* OPS antigen in naturally infected natural hosts has been, quite assertively, reported to be dominated by the common epitopes (Alonso-Urmeneta et al., 1998), 'Obviously, the practical implications are that the epitopic structure of the LPS used in the iELISA is not relevant in the diagnosis'.
- 9) Antibodies from *Brucella* infected animals can be detected very effectively using the OPS from *Y. enterocolitica* O:9 (Bundle et al., 1989b).

In the face of this unsupportive information, attempting to discover, generate and apply oligosaccharides derived from the *Brucella* OPS may not have outwardly seemed to be a rational pursuit. However, within the detail the evidence is not so dissuasive. For example, the addition of too much monospecific anti-M sera will agglutinate A dominant *Brucella* strains. The observations on the dominance of the common antibodies was based largely on qualitative rather than quantitative data (Alonso-Urmeneta et al., 1998). Even a relatively low M epitope abundance on OPS, of approximately 2%, still translates into a large quantity of exposed antigen in relation to those that may be available on, or within, proteins. Antibody absorbance data from infected cattle supports the theory that, in some cases, antibodies against *Brucella* specific OPS epitopes are present (Kittelberger et al., 1998).

Due to the relatively high level of uncertainty inherent in the project hypothesis a pilot 'proof-of-concept' study was conducted prior to progression into the main objective – the immunoselection and structural identification of *Brucella* O-chain epitopes and their use in the serological diagnosis of porcine and bovine brucellosis. This 'proof-of-concept' study is described in Chapter 3.

7.1. Discussion: Chapter 3

The aim of the work described in chapter 3 was to demonstrate that the OPS derived from *Brucella* A dominant and M dominant strains and from *Y. enterocolitica* O:9 display different levels of reactivity against polyclonal antibodies within bovine sera that are truly or falsely positive by standard serology.

The successful development of the OPS iELISA using the Carbo-BIND™ plates allowed for the specific quantitative evaluation of the interaction of the OPS antigens with the different antibody populations tested. Although *Brucella* OPS iELISAs have been described previously (Nielsen et al., 1989) they have been based on the passive absorption of OPS to polystyrene plates. Given the highly hydrophilic nature of the OPS this process is of low efficiency and results in comparatively low titres and reproducibility. The method of covalent attachment of OPS to ELISA plates developed in this study provided a robust method for the highly sensitive determination of the binding of antibodies to the OPS.

The findings from the OPS iELISA results from both the cattle and pig sera demonstrated the affect of the different OPS structures, from both A and M *Brucella* types and also *Y. enterocolitica* O:9 on antibody binding. If the differences in OPS structures were of no consequence to antibody binding then there would be no difference in the degree to which the antibodies from the different serum populations, true or false positives, did indeed bind these different antigens. Although the serum populations would vary in the magnitude of their antibody binding response from each other, commensurate with their individual titres, this difference would remain the same irrespective of the OPS antigen used. This was not the case. The data clearly demonstrated, with a high degree of statistical confidence, a difference in antibody binding. Therefore one must conclude that the OPS antigens are not recognised in the same manner by the different antibody populations. The data from the rLPS iELISAs helped to support the hypothesis by demonstrating that any effects that extraneous core oligosaccharides may have exerted did not negatively influence the data.

The difference between the OPS from *Brucella* and *Y. enterocolitica* O:9 was manifested by a relatively greater attraction for antibodies from within true positive samples to bind to *Brucella* OPS compared to those within false positive samples. This was demonstrated using pig sera (from animals infected with *B. suis* biovar 1) with *Brucella* A type OPS and may be due to the presence of *Brucella* specific epitopes not found in the OPS of *Y. enterocolitica* O:9 such as the 'C' and 'M'

epitope. Given the relatively low abundance of the M epitope on the OPS of the infective *Brucella* strains in this case, the difference might be assumed to be largely due to the presence of the 'C' epitope.

However, what the data from the pig sera (from animals infected with *B. suis* biovar 1) also demonstrated that the M type OPS iELISA was even more different from that of the *Y. enterocolitica* O:9 OPS iELISA than that derived from the A type OPS. Furthermore, in the case of the pig sera (where the two *Brucella* type OPS antigens were compared) there was also a highly significant difference between the results from the *Brucella* A and M OPS types. Given that the only structural difference between these two antigens is the frequency of the M epitopes and the presence of the A epitope the observations suggest a significant role for these epitopes in the binding of polyclonal sera. Even extraneous antigenic material such as core sugars or the OPS primer sequences should be the same for both A and M types. This reinforces the conclusion that the presence of the M and the absence of the C/Y antigen is the significant basis for the observations.

There was no significant difference between the relative OPS iELISA results from sera from pigs infected with *B. suis* biovar 2 (which does not possess M epitopes) and pig FPSR samples. This absence supports the theory that the differences in antibody binding profiles are due to the fine structural elements within the OPS that differentiate the epitopes rather than alternative and undefined properties within the antibodies that may be induced by infection with *Brucella* or other, cross-reactive, Gram-negative bacteria.

As the more significant differences were observed when testing the pig sera (from animals infected with *B. suis* biovar 1) using the *Brucella* M type OPS this was used in preference to the A type to evaluate the cattle sera. There were highly significant differences in the binding patterns of the antibodies within the sera of true positive and false positive sera to the difference OPS types. In addition, sera from cattle experimentally infected with either *Y. enterocolitica* O:9 or *B. abortus* biovar 1 (A dominant) were tested with the *Brucella* M type OPS and OPS from *Y. enterocolitica* O:9. Once again a highly significant difference in the antibody binding profile of the two serum populations was observed.

It is worth emphasising that the discovery of the significance of the M epitope and its potentially important role in diagnosis has been demonstrated using sera from cattle infected with A dominant strains of *Brucella*. It has been known for many years that the M epitope is likely to be present, on

average only once or twice per type A type OPS molecule (Meikle et al., 1989). Although low in comparison the frequency of the A epitope, relative to many other structures on the surface of the *Brucella* cell, the M epitope is comparatively abundant. The recent publication revisiting the structure of the *Brucella* OPS (Kubler-Kielb and Vinogradov) found that the M epitope, if present on the OPS, is always at the terminal end and will therefore be most exposed to the extracellular environment and host immune system. This may help to explain why even sera from animals infected with A dominant strains of *Brucella* react in the manner described above. This data refutes some of the preconceptions, described earlier in the discussion, relating to the value of the M epitope structure to the universal diagnosis of brucellosis. An important caveat to this is the case of *B. suis* biovar 2 because, as mentioned above, this has no C specific or M epitopes.

The OPS iELISA methods developed and the results generated met the first specific aim of the project: to demonstrate that the OPS derived from A and from M dominant strains of *Brucella* and from *Y. enterocolitica* O:9 display different levels of reactivity against polyclonal antibodies within bovine sera that are true positives compared to those that are false positives by standard serology. Although fulfilling this aim, the native OPS iELISAs could only determine differences between serum types at the population level. At the level of the individual sample, which is what is needed for effective diagnostic assays in this case, these native OPS iELISAs lacked sufficient diagnostic specificity.

The ability of specific structures in the OPS to generate specific monoclonal antibodies has been known for many years but this is the first time that these structural differences have been shown to evoke polyclonal antibody populations of differing specificity in naturally infected hosts or in any livestock species. The existence of these antibodies, one of the main hypotheses of this project, suggests that if the specific antigen(s) against which they are reacting can be isolated for diagnostic use then a more effective diagnostic assay to resolve FPSRs in cattle may be developed. These methods and the results presented are novel and in contradiction of the current paradigm that for FPSRs the OPS antigen only represents the problem and has no part in the solution.

7.2. Discussion: Chapter 4

The aim of the work described in chapter 4 was to demonstrably generate oligosaccharides of comparable length and structure to those proposed to form discrete antibody epitopes from native *Brucella* and *Y. enterocolitica* O:9 OPS antigens.

Meeting this aim presented many technical challenges due to the specific nature of the OPS under study. The key elements in the successful generation and analysis of the D-Rha4NFO homo-oligosaccharides were: the development of methods of controlled hydrolysis by hot concentrated HCL, the separation of intact D-Rha4NFO homo-oligosaccharides by the combined use of size exclusion and ion exchange chromatography and the application of graphitised carbon column HPLC online with ESI-MS/MS.

Owing to the structure of the OPS it is particularly difficult to hydrolyse without loss of the N-formyl groups that are critical to antibody binding (Biermann, 1989; Isshiki et al., 1995). The most effective method to adopt for this is solvolysis by hydrogen fluoride (Knirel et al., 1989) but the use of this chemical requires highly specialist equipment in order to be done safely and this facility was not available. Despite the challenges the partial hydrolysis of intact oligosaccharides from *Brucella* A and M type OPS and from the OPS of *Y. enterocolitica* O:9 was achieved. This was done using strong hydrochloric acid and following methods adapted from the hydrolysis of *V. cholerae* (Redmond, 1979) and chitin (Einbu and Vårum, 2006). The delicate balance of conditions required to achieve optimal partial hydrolysis was determined by the evaluation of the complex resultant polymer mix through the use of MALDI-ToF and modified FACE.

The use of a dual size exclusion chromatography and ion exchange HPLC column coupled to ESI-MS made possible the identification and separation of the desired short intact oligosaccharides due to their smaller size and uncharged state (having retained their N-Formyl groups). Despite the complex and convoluted data sets derived from the ESI-MS the presence of OPS ions could be verified by virtue of the regular repeating series of peaks observed. The degree of convolution was minimised by the introduction of NaI to the running buffer in order to promote the dominance of sodiated ion species. Once the larger OPS polymers were separated off from the smaller intact oligosaccharides the mass spectra became less complex, highly sensitive and generated a very clear picture of the oligosaccharide composition. Fragmentation MS/MS confirmed the structural identity of key ions derived from the D-Rha4NFO homopolymers - albeit limited by the inability to

resolve glycosidic linkage type. Evaluation of linkage was attempted via cross-ring fragmentation at high collision energy levels within a quadrupole chamber of the ESI-QToF but this did not yield the necessary level of detail.

Additional information relating to the structure of the oligosaccharides was derived from the application of the graphitised carbon HPLC column with online ESI-MS. The separation of oligosaccharides within such columns occurs not only by size but also by conformation by an as yet undefined mechanism (Ruhaak et al., 2009). This method of separation provided some surprising but very useful ion specific chromatograms. This was amply demonstrated by the chromatogram for the D-Rha4NFo tetrasaccharides from the A dominant *B. abortus* S99, the M dominant *B. melitensis* 16M and the exclusively $\alpha(1\rightarrow2)$ linked *Y. enterocolitica* O:9. These chromatograms show a peak that is specific for *Brucella* and more abundant in *B. melitensis* 16M than in *B. abortus* S99. Given that the only known difference between the structures of the selected precursor molecules is due to the occurrence of the $\alpha(1\rightarrow3)$ linkage it was hypothesised that this feature was responsible for the peak unique to *Brucella*. The presence of multiple elution peaks for the same ion may be due to the formation of oligosaccharide dimers. It was striking, and unexpected, that the most simple tetrasaccharide structure, that exclusively $\alpha(1\rightarrow2)$ linked, produced such a complex elution spectrum.

The oligosaccharides derived from *B. abortus* biovar 1 (OPS type A) and analysed within the study described in chapter 4 were used in the next phase of the project, antibody affinity chromatography, with the GC-HPLC-ESI-MS method used to monitor the outcome.

7.3. Discussion: Chapter 5

The aim of the study described in chapter five was to evaluate the epitopes within the *Brucella* type A OPS derived oligosaccharides by affinity chromatography using polyclonal and monoclonal antibodies and HPLC-ES-MS in order to investigate the nature of their binding.

The polyclonal sera used for the affinity chromatography of the *Brucella* type A OPS derived oligosaccharides was derived from a cow experimentally infected with *B. abortus* stain 544, a biovar 1 strain that has A dominant OPS. Prior to the use of the sera for selection, it was purified by affinity chromatography against *Brucella* and *Y. enterocolitica* O:9 OPS. As anticipated the purification of

the anti-OPS antibodies from the non-specific serum components was largely successful. It was hypothesised that the affinity purification may also enable the production of separate populations of polyclonal sera with differing response profiles to the different OPS types. In particular a population of antibodies specific to the 'C' epitope was sought. To some extent this was achieved by a process of positive and negative selection through columns conjugated with *Brucella* A type OPS and with OPS from *Y. enterocolitica* O:9. Two populations of anti-OPS polyclonal sera – derived from the same source – were prepared. These had different reactivity profiles against the different OPS types, however a notable degree of cross reactivity between the OPS types remained. Nonetheless these two antibody populations were conjugated to affinity chromatography columns for the selection of the D-Rha4NFO oligosaccharide homopolymers generated as described in chapter 4. In addition an M specific mAb was also conjugated to an affinity chromatography column.

The data relating to the retention of the D-Rha4NFO oligosaccharide homopolymers demonstrated that antibody based binding had been successful as elution from the columns yielded oligosaccharides that had clearly been retained throughout the washing process. The longer oligosaccharides and polysaccharides were more retained within the columns as demonstrated by their comparatively increased yield under more stringent elution conditions. This information helped to verify that the affinity chromatography method was working as may be expected based upon the presence of multiple epitopes and bivalent antibodies promoting more effective binding.

However the results relating to the shorter oligosaccharides were of more interest. The shorter the better as such oligosaccharides will not allow multiple attachment of antibody binding sites and are more likely to present only one type of epitope. Both the columns to which polyclonal antibodies were conjugated effectively retained, until elution, specific oligosaccharides. Yet, despite the differences in the binding preferences of the antibody populations there were no observable differences in oligosaccharide composition or in their elution profiles from the graphitised carbon columns. These profiles did not look much different from the profile of the oligosaccharide sample prior to affinity chromatography. It is probable that the binding and elution methods employed were insufficiently subtle to tease out any differences in binding profile that may have been present in the antibody populations. What the data did show however is that the polyclonal antibodies very effectively bound small oligosaccharides. This included tetrasaccharides, the elution profiles for which included the peak found only in the material derived from the *Brucella* OPS. This is significant as it means that short oligosaccharides may be recognised by other polyclonal sera from livestock and thus have at least some potential to make useful diagnostic antigens.

The information obtained after affinity chromatography against the anti-M epitope mAb was very different. This was best exemplified by the elution profile from the graphitised carbon column of the tetrasaccharide. Whereas the profiles arising from the material eluted from the polyclonal antibodies did not look much different from the profile of the sample prior to affinity chromatography, the profile after binding to the anti-M epitope mAb was very different. This profile had a high resemblance to the profile derived from the (non-affinity selected) D-Rha4NFO oligosaccharide homopolymers derived from the M type *Brucella* OPS. Given the known specificity of this mAb the derived elution profile strongly suggests that the peak observed previously that is unique to the *Brucella* derived oligosaccharides is indeed related to the presence of an M epitope. Furthermore, the evidence from the elution profiles from the polyclonal sera demonstrates that they bind this M epitope tetrasaccharide.

The data from chapter 5 provided strong evidence for the a significant role for the M epitope and $\alpha(1\rightarrow3)$ linkage in *Brucella* specific antibody interactions. In contrast there was a lack of evidence for the role of a 'C specific' epitope (and still no clear evidence for what the structure for this epitope - if it exists - may be). On this basis the project continued by pursuing the development of a diagnostic tetrasaccharide antigen based on elements that comprise the M epitope.

7.4. Discussion: Chapter 6

The aim of the study described in chapter 6 was to develop an indirect ELISA using the oligosaccharide structures indicated by the preceding study in order to generate a serodiagnostic assay that may be used to assist in resolution between sera that are true or false positives as determined by standard serology. Then to evaluate the diagnostic efficacy of the developed iELISA using a panel of cattle sera containing those that are either true or false positive by standard serology.

The evidence gathered in the studies described in the preceding chapters indicated a potential role for a discrete M epitope antigen in the more specific serodiagnosis of brucellosis. In particular a tetrasaccharide antigen form. On the basis of this evidence the production of a synthetic D-Rha4NFO tetrasaccharide 'M' (TSM) antigen was commissioned. This had a central $\alpha(1\rightarrow3)$ linked flanked on either side by $\alpha(1\rightarrow2)$ links. Although this exact form was not suggested by the

experimental data, it was selected based on a hypothesis that it was the tetrasaccharide that, whilst presenting a complete M epitope, presented minimal A like epitopes (i.e. no contiguous $\alpha(1\rightarrow2)$ links).

The TSM antigen bound effectively to the highly M specific BM40 mAb and also bound to polyspecific anti-M sera as was demonstrated by cELISA. Attempts to use the TSM antigen in iELISA, a more analytically sensitive assay format, were impeded by the highly hydrophilic nature of the structure. This eliminated passive binding as an option for the coating of ELISA plates. To facilitate evaluation of the TSM antigen by iELISA, the structure had to be compromised by a process of oxidation in order to generate the aldehyde groups that would bind the surface of Carbo-BIND™ ELISA plates. This procedure was similar to that performed on the native OPS (as applied in chapter 3) but was conducted under much more controlled conditions due to the relative lack of epitope redundancy between the native OPS and the synthetic TSM antigen. Having achieved the desired oxidation, the TSM was conjugated to ELISA plates and a serodiagnostic assay developed. This assay was applied to the cattle sera classified as true or false positive to standard serodiagnostic assays. In addition to the oxidised TSM antigen, pentasaccharide and nonosaccharide D-Rha4NFo antigens (produced by Professor D. Bundle), each containing a single $\alpha(1\rightarrow3)$ amongst multiple $\alpha(1\rightarrow2)$ links, were also evaluated against the serum panel. These antigens required no additional modification as they were linked to bovine serum albumin. This acted as a convenient and benign carrier with which to attach the antigens to standard ELISA plates by passive absorption.

The diagnostic efficacy of each of the antigens was quantified using ROC analysis to quantify the AUC and also the YI. The results showed that the oxidised TSM antigen was the best performer. This evidence indicates that the presence of the $\alpha(1\rightarrow3)$ link in the TSM antigen and the absence of contiguous $\alpha(1\rightarrow2)$ links is the basis for this superior performance. The next best antigen, the pentasaccharide, contained, in addition to the $\alpha(1\rightarrow2)$ flanked $\alpha(1\rightarrow3)$ linkage, one additional $\alpha(1\rightarrow2)$ link. The contiguous $\alpha(1\rightarrow2)$ links within the pentasaccharide may be the reason for the lower, albeit insignificantly, AUC and YI values compared to the TSM antigen. The nonasaccharide contained 4 contiguous $\alpha(1\rightarrow2)$ links on one side of the $\alpha(1\rightarrow3)$ link and 3 contiguous $\alpha(1\rightarrow2)$ links on the other, i.e., the $\alpha(1\rightarrow3)$ link is centrally positioned in the oligosaccharide. This is consequentially likely to be the reason for the relatively (and significantly when compared to the TSM antigen) reduced AUC and YI values for the iELISA with this antigen compared to those with the two other synthetic antigens. It is less apparent why the nonasaccharide iELISA should have a

significantly higher AUC value than the *B. melitensis* OPS iELISA. This may be to do with the regular, or otherwise, order of the two linkage types within the native structure. The multivalent nature of the native OPS antigen may also play a role compared to the monovalent nonasaccharide.

7.5. Discussion: Summary

The study presented has been successful but is not complete. The aim of the study was to identify, select and apply discrete single epitope antigens from the OPS of *Brucella* and apply these to the serodiagnosis of brucellosis. It was hypothesised that epitopes specific to the *Brucella* antigen, if harnessed correctly, could be used to improve the specificity of serodiagnosis and assist in the resolution of false positive serological reactors. The data collected in this study has proved the underlying theory behind this hypothesis to be correct. An iELISA based on a discrete M epitope antigen has significantly ($P < 0.0001$) improved diagnostic capability to resolve FPSR samples from those from truly infected animals. The less 'M like' the antigen becomes, the less specific it becomes. But what does this mean in practice? Although the oxidised TSM antigen was demonstrably more effective at discriminating between true and false positives, the low sensitivity is a point for concern. From a practical perspective the risk of declaring false negatives may be at a level that is unacceptably high. Nevertheless, there is room for optimism. The presented study is not complete. The most obvious gap to fill is the evaluation of a complete and appropriately presented TSM antigen. This, and other issues and opportunities will be discussed in the next section.

The results presented in this thesis take several steps forward with regards to the development of an effective confirmatory serodiagnostic assay for bovine brucellosis and points the way towards further improvements. The significance of the ultimate development of an effective confirmatory serodiagnostic assay is the improved ability to determine brucellosis free status. This would be achieved in a manner that has little additional logistical and financial burden for the additional assay would be completed within a few hours, using simple ELISA methods, on the serum sample already available. This will lead to a reduction in costly epidemiological investigations, restrictions on animal movements, unnecessary animal slaughter and compensation. The successful development of such assay based upon the OPS antigens described here would also have implications on the design and use of vaccines for the control of brucellosis, a topic that will be returned to in the following sections.

Chapter 8

FUTURE DIRECTIONS

The evaluation of the synthetic oligosaccharide antigen iELISAs on the cattle sera demonstrated the improvement of resolving capability compared to the use of the native OPS. However, the partitioning of the two serum populations, FPSR samples and those from *Brucella* infected animals, is not perfect. Whilst it may be ambitious to hope that this will ever truly be the case there is obvious potential for improvement. A major gap in the study is that a D-Rha4NFo tetrasaccharide with a central $\alpha(1\rightarrow3)$ link flanked by $\alpha(1\rightarrow2)$ links (TSM antigen) was never truly tested in indirect iELISA format against polyclonal sera from the target hosts. The oxidised version of this antigen that was applied in iELISA, whilst demonstrating at least some retained specific M antibody binding capability, was not the complete article. Some of the sera from the truly infected cattle did not show elevated titres against this antigen despite possessing anti-*B. melitensis* 16M OPS antibodies. In contrast to this, the data from the pentasaccharide D-Rha4NFo, essentially the TSM antigen with an additional $\alpha(1\rightarrow2)$ link at the reducing end, gave rise to elevated titres from all samples from *Brucella* infected cattle, but also from most of the FPSR samples. It is reasonable to predict that the use of an intact TSM antigen will generate data that lies somewhere between these two distributions, potentially extracting the best features of each – the specificity of the oxi-TSM antigen and the sensitivity of the pentasaccharide.

A BSA conjugated TSM antigen is currently being synthesised by Professor Bundle and his team. The plan is for this antigen to be sent the Brucellosis Reference laboratory at the Animal Health and Veterinary Laboratories Agency for evaluation against the same panel of cattle sera as was tested by the other synthetic oligosaccharides. It may have been noted that neither the sera from the experimentally infected cattle nor the swine sera has been tested using the oligosaccharide antigens. Little volume of these samples remain and they are a precious commodity. For this reason, they are being saved for application to the BSA conjugated TSM antigen when this arrives, not only for iELISA but for other potential permutations. These are described below.

The nature of the TSM antigen presents further interesting serological opportunities. The proven interaction between the BM40 mAb and the TSM will enable the development of a cELISA akin to the standard cELISA that utilises a solid phase sLPS antigen derived from *B. melitensis* 16M. As shown in chapter 1, the standard cELISA is still prone to FPSRs. This would appear to be due to the co-existence and overlapping nature of the common (C/Y) epitopes on this antigen. By removing these through the use of the TSM antigen, the specificity of the cELISA may also be improved in a manner analogous to the improvement of the iELISA. The element of antibody affinity selection that is inherent in the cELISA owing to the integral competition for antigen binding may

further enhance the specificity. Antibodies with high affinity for C/Y epitopes may effectively out compete the BM40 mAb for antigen binding to *B. melitensis* 16M OPS owing to steric hindrance. Such antibodies, as may be raised by infection with *Y. enterocolitica* O:9, will have a reduced affinity to the TSM antigen and therefore be less able to bind and less able to compete. There is an ongoing supported research programme within my laboratory at the Animal Health and Veterinary Laboratories Agency that encompasses this project and, as it has proven to be a promising line of enquiry, this future work on the TSM antigen will be completed.

The development and evaluation of a TSM cELISA is a high priority. Less of a priority but interesting nonetheless is the development of homogeneous serodiagnostic assays using Fluorescent Polarisation (FPA) (Nielsen and Gall, 2001) and Time-Resolved Fluorescent Resonance Energy Transfer (TR-FRET) assays (McGiven et al., 2009). The small size of the TSM antigen, even when conjugated to fluorescein, make it almost perfect for FPA. When unbound by antibody it will spin rapidly and light emitted will be highly depolarised (more so than with the conventional native *Brucella* OPS antigens used currently). When bound by antibody the rate of spin will slow and the depolarisation of light will be much reduced. The relationship between size, spin and the depolarisation of light favour the use of small antigens for antibody detection (Nasir and Jolley, 1999). The same fluorescein conjugated TSM antigen would also be a perfect partner in TR-FRET assay with lanthanide conjugated BM40 mAb. Both of these rapid homogenous assays could offer interesting options for high-throughput serological testing.

The additional serological and TSM cELISA results will complete the laboratory project (the FPA and TR-FRET variants offer less fundamental knowledge but are an interesting applied option). Application of the oligosaccharide antigen iELISAs to sera from uninfected sheep infected with *B. melitensis* biovar 3 (mixed A and M type OPS) demonstrated their diagnostic effectiveness beyond use in cattle sera. Despite the increased frequency of M epitopes on the OPS of the aetiological agent, there was no notable generalised increase in the response to the pentasacchride and TSM antigens compared to the sera derived from the cattle infected with A dominant *B. abortus*. In the absence of any data that was truly supportive of the existence of the 'C specific' epitope, the TSM antigen appears to be the optimal unique epitope from within the *Brucella* OPS. It may be that the 'C specific' epitope contains elements of the non-reducing portion of the OPS, or that it overlaps with the core. Such sugar sequences could indeed be unique to *Brucella*. However they remain sequestered beneath the OPS which is in stark contrast to the information recently published that

suggests the M epitope is presented at the tip of the OPS – even in the OPS from A dominant strains. *B. suis* biovar 2 being exceptional. Or is it?

The efficacy of proposed TSM antigen assay is based on the assumption that all *Brucella* OPS contains M antigens - $\alpha(1\rightarrow3)$ links. This has now been shown not to be the case for at least two strains: the type strain of *B. suis* biovar 2 (Zaccheus et al., 2013) and the strain designated *B. inopinata* BO2 (Wattam et al., 2012). Whilst the OPS of *B. inopinata* is undeniably interesting, the strain itself is a distant member of the *Brucella* genus and of little epidemiological significance. This is not the case for *B. suis* biovar 2. It is the most common aetiological agent of swine brucellosis in Europe. Given their existence it would be presumptuous to assume, without further research, that these are the only members of the genus to lack the $\alpha(1\rightarrow3)$ linkage.

Several OIE Reference Laboratories for Brucellosis, including the AHVLA (UK), have large and well curated strain collections. Evaluation of a significant number of strains for evidence of the M epitope would be a worthwhile, although not inconsiderable, objective within this context. This might be most efficiently expedited by immunodetection with anti-M specific mAbs such as BM40. Developing a DNA based assay would be most useful although given the imprecise knowledge of the origin of the M epitope this would be challenging. The similarity of the gene sequences, and promoters, that are believed to be involved in the genesis of the different linkage types within the OPS illustrates the challenge that further research into this subject faces. Evaluating the transcription of some of these genes may provide clues. Despite the challenges, if successful, the knowledge gained may provide some valuable insights into mechanisms of virulence and prove useful in the generation of future vaccine strains.

The development of the TSM assay may have solved the following paradox. The incorporation of OPS is necessary for the most efficacious *Brucella* vaccines but its incorporation results in the production of anti-OPS antibodies that react in serology and confound diagnosis. The OPS thus simultaneously presents both the solution (sufficient protective efficacy) and the problem (inability to distinguish infected from vaccinated individuals). If a vaccine were to contain OPS with only $\alpha(1\rightarrow2)$ linkages, then protection may be facilitated via the common C/Y epitopes and the ability to differentiate between infected and vaccinated animals could be achieved by the application of a TSM antigen in serodiagnosis. In essence, the vaccine would induce anti-OPS antibodies similar (if not identical) in specificity to those generated by infection with *Y. enterocolitica* O:9.

As has been shown, oligosaccharide discrete M antigens have significantly enhanced ability to discriminate between samples presenting either false positive or true positive results in conventional and contemporary serology. A potential vaccine of this type may be developed from the bottom up as a protein glycoconjugate (Moxon et al., 2011) similar in principle to Prevnar and Synflorix pneumococcal conjugate vaccines and MenAfriVac, a tetanus toxoid glycoconjugate vaccine that protects against meningitis. Or from the top down, starting with a whole cell antigen. *B. suis* biovar 2 would be a good place to start looking. It infects but does not cause disease in ruminants and is self limiting. It appears to be non-pathogenic for humans. The OPS contains no $\alpha(1\rightarrow3)$ linkages. Modification of the LPS (Conde-Álvarez et al., 2013) core may create sufficient attenuation to prevent it from causing disease in swine. The attenuation and structural changes may also prevent the modified strain from raising anti-rLPS antibodies enabling the continuance of the rLPS iELISA to be an effective confirmatory assay for brucellosis in swine (McGiven et al., 2012).

Chapter 9

OVERVIEW AND CONCLUDING REMARKS

The results from this project provide additional evidence of the important role of glycans in disease. Within the context of this study that role is in the induction and detection of the humoral immune response. That it was felt necessary to return to the OPS for a solution to the problem of false positive serological reactions in the serodiagnosis of brucellosis is testimony to the dominance of the immune response by this highly significant molecule. That this is the first return to synthetic oligosaccharides whose form is derived from the structure of the *Brucella* OPS since the late 1990s (Bundle et al., 1989a) demonstrates not only the novelty of this approach but also the challenges. In an era of high throughput genomics and proteomics the carbohydrate is proving to be substantially less amenable to such automated approaches.

However, as demonstrated by this study, carbohydrates cannot be ignored and their study may be highly rewarding. Although less high throughput, techniques such as HPLC-MS/MS are incredibly powerful analytical tools that facilitate the resolution of the complex structures of glycans. Their structural permutations, especially in bacteria, is many times greater than those of DNA, RNA and proteins. Their synthesis is frequently complex and achieved via a web of protein driven processes. Furthermore, even apparently innocuous and minor structural differences, such as the difference between an $\alpha(1\rightarrow2)$ and an $\alpha(1\rightarrow3)$ link, may have significant ramifications.

Tetrasaccharide antigens have been successfully applied in ELISA for the detection of specific antibodies before (Kuehn et al., 2009) and pentasaccharide vaccines shown to induce protection against bacterial infection (Gening et al., 2010). Yet many diagnosticians would be surprised that such a small simple and seemingly innocuous structure as the TSM antigen could make a difference to the detection and diagnosis of disease and, potentially, create a new pathway towards the development of improved vaccines for brucellosis.

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