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MOLECULAR VARIATION OF BOVINE ROTAVIRUS

by

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ABSTRACT

MOLECULAR VARIATION OF BOVINE ROTAVIRUS

The thesis starts with a general overview of the microbiological causes of neonatal enteritis of calves with relevant cross-reference to other animal species. This is followed by a detailed description of the pathology, immunology and molecular biology of rotavirus.

The experimental results describe the evaluation of various methods for analysing the genome profiles of rotavirus, with due regard to sensitivity and ease of operation. The optimum method was then used to determine, over a 1-2 year period, the extent of variation in genome profiles of rotaviruses excreted asymptomatically by calves on 3 farms. Rotavirus strains showing different genome profiles, from 1 farm, were isolated and cloned in cell culture. Further analyses conducted on these isolates included antigenic variation, comparison of the virus induced intracellular polypeptide profiles and, with some isolates, the ability to cause disease in experimentally infected calves. One isolate was shown to have an atypical genome profile, containing a genome segment of increased size. To determine the possible origin of the anomolous segment, viral mRNA species, from *in vitro* transcription using viral cores, were translated in an *in vitro* cell-free system and the products analysed. Another isolate was shown, experimentally, to infect calves without causing disease. This isolate and a rotavirus known to cause disease were studied in parallel to observe any biological features relevant to virulence. This pair of viruses were also used to produce reassortant viruses by co-infection of cell cultures. These reassortants were characterised genotypically and phenotypically to identify the role of individual viral genes in virulence variation of bovine rotaviruses.

1.0 INTRODUCTION

1.1 NEONATAL CALF GASTROENTERITIS

Gastroenteritis and respiratory disease are the principle causes of morbidity and mortality in the neonate. The most frequent clinical feature of enteric diseases is diarrhoea often leading to dehydration and acidosis, which if not treated, may result in death. Although recovery from these diseases is common, impaired growth may occur. Gastroenteritis can be caused by a variety of microbial agents including bacteria, protozoa and viruses.

1.1.1 Calf Enteritis - Causative Agents

1.1.1.1 Bacteria

The normal flora of the calf intestine contains numerous strains of *Escherichia coli* (*E. coli*) of which some strains have been shown to cause enteritis. The pathogenic strains differ from the strains in the normal flora in their possession of virulence factors which include attachment proteins, necessary for the organism to colonise the gut, and toxins which cause the clinical disease (Moon, 1974). The pathogenic *E. coli* strains can be separated into two groups, the enterotoxigenic strains (ETEC) and the enteropathogenic strains (EPEC). ETEC strains produce one of the two types of enterotoxin, heat labile or heat stable toxin, which stimulate the intestinal crypt cells to secrete fluids and electrolytes resulting in a watery diarrhoea complicated by severe dehydration (Moon, 1978). A large proportion of the ETEC strains also produce a pilus antigen, K99, which along with the toxins are plasmid encoded. K99 acts as an adhesin allowing the bacteria to colonise the gut by reacting with specific enterocyte receptors. The EPEC strains also produce an adhesin and a toxin, but in this case the toxin is a cytotoxin which destroys the epithelial cells which are shed causing villous atrophy and a malabsorption syndrome.

Although ETEC and EPEC are the most common cause of bacterial enteritis in calves other bacteria including *Salmonella sp.* (salmonellosis) and *Mycobacterium sp.* (Johne's disease) are also found. These bacteria are enteroinvasive and cause chronic haemorrhagic diarrhoea.

1.1.1.2 Protozoa

Cryptosporidium was first detected in calves by Panciera *et al.* (1971) and subsequently described in association with calf enteritis by Pohlenz *et al.*, (1977). Initially it was difficult to assess the significance of *Cryptosporidium* as an enteric pathogen because these protozoa were often isolated with other known enteropathogenic agents (Snodgrass *et al.*, 1980a). However Tzipori *et al.*, (1980a) isolated *Cryptosporidium* as the sole agent in an outbreak of calf diarrhoea and successfully produced disease in an experimentally infected calf.

1.1.1.3 Viruses

The first association of viruses with calf enteritis was described by Mebus *et al.*, (1969) when the group reproduced disease in calves experimentally infected with a faecal filtrate from an outbreak of calf enteritis. The viral agent was subsequently found to be rotavirus. Shortly after this Stair *et al.*, (1972) reported a second virus, bovine coronavirus, identified by electron microscopic examination of diarrhoeic faeces. Both these viruses were subsequently recognised in the UK (Woode *et al.*, 1974; Bridger *et al.*, 1978). Other viruses have also been implicated and these include a calici-like virus and an astrovirus (Woode and Bridger, 1978), Breda virus (Woode *et al.*, 1982), parvovirus (Huck *et al.*, 1975) and other small round, as yet unidentified, viruses.

Rotavirus and coronavirus infect the enterocytes at the tips of the villi in the small intestine resulting in villous stunting (Mebus *et al.*, 1971b, 1973). Infection of the epithelium destroys the cells which synthesise disaccharidases, maltase, sucrase and lactase (Bishop *et al.*, 1973). Lack of these enzymes causes lactose

and other disaccharides to accumulate in the gut. As only monosaccharides are absorbed by the healthy gut, malabsorption occurs. The accumulation of disaccharides in the gut causes an osmotic drain, absorbing fluids from the body into the gut lumen leading to dehydration (Flewett, 1977). Absorption of D-xylose is impaired in this way and has been used as an index of upper small intestine function (Mavromichalis *et al.*, 1977). This malabsorption syndrome also leads to a reduction of glucose-coupled sodium transport leading to an imbalance of sodium/potassium levels in the blood (Davidson *et al.*, 1977). After the loss of the infected epithelial cells, the villi are refoliated by cells from the crypts which are refractile to infection by these viruses and hence recovery is common. Parvovirus, however, infects the cells of the crypt so when the villous epithelium is shed, as part of the normal cellular turnover, there are no cells migrating from the crypts to replace them, resulting in villus collapse. The clinical feature of parvovirus infection is a sudden onset of diarrhoea with death ensuing shortly afterwards (Moon, 1978).

The severity of the disease caused by these viruses largely depends on the site and extent of infection. The primary site of rotavirus infection was found to be the jejunum and ileum (Mebus and Newman, 1977), and for coronavirus, the same areas of the small intestine and also the colon (Mebus *et al.*, 1973). Because the colon absorbs fluids from the gut lumen, damage to this function by coronavirus infection results in a watery diarrhoea, whereas the diarrhoea produced by a rotavirus infection is "pasty" or semi-solid. Astrovirus mainly infects the duodenum and on its own causes only a mild diarrhoea (Woode and Bridger, 1978). The reason for this is thought to be that any loss of absorption in the proximal small intestine is compensated for by the colon. However, multiple infections with viruses are common, resulting in a more severe disease due to the extensive infection along the length of the intestine. Moon (1978) also suggested that there may be an association between the extent of virus infection of the epithelial cells lining the sides and tips of the villi and the severity of the resulting diarrhoea. It was proposed that rotavirus, which mainly infects the apical half of the villi, produces a milder disease than

coronavirus which infects all the villous epithelial cells. This may be an over simplification because the extent of villus infection may depend on the virus strains used.

Although rotaviruses are frequently associated with acute diarrhoea in calves, sub-clinical infections are common (De Leeuw *et al.*, 1980). In a longitudinal survey McNulty and Logan (1983) reported the detection of rotavirus in 79% of calves during the first 3 weeks of life and of these 80% showed no clinical signs of infection. The

reasons for sub-clinical infections are not fully understood and therefore characterisation of the viruses associated with such infections would give a clearer picture of the role of rotavirus in neonatal calf diarrhoea.

1.2 ROTAVIRUS

1.2.1 Rotavirus - the genus

The genus *Rotavirus*, within the family Reoviridae, was established by the International Committee on Taxonomy of Viruses in 1978 (Matthews, 1979). The family Reoviridae comprises five other genera, *Orthoreovirus*, *Orbivirus*, *Phytoreovirus*, *Fijivirus* and an unnamed group of cytoplasmic polyhedrosis viruses. It has been suggested that the family name should be changed to Diplornaviridae to emphasise the double stranded nature of the genomic RNA. The *Rotavirus* genus was created to include a number of antigenically related and morphologically identical viruses that caused enteritis, mainly in young mammals and birds. The name rotavirus was first proposed by Flewett *et al.*, (1974), derived from the Latin word rota meaning wheel, because of the resemblance of the virions, by microscopy, to small wheels with a wide hub, radiating short spokes and a thin rim. A calf rotavirus, Nebraska calf diarrhoeal virus (NCDV) described by Fernelius

et al., (1972), was suggested initially as the reference strain but the detection of multiple serotypes and the ability of some strains to cross infect heterologous species has caused the naming of type species to be abandoned.

Probably the earliest recorded work on rotavirus was made by Light and Hodes (1943,1949) who inoculated calves with a filtered stool preparation from children suffering from gastroenteritis and produced diarrhoea. At that time they were unable to determine the link between the agent and rotavirus, but subsequent electron microscopic examination of stored material from these experiments by Hodes (1977) revealed the presence of rotavirus. Likewise, other workers have retrospectively implicated rotavirus from outbreaks of diarrhoea in the young of mice (Cheever and Mueller, 1947; Kraft, 1957; Adams and Kraft, 1967; Banfield *et al.*, 1968), monkeys (Malherbe and Strickland-Cholmley, 1967) and cattle (Mebus *et al.*, 1969). Since the classification of rotavirus as a separate genus, these viruses have been isolated from many species of mammals and birds, including mice, monkeys, calves, man, foals, sheep, goats, rabbits, pigs, deer, antelope, apes, dogs, kittens, turkeys and chickens (reviewed by McNulty, 1978; Flewett & Woode, 1978; Estes *et al.*, 1983).

1.2.2 The physicochemical properties of rotavirus

During purification of rotavirus from faecal samples it was found that virus particles banded at two different densities after caesium chloride (CsCl) density gradient centrifugation. The particles with a density of 1.36g/ml were 70nm in diameter and had a smooth outer rim, while the particles with a density of 1.38g/ml were rough in appearance with a diameter of 60-65nm (Petric *et al.*, 1975; Bridger and Woode, 1976; Kapikian *et al.*, 1976; Elias, 1977). The smooth particles were described as the complete virions and the rough particles as lacking the outer rim or capsid layer.

As would be expected for an enteric virus, rotaviruses were found to be stable at low pH (pH 3.5) but labile at high pH (pH 10). Also, treatment of the virus with low concentrations (5mM) of either ethylenediamine tetra-acetic acid (EDTA) or ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA) significantly reduced infectivity (Estes *et al.*, 1979) suggesting the crucial role of Ca^{++} in stabilising the virus. With this information Shirley *et al.*, (1981) reported that rotavirus could be stabilised during purification by the addition of low levels of $CaCl_2$. Removal of the Ca^{++} ions by chelation was shown to remove the outer capsid layer (Cohen, 1977) and supported the observations of Bridger and Woode (1976) and Elias (1977) that only the complete, smooth virions were infectious. Removal of the outer capsid layer activated an RNA-dependant RNA polymerase present within the virus particle (Cohen *et al.*, 1979). This method of activation has been used for the *in vitro* transcription of single stranded viral mRNAs (Cohen *et al.*, 1979; Flores *et al.*, 1982a). Transcription has been shown to be asymmetric, with all transcripts being full length positive strands made from the negative strand of the viral double stranded (ds-) RNA (McCrae & McCorquodale, 1982).

Treatment of virus with higher concentrations of $CaCl_2$ (2M) reduced infectivity (Estes *et al.*, 1979) releasing icosahedral core structures having a diameter of 38nm (Palmer *et al.*, 1977) and a density of 1.44g/ml (Bican *et al.*, 1982).

1.2.3 The rotavirus genome

The rotavirus genome was shown to comprise RNA (Welch,1971; Much and Zajac, 1972; Petric *et al.*, 1975) and it was demonstrated subsequently that the RNA was double stranded (Welch and Thompson, 1973; Todd and McNulty, 1976). The segmented nature of the genome was reported by Rodger *et al.*, (1975) and Newman *et al.*, (1975) after separation of the viral RNA by polyacrylamide gel electrophoresis into 8 discrete bands. However, after calculating the molar ratios of RNA in each band it was concluded that 2 of the bands, namely 2 and 6, contained

2 and 3 RNA species, respectively. The rotavirus genome therefore contained 11 segments of double stranded RNA (Fig 1.1), which contrasted with reoviruses (Bellamy *et al.*, 1967; Shatkin *et al.*, 1968) and orbiviruses (Vervoerd *et al.*, 1970) which contain 10 segments. The 11 rotavirus segments range in molecular weight from 2 x 10^5 to 2.2 x 10^6 with a total genome size of between 11 x 10^6 and 14 x 10^6 (Kalica *et al.*, 1976, 1978a, 1978b; McNulty *et al.*, 1981; Obijeski *et al.*, 1977; Rodger *et al.*, 1975; Todd & McNulty, 1977). The rotavirus RNA segments fall into 4 size classes, comprising segments 1, 2, 3 and 4, segments 5 and 6, segments 7, 8 and 9 and segments 10 and 11 (Fig 1.1), based on their electrophoretic migration and contour length measurements by electron microscopy (Rixon *et al.*, 1984).

It became evident that rotavirus strains could be distinguished by differences in the migration of the RNA segments after electrophoresis (Kalica *et al.*, 1976; Rodger and Holmes, 1979) and a scheme to use this information to characterise different strains of virus was proposed by Lourenco *et al.*, (1981). The scheme divided the segments into the 4 size classes containing segments 1-4, 5-6, 7-9 and 10-11 respectively, and each pattern within each group was given a lower case letter to identify it. It soon became apparent that this scheme was too elaborate, did not take into account variation of segment migration due to changes in electrophoresis conditions such as gel strength and buffer components and therefore, when used to compare strains from different laboratories, could not cope with the complexity of the migration changes. This scheme and a similar one devised by Moosai *et al.* (1984), called 'rotacode', have not been adopted.

However, it was recognised that the electrophoretic pattern, or genome profile (often called electropherotype), was a useful epidemiological tool which provided a fingerprint for a particular virus isolate. This type of analysis provided useful data on the introduction of rotavirus strains into a population and their spread (Albert *et al.*, 1983; Follett and Desselberger, 1983). However, attempts to correlate the genome profile with other viral characteristics has been disappointing. For example, it has been shown that genome profiles do not correlate with serotypic

data. Beards (1982) found that some human rotaviruses with differing genome profiles belonged to the same serotype; conversely, some strains with identical genome profiles belonged to different serotypes. Also, the analysis of the partial digestion products of individual genome segments by base specific ribonucleases led Clarke and McCrae (1982) to the conclusion that variation in genome segment mobility was always associated with detectable changes in nucleotide sequence and that segments of similar mobility could also show significant sequence changes. This was confirmed by Flores *et al.* (1982b) with RNA-RNA hybridisation and by Desselberger *et al.* (1986) using two dimensional oligonucleotide mapping of genome segments. Flores *et al.* (1982b) found that genome segments which had a similar migration in polyacrylamide gels did not necessarily exhibit homology by hybridisation and, conversely, some segments homologous by hybridisation had different mobilities. Desselberger *et al.* (1986) also showed considerable sequence variation in genome segments from different virus strains which co-migrate in polyacrylamide gels.

The genome diversity of rotaviruses is analogous to the heterogeneity observed in other segmented genome viruses, e.g. reovirus (Hrdy *et al.*, 1979) and influenza virus (Palese, 1977).

Until recently all rotaviruses were thought to contain a common group-specific antigen (see Section 1.2.5) but then several viruses were described which did not possess this antigen and were called atypical. Pedley *et al.* (1983) defined three rotavirus groups, group A containing the original viruses and groups B and C the atypical viruses. Since then two further groups have been described (Pedley *et al.*, 1986). Besides serological differences between the group A viruses and viruses from other groups, Pedley *et al.* (1986) found that the genome profiles were distinct. The genome of the group A viruses was defined as comprising four high molecular weight ds-RNA segments (1 to 4), five middle-sized segments (5 to 9) including a distinctive triplet of segments (7 to 9), diagnostic for group A rotaviruses, and two smaller segments (10 and 11). Although variation in genome profiles of group A rotaviruses is common, as described earlier, in most cases this

basic pattern has been maintained. However, Espejo *et al.* (1980b) and Rodger *et al.* (1981) described rotavirus isolates in which the electrophoretic migration of segments 10 and 11 was less than that seen in other isolates and called them short profile viruses. This was followed by a description of the "super short" profile (Hasegawa *et al.*, 1984; Albert, 1985) in which segment 10 migrated just below the segments 7, 8 and 9 triplet. In the same year Pedley *et al.* (1984) described the isolation of group A rotaviruses, from chronically infected immunodeficient children, whose genome profiles contained additional RNA segments migrating between segments 1 and 7 which had sequences homologous to normal rotavirus segments. These RNA segments of increased size were derived from the segments of normal size by concatemer formation. Group A rotaviruses with concatemeric segments have also been recovered from calves (Pocock, 1987), pigs (Bellinzoni *et al.*, 1987), rabbits (Thouless *et al.*, 1986) and from asymptomatically infected immunocompetent children (Besselaar *et al.*, 1986)

Sequence data is now available for all the rotavirus genome segments from at least one virus strain and some segments have been sequenced from several strains. One interesting finding is that although heterogeneity in segment electrophoretic mobility is common, sequence data has revealed that the same segment from different strains almost always contain the same number of nucleotides. Therefore, the variation in segment mobility would appear to be a function of nucleotide sequence and/or secondary structure and not molecular size.

1.2.4 Rotavirus proteins

The proteins of both human and bovine rotaviruses were first analysed in 1975 (Rodger *et al.*, 1975; Newman *et al.*, 1975). The viruses were extracted from faecal preparations, the proteins dissociated with sodium dodecyl sulphate (SDS) and separated by polyacrylamide gel electrophoresis. These, and several subsequent reports (Todd and McNulty, 1977; Monette *et al.*, 1979; Obijeski *et al.*, 1977; Rodger *et al.*, 1977; Cohen *et al.*, 1978; Kalica and Theodore, 1979; Matsuno and Mukoyama, 1979; Thouless, 1979; Espejo *et al.*, 1980a,

1981; Mason *et al.*, 1980; Smith *et al.*, 1980; Estes *et al.*, 1981; McCrae and Faulkner-Valle, 1981; Novo and Esparza, 1981; Bican *et al.*, 1982), have analysed the proteins of rotaviruses from many host species and purified them from either faecal preparations or, in the case of the few cultivatable strains, from tissue culture fluids. Many of these reports produced conflicting data on the number and sizes of the virion proteins. The main reasons for this were the different buffer systems and gel concentrations used for electrophoresis, the different molecular weight standards used to estimate the sizes of the proteins and the inherent variation of molecular weights of the viral proteins of the different rotavirus strains examined. The general consensus from all these reports was that complete virions contained 6 proteins. Two of these proteins were located in the virion outer capsid layer and could be removed by treatment of the virus with EDTA (Estes *et al.*, 1981). One of the outer proteins was shown to be glycosylated. The non-glycosylated outer protein was shown to be cleaved by proteolytic enzymes such as trypsin (Estes *et al.*, 1981; Espejo *et al.*, 1981) and this may have contributed to the variability in the number of viral proteins reported.

Subsequent work describing the analysis of the viral proteins synthesised by rotavirus in infected cells and the *in vitro* translation of the genomic segments has given a clearer picture (McCrae and Faulkner-Valle, 1981; Arias *et al.*, 1982; Ericson *et al.*, 1982). These reports indicated that each of the rotavirus genome segments is monocystronic, encoding a single viral protein; the whole genome thus produces 11 proteins (Fig 1.1). There is still some debate as to the number of structural proteins but most workers agree that the virus contains 2 major surface proteins, 4 inner capsid proteins and 5 non-structural proteins (Table 1.1). Recent sequence data has shown that all the rotavirus genes possess at least one long open reading frame following the first initiation codon. Although some genes contain additional open reading frames either in phase (genes 7, 9 and 10) or out of phase (gene 11) the first initiation codon was usually found to be a strong initiation codon, based on Kozak's rules (Kozak, 1981;1986). This evidence confirmed that all of the rotavirus gene segments are monocistronic, with the exception of the vp7 gene which is possibly bicistronic (Chan *et al.*, 1986). The nomenclature of the viral

proteins used throughout the thesis is that of McCrae & McCorquodale (1982). However, initial studies on the simian rotavirus SA11 by Mason *et al.* (1980) failed to identify a protein product from genome segment 3 and therefore vp3 related to the product of segment 4. This confusion has since been resolved by the detection of the segment 3 product of SA11 by Liu *et al.* (1988). The protein product of SA11 segment 4 has therefore been renamed vp4, however, it should be noted that in many publications prior to 1988, vp3 referred to the product of gene 4.

The modifications and known functions of the viral proteins are shown in Table 1.1. Except for their location in the inner core of the virus little is known of the functions of vp1, vp2 and vp3. It has been shown that vp2 can bind RNA (Boyle & Holmes, 1986) but whether this protein is involved in the packaging of the RNA in the virion, acts as a polymerase, or has another enzymatic activity is not known. Evidence that vp1 and vp3 may be involved in transcription came from work with rotavirus ts-mutants (Gombold *et al.*, 1985; Gombold & Ramig, 1987) in which lesions mapped to genome segments 1 and 3 led to a failure to synthesise ssRNA.

The major rotavirus structural protein, vp6, comprising 51% of the total virion protein, is located on the surface of the inner capsid. Most monoclonal antibodies (Mabs) directed against vp6 react with all group A rotaviruses (see Section 1.2.5) indicating that vp6 specifies the rotavirus serogroup A (Gary *et al.*, 1982; Singh *et al.*, 1983). Also, Mabs have been used to show that subgroup specificity (see Section 1.2.5) is located on vp6 (Greenberg *et al.*, 1983a; Singh *et al.*, 1983). Vp6 readily forms trimers (Gorziglia *et al.*, 1985; Sabara *et al.*, 1987), which is thought to contribute to the highly immunogenic and antigenic nature of this protein (Estes *et al.*, 1987; Sabara *et al.*, 1987).

There are two proteins in the rotavirus outer capsid, vp4 and vp7. Vp7 constitutes 30% of the total virion protein, making it the second most abundant virion protein and also the major component of the virion outer capsid. This protein is encoded by one of the triplet of genome segments and therefore can be derived

from either segments 7, 8 or 9, depending on the virus strain (Greenberg *et al.*, 1983a; Kalica *et al.*, 1981; Matsui *et al.*, 1989). Biochemical analysis showed that vp7 is a glycoprotein that contains N-linked high mannose oligosaccharides which are further processed by trimming (Kabcenell & Atkinson, 1985). From sequence data it has been shown that vp7 contains a cleavable signal sequence at the amino terminus so that the protein is co-translationally glycosylated in the endoplasmic reticulum (Both *et al.*, 1983; Kabcenell *et al.*, 1988). However, glycosylation is not essential for infectivity as shown by Estes *et al.* (1982) who isolated a fully infectious mutant of SA11 containing a non-glysolated vp7. Vp7 is the major, and at first thought to be the only, neutralising antigen of rotavirus (Greenberg *et al.*, 1983a; Kalica *et al.*, 1981). Analysis, using Mabs directed against vp7, has shown that this protein defines the rotavirus serotype (see Section 1.2.5; Greenberg *et al.*, 1983b; Taniguchi *et al.*, 1987). Also, it has recently been reported that vp7 is the protein responsible for the initial attachment of the virus to host cells (Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988).

Vp4, the product of genome segment 4, was shown to be a non-glycosylated component of the outer capsid (Arias *et al.*, 1982; Mason *et al.*, 1980; McCrae & McCorquodale, 1982). The proteolytic cleavage of vp4 into polypeptides of molecular weights 60,000 and 28,000 Daltons results in the enhancement of infectivity (Espejo *et al.*, 1981; Estes *et al.*, 1981). This cleavage does not affect the binding of the virus to the host cell but enhances penetration (Clark *et al.*, 1981; Fukuhara *et al.*, 1988; Kaljot *et al.*, 1988). Although vp4 is only a minor component of the rotavirus outer capsid it has been shown to perform a number of functions. The restriction of growth in cell culture by some human rotaviruses was shown by analysis of reassortants from human and a cultivatable bovine rotavirus to be a function of the genome segment 4, vp4 (Greenberg *et al.*, 1983a). Some rotavirus strains show haemagglutinating activity and this has been shown to be a property of vp4 by both reassortant analysis (Kalica *et al.*, 1983) and the inhibition of haemagglutination by vp4 Mabs (Greenberg *et al.*, 1983b). Some of these Mabs were also found to neutralise virus infectivity, a function thought exclusive to vp7 (Greenberg *et al.*, 1983b). A further function of vp4 appears to

be related to virulence. Offit & Blavat (1986) used reassortants between two rotaviruses, showing differences in virulence for newborn mice, and found that the virulence of these reassortants co-segregated with genome segment 4. Also, the analysis by hybridisation and sequence comparison of two groups of rotaviruses, isolated from human infants with either symptomatic or asymptomatic infections, showed significant conservation of segment 4 in the viruses within each group, but considerable variation between the groups suggesting a role of vp4 in virulence determination (Flores *et al.*, 1986; Gorziglia *et al.*, 1986, 1988a).

The functions of the non-structural proteins, vp5, vp8, vp9 and vp11, are poorly understood. However, the fact that they are basic proteins, as predicted from sequence data, also the demonstration of their RNA binding ability (Boyle & Holmes, 1986), their presence in intracellular subviral particles exhibiting replicase activity (Patton, 1986) and that ts-mutants whose lesions map to the genome segments which encode these proteins exhibit a dsRNA$^-$ phenotype, all suggest that these proteins are involved in RNA replication. The other non-structural protein is encoded by genome segment 10 and is synthesised as a polypeptide of 20,000 Daltons, vp12. As with vp7 this polypeptide is co-translationally glycosylated to give a product of 28,000 Daltons, vp10 (Ericson *et al.*, 1982; Kabcenell & Atkinson, 1985). Evidence suggests that this protein contains a non-cleavable signal sequence (Kabcenell & Atkinson, 1985). It has been suggested that vp10 acts as a receptor to bind single shelled virus particles and mediate their budding into the lumen of the endoplasmic reticulum (Au *et al.*, 1989). It has also been reported that glycosylation of vp10 is essential for the shedding of the transient envelope from the budding virus particles (Petrie *et al.*, 1983).

1.2.5 Rotavirus serology

Because of the lack of suitable numbers of isolates that could be grown in cell culture, initial serologic studies with rotaviruses were performed by either immune electron microscopy (IEM; Bridger and Woode, 1975; Saif *et al.*, 1977), ELISA (Yolken *et al.*, 1978; Thouless *et al.*, 1982), complement fixation (CF; Kapikian

et al., 1976; Thouless *et al.*, 1977; Zissis and Lambert, 1978) or the neutralisation of foci, detected by fluorescence, of abortive infection in cell culture (Beards *et al.*, 1980; Wyatt *et al.*, 1982). At first distinct groups were demonstrated by all these techniques which were themselves thought to be comparable. Initially, this led to the erroneous assumption that all rotaviruses isolated from one species would differ serologically from those of another species (Thouless *et al.*, 1977; Estes and Graham, 1980; Murakami *et al.*, 1981; Sato *et al.*, 1982). However, following the introduction of another assay, immune adherence haemagglutination assay (IAHA; Kapikian *et al.*, 1981), it was found that the CF, ELISA and IAHA tests were detecting a different antigenic property from the neutralisation test and these properties were designated subgroup and serotype, respectively. The subgroup specificity was shown by Kalica *et al.* (1981) to be a property of vp6, the product of genome segment 6, the major inner capsid protein which also contains the rotavirus common group antigenic site. The neutralisation test was at first thought to be detecting an exclusive function of vp7 (Greenberg *et al.*, 1983a); more recent work has shown neutralisation to be a function of both the viral proteins in the outer capsid of the virus, vp4 and vp7. However, the neutralisation test has produced useful epidemiological data when comparing field isolates. With the advent of techniques for the propagation of rotaviruses in cell culture there have been many studies which defined serological groups of rotaviruses from neutralisation data. The human viruses tested fell into clearly defined serogroups and, with the addition of viruses from other mammalian species, nine serotypes have been suggested (see Table 1.2; Kapikian and Chanock, 1985). The criterion laid down for the definition of a serotype was a 20 fold or greater difference in serum antibody titre between members of different serotypes (Wyatt *et al.*, 1982). Serotypes 1, 2, 8 and 9 contained only human rotaviruses, serotype 4 contained viruses from humans and pigs, whereas serotype 3 viruses had the broadest host range and included strains from humans, monkeys, dogs, cats, horses, pigs, mice and rabbits. The bovine viruses when tested against representative strains from the existing serotypes did not cross-neutralise and all the bovine rotaviruses, to date, have been placed in serotype 6. The finding that virus neutralisation was a function of both vp4 and vp7 and the discovery of intertypic

virus strains i.e. isolates which have neutralisation specificities of more than one serotype, has led to a re-evaluation of the rotavirus serotype classification. One such human rotavirus, strain M37, was found to belong to both serotypes 1 and 4 (Hoshino *et al.*, 1985a). The serotype 1 specificity was a function of vp7 and the serotype 4 specificity that of vp4. The independent segregation of the vp7 and vp4 neutralising antigens will require a serological classification of rotaviruses similar to that defined for influenza viruses in which the haemagglutinin and neuraminidase neutralising antigens are also independently segregated. This will await the production of the necessary serological reagents.

The serotypic characterisation of bovine rotaviruses was again slow at first because of the difficulty of adapting the viruses for growth in cell culture. However, with the advent of the fluorescent focus neutralisation assay, two groups reported the existence of two bovine rotavirus serotypes (Murakami *et al.*, 1983; Woode *et al.*, 1983). The results of Bridger *et al.*, (1983) suggested a third serotype existed and Murakami *et al.*, (1983) detected viruses that were intermediate between serotypes. These 'bovine' rotavirus serotypes as yet have not been integrated into the overall rotavirus serology. However, these data along with the fact, mentioned above, that some rotaviruses from different species cross react serologically suggest that a whole range of rotavirus serological types may exist. The fact that considerable variations do occur within serotypes suggests that the antigenic differences might be enough to give one strain an advantage over another in the field and therefore serological data should not be confined solely to the definition of specific groups.

Besides the neutralising antigens, it has been reported that rotaviruses contain a common antigen which can be detected by immunofluorescence, complement fixation, immune electron microscopy, enzyme-linked immunosorbant assay and radio-immune assay (Kapikian *et al.*, 1975; Schoub *et al.*, 1977; Bishai *et al.*, 1979). This common antigen was shown to be on the inner capsid layer as only rough particles were agglutinated by antisera against heterologous strains (Bridger, 1978) and later shown to be a function of vp6. It was originally thought that all

rotaviruses contained a common antigen until several groups of workers described viruses which were similar in size and morphology but distinct serologically. They were observed in the faeces of pigs (Saif *et al.*, 1980; Bridger, 1980; Bridger *et al.*, 1982; Bohl *et al.*, 1982), chickens (McNulty *et al.*, 1981) and humans (Rodger *et al.*, 1982; Nicolas *et al.*, 1983). These newly observed viruses were initially called pararotaviruses but Pedley *et al.*, (1983) suggested the formation of separate serogroups for rotaviruses with the original isolates being assigned to group A. There are now 5 rotavirus serogroups A - E, with group B viruses isolated from humans, pigs, cattle, sheep and rats, group C from humans, pigs and ferrets, group D from chickens and group E from pigs (Bridger, 1987; Torres-Medina, 1987). However, many rotavirus isolates known to be non-group A have yet to be serogrouped. Although the viruses in the other serogroups have a genome comprising 11 double stranded RNA segments, their origin and significance remain obscure. Their prevalence, however, based on serological evidence suggests a widespread distribution and more work is needed to determine their importance. Once again, progress has been hampered by the difficulty in adapting the non-group A rotaviruses to growth in cell culture; however, recently Saif *et al.* (1988) have successfully propagated a porcine group C rotavirus.

1.2.6 Growth of rotaviruses in cell culture.

Until fairly recently, rotaviruses were considered fastidious viruses, difficult to adapt to growth in cell cultures. Some rotaviruses, however, grew readily in primary or secondary cultures of the species they infected, e.g. Simian rotaviruses SA11 and "O" agent (Malherbe & Strickland-Cholmley, 1967). Even up to 1981, only a limited number of rotaviruses had been adapted and those were isolated from cattle, pigs and birds (Mebus *et al.*, 1971a; Fernelius *et al.*, 1972; Welch & Twiehaus, 1973; Woode *et al.*,1974; McNulty *et al.*, 1976, 1977, 1979; L'Haridon & Scherrer,1976; Theil *et al.*, 1977; Babiuk & Mohammed, 1978). During this period Babiuk *et al.* (1977), acting on the observation that certain viruses, including influenza (Appleyard & Maber, 1974), Sendai virus (Homma, 1971) and reovirus (Spendlove & Schaffer, 1965), required proteolytic enzyme

treatment to enhance infectivity, showed that treatment of faecal rotaviruses with trypsin greatly improved the isolation rate into cell culture. In 1977 two groups showed that either BSC-1 or MA104 cell lines, both derived from monkey tissue, could be used for primary viral isolations (Babiuk *et al.*, 1977; Matsuno *et al.*, 1977). Kurugi *et al.* (1976) and McNulty *et al.* (1977) found that the cytopathic effect of rotavirus was more extensive if the cell cultures were rolled, but yields of virus were similar to those of stationary cultures. However, Koves (1979) and Fukusho *et al.* (1981) reported that isolations of bovine and porcine rotaviruses were only successful when rolled cultures were used. Since the introduction of trypsin treatment of virus, susceptible cell lines and the rolling of cultures, rotavirus isolation from many animal species has become routine (Fukusho *et al.*, 1981; Sato *et al.*, 1981; Urasawa *et al.*, 1981; Taniguchi *et al.*, 1982; Wyatt *et al.*, 1983; Ward *et al.*, 1984; Albert *et al.*, 1987).

1.2.7 Host range

As described above, rotaviruses have been shown, either by virus isolation or by the direct visualisation of virus particles, to infect many avian and mammalian species. Serological evidence has revealed that many more species can be infected and it has been suggested that rotaviruses may infect all mammalian and avian species (Estes *et al.*, 1983). Although there is at present no conclusive data to suggest that, in nature, rotaviruses from one species will infect other species, results from experimental infections and serological evidence would suggest they do. For example human rotaviruses have been shown to infect monkeys, pigs, calves, lambs and dogs (reviewed by Estes *et al.*, 1983), also simian rotaviruses can infect piglets and calves (Rodger *et al.*, 1977) and bovine rotaviruses will infect piglets (Woode *et al.*, 1974, 1975; Hall *et al.*, 1976) and mice (Noble *et al.*, 1980), all under experimental conditions. The majority of these cross-species infections are sub-clinical and therefore may occur naturally but remain undetected. However, some simian and canine rotaviruses have been reported to be serologically closely related to some human strains (Wyatt *et al.*, 1982; Gaul *et al.*, 1982) providing further evidence of cross-species transmission.

1.2.8 Pathogenesis of bovine rotavirus

Rotaviruses have been associated with disease in calves aged under 4 weeks, with the highest frequency occurring in the second week of life (Woode and Bridger, 1975; Acres and Babiuk, 1978). The virus infects calves by the oral-faecal route. Multiplication occurs in the columnar epithelium cells of the small intestine (Mebus and Newman, 1977) resulting in the loss of these absorptive cells and leading to denudation and shortening of the villi. Refoliation of the villi occurs by the migration of immature cuboidal cells from the crypts. These replacement cells appear to be refractile to infection with rotavirus and therefore the infection is self-limiting. The loss of the epithelial cells leads to a loss of intestinal disaccharidase activity and a consequent malabsorption syndrome (Pearson *et al.*, 1978). The loss of this enzyme activity, important for the metabolism of ingested sugars, results in an osmotic imbalance which can lead to fluid being secreted into, rather than absorbed from, the large intestine. The subsequent increase in the secretion of fluids leads to an increased output of faeces of pasty consistency and light brown, yellow or white in colour, depending on the severity of the infection.

Pearson *et al.*, (1978) found that the number and size of intestinal lesions in calves infected with rotavirus varied between isolates. Also Argenzio (1978) reported that when infection was confined to the upper part of the small intestine fluid loss could be compensated by the uninfected lower small and large intestine. The implication of this work was that rotavirus infections of calves may be sub-clinical. The finding that 100% of adult cattle have serum antibody to rotavirus would support the view that sub-clinical rotavirus infections are common (De Leeuw *et al.*, 1980; Schusser *et al.*, 1982; McNulty and Logan, 1983). The possible reasons for sub-clinical infections include the passive transfer of maternal antibody via colostrum and milk which would limit the infection (Snodgrass and Wells, 1976; Bridger and Brown, 1981), the existence of rotavirus strains of differing virulence (Carpio *et al.*, 1981) or an age related resistence to infection (Tzipori *et al.*, 1981).

1.3 OBJECTIVE

The objective of this project was to characterise, both biologically and at the molecular level, rotavirus isolates shed asymptomatically by calves reared on farms which did not have a history of bovine enteric disease. The data would be compared with the published work on the characteristics of rotaviruses isolated from outbreaks of calf enteritis in order to investigate the observed variation in pathogenesis during rotavirus infections of calves. Such information could assist in the formulation of methods for the control of disease caused by rotavirus in calves and other animal species.

Table 1.1 Rotavirus proteins

[Data from Estes (1990)]

Viral protein	Molecular weight (1)	Modification	Virion location	% of virion protein	Function
vp1	125,005		Inner core	2	Slightly basic
vp2	94,000		Inner core	15	RNA binding?
vp3	88,000		Inner core	0.5	?
vp4	86,751	Trypsin cleavage	Outer capsid	1.5	Haemagglutinin, neutralising antigen protease enhanced infectivity, virulence
vp5	58,654		Non-structural		Slightly basic
vp6	44,816		Inner capsid	51	Serogroup antigen, Subgroup antigen
vp7	33,919	Cleaved signal sequence, high mannose glycosylation	Outer capsid	30	Neutralising antigen cell attachment protein
vp8	36,700		Non-structural		RNA binding?
vp9	34,600		Non-structural		Basic protein, role in RNA replication?
vp10	20,290 (vp12)	Uncleaved signal sequence, high mannose glycosylation	Non-structural		Role in morphogenesis
vp11	21,700	Phosphorylated	Non-structural		Slightly basic

(1) Mol. wt of nascent polypeptide derived from sequence data

Table 1.2 Host range of rotavirus serotypes

Serotype	Human	Simian	Equine	Porcine	Canine	Feline	Lapine	Murine	Bovine	Avian
1	+									
2	+									
3	+	+	+	+	+	+	+	+		
4	+			+						
5			+	+						
6									+	
7										+
8	+									
9	+									

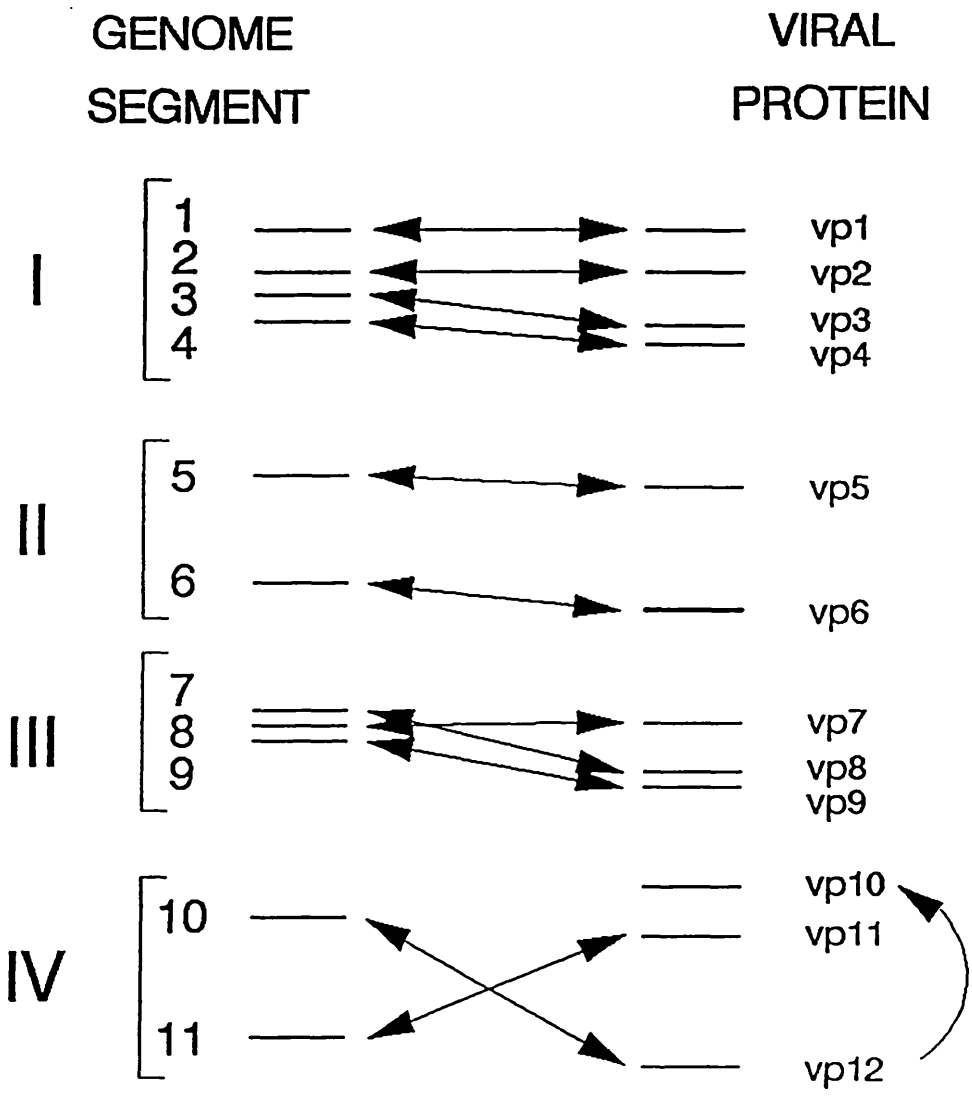


Fig 1.1: Coding assignments of the rotavirus genome segments. The arrows denote the coding assignments. The roman numerals denote the groupings of the genome segments into size classes. The viral proteins are labelled vp1 to vp12 according to McCrae & McCorquodale (1982).

2.0 ROTAVIRUS GENOME PROFILES: ANALYSIS OF FAECAL SAMPLES FROM 3 FARMS

2.1 MATERIALS AND METHODS

2.1.1 Methods for genome profile analysis.

2.1.1.1 Method 1: Growth of rotavirus and labelling of viral RNA in cell culture.

2.1.1.1.1 Preparation of faecal samples.

A 10% suspension of faeces in cell culture medium (Earle's salt solution containing lactalbumin hydrolysate and galactose; 0.2% sodium bicarbonate; 60 μg/ml penicillin; 100μg/ml streptomycin and 50U/ml mycostatin) was mixed with an equal volume of trichlorotrifluoroethane (Arclone P; ICI, Mond Division, Runcorn, Cheshire, UK.). The mixture was sonicated and centrifuged at 1,000g for 10min. The aqueous phase was removed and passed sequentially through sterile 1.2nm and 0.45nm Millipore filters. The filtrate was treated with trypsin (Type IX; Sigma Chemical Co., Dorset, U.K.) at a final concentration of 2.5μg/ml at 37^{0}C for 15min.

2.1.1.1.2 Growth and labelling of virus in cell culture.

Primary cultures of calf kidney (CK/1) cells were prepared by the method described by Luther (1972) for the preparation of primary porcine kidney cells. Five millilitres of the treated faecal preparation were added to each 20oz flat glass bottle containing a confluent monolayer of CK/1 cells and incubated at 37^{0}C for 1h. The inoculum was removed and the cell sheet washed x2 with 20ml medium prior to the addition of 20ml medium containing 1/10th the normal phosphate concentration and 0.25μg/ml trypsin. At 7h post infection (p.i.), 1mCi [^{32}P]-sodium orthophosphate was added to each bottle and the incubation continued at 37^{0}C. The cultures were harvested after 48h at 37^{0}C.

For comparison, a cell culture adapted UK rotavirus strain (Bridger and Woode, 1975) was also grown in CK/1 cells.

2.1.1.1.3 Extraction of viral RNA from infected cells.

At harvest the cell sheet was scraped off the bottle into the medium. The cells and medium were centrifuged at 100,000g for 1h and the pellets resuspended in a small volume of TS (0.14M NaCl; 10mM Tris/HCl pH 7.4). One tenth volume of a solution containing 3.5% Triton X-100 and 3.5% sodium deoxycholate was then added and incubated at 4^0C for 30min. The cell nuclei were removed by centrifugation at 2,000g for 30min and the supernatant kept for RNA extraction.

2.1.1.1.4 Virion RNA extraction

RNA was extracted from the virus samples by disruption with SDS and protein digestion by proteinase K (Smith *et al.*, 1977) prior to deproteinisation with phenol/chloroform and ethanol precipitation (Goldstein *et al.*, 1976).

Virus samples were mixed with an equal volume of a solution containing 0.04M NaCl; 4mM $CaCl_2$; 1% SDS. Proteinase K (Protease Type XI; Sigma Chemical Co., Dorset, UK.) was added to a final concentration of 500μg/ml and the mixture was incubated at 37^0C for 30 min. One tenth volume each of 100mM EDTA and 1M NaCl were then added, followed by 2 volumes phenol/chloroform (1:1 $^v/_v$). The samples were mixed by vortexing for 30sec, then the two phases were separated by centrifugation at 3,750g for 20 min. The aqueous phase was removed and the organic phase re-extracted with 1 volume SSTE buffer (0.2% SDS; 0.1M NaCl; 2mM EDTA; 25mM Tris/HCl pH 7.4). The combined aqueous phases were re-extracted with 2 volumes phenol/chloroform before the addition of $^1/_{10}$th volume 3.0M potassium acetate and $2^1/_2$ volumes ethanol. After overnight storage at -20^0C the RNA was pelleted by centrifugation at 1,000g for 30 min. The pellets were dissolved in a small volume of sterile distilled, deionized water (DDW) and stored at

-70^0C. Where necessary, RNA concentrations were determined by spectrophotometry: 1 E_{260} was assumed equivalent to 50μg/ml ds-RNA (Gaillard and Joklik, 1982).

2.1.1.1.5 Purification of rotavirus RNA by CF11 cellulose chromatography.

The differential adsorption of single-stranded RNA (ss-RNA) and ds-RNA to CF11 cellulose in buffer containing different proportions of ethanol (Franklin, 1966) was used to separate rotavirus genomic ds-RNA from any contaminating ss-RNA.

CF11 cellulose (Whatman LabSales Ltd., Maidstone, Kent) was suspended in STE buffer (0.1M NaCl; 1mM EDTA; 50 mM Tris/HCl pH 6.8) and the fines removed by decantation. Columns (8 x 102mm disposable polystyrene columns, Pierce & Warriner, Chester, U.K.) containing 2ml packed CF11 were prepared and equilibrated with STE containing 15% ethanol. RNA samples were made up in STE 15% ethanol and applied to the column, which was then washed with 25ml of the same buffer to remove the ss-RNA. The ds-RNA was then eluted with 25ml STE buffer without ethanol and collected as 1ml fractions. A 25µl aliquot of each fraction was removed for scintillation counting. The peak fractions were pooled and the RNA precipitated with $2^1/_2$ volumes of ethanol at -20^{0}C overnight. The RNA was pelleted at 10,000g for 30min and redissolved in 100µl DDW.

2.1.1.1.6 Electrophoresis of viral RNA

Gel electrophoresis was performed in 10% polyacrylamide vertical slab gels with a 3% polyacrylamide stacking gel using the discontinous buffer system of Laemmli (1970). The acrylamide was purchased as a preweighed mixture of acrylamide and N,N'-methylene-bis-acrylamide in the ratio 37.5:1 (Bio-rad Laboratories Ltd., Watford, Herts., UK.). Electrophoresis was performed at either 50mA constant current for 5h or at 20mA for 16h. Samples for electrophoresis were prepared by mixing equal volumes of the RNA preparation in DDW and 2x sample buffer (0.125M Tris/HCl pH 6.8; 4% SDS; 4% 2-mercaptoethanol; 20% glycerol) and incubation at 50^{0}C for 2min.

After electrophoresis, the gels were dried under vacuum onto Whatman No. 1 filter paper and autoradiographs produced by placing the dried gel in contact with Fuji RX Xray film.

2.1.1.2 Method 2: 3'-terminal labelling of viral RNA.

The method for labelling the 3'-OH terminus of rotavirus genome segments was essentially that described by Clarke and McCrae (1981).

2.1.1.2.1 Partial purification of virus from faeces

Ten percent suspensions of bovine faecal samples were prepared in 0.02M Tris/HCl pH 7.4. To 1 volume of faecal suspension was added $^1/_2$ volume Arklone P and the mixture sonicated for 30sec at 75W (Sonifier B-12, Branson Sonic Power Co., Danbury, Conn., USA.). The hydrocarbon and aqueous phases were separated by centrifugation at 1,000g for 5min. The upper aqueous phase was removed and re-extracted with a further $^1/_2$ volume Arklone P. The lower hydrocarbon phase was re-extracted with an equal volume of 0.02M Tris/HCl pH 7.4. After centrifugation at 1,000g for 5min, both aqueous phases were combined and centrifuged at 2,500g for 30min. The supernatants were centrifuged at 75,000g for 1h and the pellets left to gently resuspend overnight at 4^0C in $^1/_{20}$th volume 0.02M Tris/HCl pH7.4, then stored at -70^0C.

2.1.1.2.2 RNA extraction.

RNA was extracted from the pelleted samples by the method described in Section 2.1.1.1.4.

2.1.1.2.3 3'-terminal labelling.

The reaction mixture (45µl), contained in a 1.5ml Eppendorf tube, comprised 10% dimethylsulphoxide, 3.3mM dithiothreitol, 5.0mM $MgCl_2$, 0.1mM ATP, 50.0mM HEPES buffer pH 8.3, 1U T_4 RNA-ligase (P.L. Biochemicals), 5µCi cytidine 3',5'-[5'-^{32}P]-bisphosphate (pCp; Amersham International plc) and 2µg RNase-free bovine serum albumin (BSA; Miles Laboratories Ltd.). The RNA sample (25µl containing 10-100ng RNA) was added and the mixture incubated at 4^0C for 16h. The reaction mixtures were then diluted 20-fold with STE buffer containing 15% ethanol and applied to CF11 cellulose columns (see Section 2.1.1.1.5) to remove unincorporated pCp. After washing the columns with 20ml of the same buffer, the labelled RNA was eluted with ethanol free STE buffer into 0.5ml fractions. The radioactivity contained in 50µl aliquots of each fraction was determined by scintillation counting. The peak fractions were pooled and the RNA precipitated with $2^1/_2$ volumes ethanol after the addition of 10µg yeast transfer RNA to act as carrier to aid precipitation. After 16h at -20^0C the RNA was pelleted at 10,000g for 30min and redissolved in 100µl DDW and 5µl taken for counting. An aliquot containing 20,000 counts/min (cpm) was used for electrophoresis.

2.1.1.2.4 Electrophoresis of viral RNA

Gel electrophoresis was performed in 10% polyacrylamide vertical slab gels as described in Section 2.1.1.1.6.

To check the sensitivity of the 3'-terminal labelling method for the detection of genome segments in comparison with the widely used ethidium bromide staining, some gels were stained with 1µg/ml aqueous solution of ethidium bromide for 1 hr prior to visualisation of the bands with UV light in a transluminator (Chromatoview, model TM-40, UVP Inc., USA) and photographed on Polaroid film (type 665).

To assess this method for the analysis of faecal rotavirus genome profiles, daily faecal samples from an experimentally infected calf were analysed. The profile data were compared with detection of virus in the faecal samples by the infectivity assay described in Section 3.1.4.

2.1.2 Analysis of Field Samples from Calves on 3 Farms.

2.1.2.1 Sampling of calves and detection of rotavirus.

Faecal samples were collected from calves on three farms, designated A, B and C. These were selected because calf enteric disease was not considered a problem on the farms during the period of the survey. The sampling periods for the three farms were as follows: Farm A, October 1979 to April 1980; Farm B, February to May 1980 and September 1980 to January 1981; Farm C, October 1979 to August 1980 and September to December 1981. Samples were collected directly from the rectum of each animal three times per week during the first five weeks of life.

Ten percent suspensions of each faecal sample were tested for the presence of rotavirus by an enzyme linked immunosorbant assay (ELISA). The collection of samples and the ELISA analysis were carried out by Dr D.J. Reynolds, at this Institute, and described by Reynolds *et al.* (1984).

2.1.2.2 Analysis of rotavirus field isolates by genome profile analysis.

At least 1 rotavirus positive faecal sample, determined by ELISA, was analysed for each calf. When more than 1 positive sample was obtained from a calf then the sample which gave the highest ELISA reading was selected. A total of 40 samples were analysed from Farm A, 43 from Farm B and 44 from Farm C.

The genome profiles of rotaviruses in the samples collected from Farm A were analysed by Method 1, which involved the growth of virus in cell culture in the presence of [^{32}P]-sodium orthophosphate, extraction of the labelled viral RNA from the cells, purification of the RNA by CF11 cellulose chromatography followed by electrophoresis and autoradiography.

The genome profiles of the viruses from farms B and C were analysed by 3'-terminal labelling of the extracted viral RNA with [^{32}P]-pCp, followed by electrophoresis and autoradiography.

2.1.2.3 Analysis of mixtures of two rotaviruses with distinct profiles.

RNA extracted from the UK bovine rotavirus strain and from an isolate from Farm C, with the C7 profile described below, was adjusted to give equal concentrations as estimated by spectrophotometry. Mixtures containing a constant amount of C7 RNA and varying amounts of UK strain RNA were prepared and 3'-terminally labelled with [^{32}P]-pCp. Samples of the labelled ds-RNA mixtures containing 10,000 c.p.m. were electrophoresed on a 10% polyacrylamide gel and the genome profiles detected by autoradiography.

2.2 RESULTS

2.2.1 Growth of rotavirus in cell culture and labelling of viral RNA.

An attempt was made to grow rotavirus, partially purified from faecal samples, in cell culture in the presence of [^{32}P]- sodium orthophosphate to label the viral RNA. In initial experiments the virus was liberated from the cells by freezing and thawing and then pelleted by centrifugation, prior to RNA extraction. After electrophoresis and autoradiography it was found that although a good profile was shown for the cell culture adapted UK rotavirus strain, no RNA bands were visible in the tracks from the two faecal viruses (Fig 2.1).

It was possible that although the production of complete virions may have been restricted, sufficient intracellular viral RNA would be produced to give a profile. Therefore, instead of freezing and thawing the cell sheet, mild detergent treatment was used to liberate cytoplasmic nucleic acid, without disrupting nuclei, from CK/1 cells infected with the same 3 viruses. As the extracted viral RNA was heavily contaminated with host cell ss-RNA, the ds-RNA was purified by CF11 cellulose chromatography. Genome profiles were produced for both faecal viruses with this method (Fig 2.2). This figure also shows the material which was not absorbed to the CF11 column in buffer containing 15% ethanol. As expected this sample contained counts which did not enter the 10% polyacrylamide gel, corresponding to ss-RNA, and also radioactivity which was spread throughout the gel, probably representing unincorporated counts or small pieces of ss-RNA. However no distinct bands in the expected position of the viral ds-RNA were visible. There were still quite a few counts which did not enter the gel in the ds-RNA tracks but this could be removed by chromatography through a second CF11 column, which suggested there was residual contamination with ss-RNA.

2.2.2 3'-terminal labelling of rotavirus virion RNA

When the method of Clarke and McCrae (1981) for the 3'-terminal labelling of rotavirus RNA was first used very few counts were incorporated. However, by adding a small amount of bovine serum albumin to the reaction mixture the level of incorporation increased a hundred fold. It was possible that the albumin prevented the T_4 RNA ligase from adhering to the wall of the Eppendorf tube which would reduce the activity of the enzyme.

To determine the sensitivity of this method in comparison with the the detection of RNA in gels by ethidium bromide staining, a large amount of viral RNA was prepared from the UK bovine rotavirus strain grown in cell culture. This RNA sample was then diluted in serial 5-fold steps and portions of these were either 3'-terminal labelled prior to electrophoresis or left unlabelled and the bands stained with ethidium bromide after separation. After 3'-terminal labelling of 5ng of viral

RNA all the rotavirus genome segments could be detected after an autoradiographic exposure of 16h (Fig 2.3a). The amount of RNA could be reduced to 1ng if the exposure time was extended to 3-4 days and an intensifying screen used. A similar gel, containing comparable amounts of RNA and stained with ethidium bromide (Fig 2.3b) showed that the smallest amount of RNA which gave a complete profile was 1μg. Therefore, 3'-terminal labelling of the viral RNA increased the sensitivity detecting all 11 genome segments 200-1000 fold when compared with the ethidium bromide staining method.

The number of counts incorporated into the ds-RNA, following 3'-terminal labelling, was directly proportional to the amount of RNA present for concentrations up to 1μg (Fig 2.4). Above this concentration the expected incorporation was lower probably because the concentration of one or more of the reactants was limiting.

To assess this method for the analysis of faecal rotavirus genome profiles, daily faecal samples from an experimentally infected calf were analysed. Fig 2.5 shows that profiles were obtained from faecal samples taken 2 days post infection up to 8 days post infection. The production of genome profiles by 3'-terminal labelling the viral RNA was, therefore, a more sensitive procedure for the detection of rotavirus than the infectivity assay. The results of this experiment are shown in Table 2.1 and reveal that no infectivity was detected on day 2 post infection but a full genome profile was produced starting with a gel loading of 3,500 counts, approximately 320 counts per genome segment.

2.2.3 Genome profile analysis of field isolates

2.2.3.1 Farm A.

Of the 40 samples tested from Farm A, 35 (87.5%) contained enough virus to produce complete profiles showing the 11 segments of ds-RNA characteristic of rotaviruses. Autoradiographs showing examples of these profiles are shown in Fig 2.6 (a and b). Tracks 20 and 31 were from two of the samples which failed to give

complete profiles, although a partial profile (segments 1-4) was obtained for sample 20 by extending the autoradiographic exposure time. Samples 13, 17, 33 and 34 gave weak bands on the autoradiograph but complete profiles were obtained. The striking feature of all the genome profiles of the rotaviruses detected on this farm was that, within the limits of the methodology, no variation was detected. This suggested that, during the 7 month observation period on this farm, all the calves excreting rotavirus had been infected with the same, or a genotypically similar, strain.

2.2.3.2 Farm B.

Of the 43 rotavirus positive samples from this farm, 40 (93%) were successfully analysed. The three negative samples were in the group of 37 obtained during the second sampling period, September 1980 to January 1981. The genome profiles of the rotaviruses from the other 34 faecal samples showed no genomic variation; examples of the profiles from 10 of the samples are shown in Fig 2.7a. Six samples, taken between February and May 1980 from calves on the same farm, were also available and were analysed to extend the observation period. The profiles of four of these (nos. 107-110) and one from the later group (no.72) are shown in Fig 2.7b. The other two samples were successfully analysed but the bands were too faint for photographic reproduction. Therefore a diagrammatic representation of all six profiles and no.72 from the later group is shown in Fig 2.8. Only two of these six profiles (nos. 105 and 107) appeared identical; the others showed variations in the mobility of segments 4, 5, 6 and 11 such that the isolates could be distinguished. The distinct profiles were labelled B1 to B5. The B6 profile of sample 72, characteristic of the isolates in the later group from this farm, was found to be similar to the B3 type shown by sample 108 except that segment 4 appeared larger.

2.2.3.3 Farm C.

All the rotavirus positive samples from this farm were successfully analysed. Examples of the genome profiles are shown in Fig 2.9a and b. Several distinct profiles were observed from this farm and these are diagrammatically represented in Fig 2.10. Eleven of the 12 samples shown in Fig 2.9a gave C1 type profiles, the exception being sample 9 which was the C3 profile. Samples 13 and 14 (Fig 2.9b) again were C1 profiles but sample 15 was a C4 and samples 17 to 21 were C5 types. Sample 16 contained 3 RNA bands which appeared as doublets and suggested that this sample contained a mixture of two rotaviruses, one with a C4 and the other with a C5 type profile. Samples 22 and 23 showed very unusual profiles, type C7, in which the normal segment 11 was apparently missing and an extra segment between segments 6 and 7 was observed.

The chronological appearance of each of these profile types is shown in Fig 2.11. From October 1979 to March 1980 the predominant profile type was C1, with only single isolates of C2 and C3. In April 1980 the C4 type appeared but after the detection of a calf dually infected with both C4 and C5 types, the C5 type became dominant and continued to infect calves in May 1980. After the single isolate of the C6 type in August 1980 no further samples were available until September 1981 when the first of the eight C7 profile type viruses was observed. In October of that year the C7 type gave way to C8 and nine isolations of these viruses were made during November and December at which time sampling was terminated.

2.2.4 Determination of the number of RNA segments in a C7 type virus.

The C7 type profile was unusual for rotavirus and so it was important to determine the total number of genomic segments in these viruses to see if this number deviated from the normal 11. The RNA bands from virus isolates C5-175 and C7-176 (samples 21 and 22, Fig 2.9b respectively) were excised from the dried polyacrylamide gel, using the autoradiograph as a template. The gel pieces were added to 3ml toluene-based scintillation fluid and the amount of radioactivity in each

band was determined by scintillation counting. The results are shown in Table 2.2. Making the assumption that the virus C5-175 contained 11 segments of ds-RNA, the sum of the counts from all the bands were divided by 11 to give an average count per segment. The number of counts in the individual bands was then divided by the average count. As the RNA segments had been 3'-terminally labelled, each segment should contain the same number of counts and therefore the final values would be expected to indicate the number of genome segments contained in each autoradiography band. All 9 bands shown on the autoradiograph for C5-175 contained a single segment except bands 2 and 6 which both contained 2. When this method was applied to the 8 bands found for C7-176, again all contained a single segment except bands 2 and 7 which contained 2 and 3 segments respectively, giving a total of 11 segments. Therefore the segment found between segments 6 and 7, labelled 6a, was a single segment of an atypical size for rotavirus.

2.2.5 Detection of mixed rotavirus infections in calves

In this study only one out of a total of 119 samples successfully analysed showed evidence of mixed infection with two rotaviruses. It was therefore important to determine whether mixed infections were rare or whether the techniques employed could not detect them. To investigate the latter possibility, mixtures containing known amounts of ds-RNA from two rotavirus isolates with disparate genome profiles (UK strain and a C7 type) were used to simulate the type of samples that might be recovered from calves concurrently infected with these two viruses. After 3' terminal labelling, the RNA segments were analysed by electrophoresis (Fig 2.12). Only samples b to f convincingly showed evidence of being mixed profiles, which meant that in a faecal sample containing two rotaviruses, if one virus was present at a concentration 10-fold less than the other, it might not be detected.

2.3 DISCUSSION

Reynolds (1983) carried out a longitudinal study of the incidence of rotavirus excretion by calves showing mild or no clinical signs of infection. Herds were selected where calves showed either mild or no enteric disturbance during the pre-weaning stages of life. The mild symptoms, including changes in faecal colour or consistancy and/or anorexia, were such that the stockman was confident that his herd was free from enteritis and no veterinary attention was necessary. Under these conditions, in the 3 herds tested, approximately 50% of all the calves excreted rotavirus during the first 5 weeks of life and of these 32% showed no clinical signs of infection. Some or all of the mild symptoms, shown by the other 68% of calves that excreted rotavirus, may not have been the result of rotavirus infection but due to other infectious agents, either alone or in combination, or even to dietary changes (Roy, 1980).

Two methods for the generation of rotavirus genome profiles were attempted. The first utilised the fact that field isolates of rotavirus, even though they could not be propagated by serial passage, would replicate to a limited extent through two serial passes in susceptible tissue culture cells. The viral RNA could then be labelled with [^{32}P] which, after extraction and the separation of individual genome segments by electrophoresis, could be detected by autoradiography. However, although this method was used to analyse the rotavirus isolates from one Farm it was replaced by the 3' terminal labelling method because of the possibility that not all rotaviruses would propagate in cell culture and also, if mixed infections with two rotaviruses were common, then only the more easily grown isolates might be detected. Other problems with this method included the time taken to produce a profile (2-3 weeks), the requirement of large amounts of [^{32}P], and the involvement of a large number of manipulative steps.

The method for labelling the 3' terminus of rotavirus RNA genome segments required the efficient separation of the virus from faecal material; this was achieved by fluorocarbon extraction. No further purification step was necessary, only the

concentration of the virus by centrifugation prior to RNA extraction. The labelling of the RNA required only small amounts of radioactivity and as many as 20 RNA samples could be labelled in 24h. This method was also found to be up to 1,000-fold more sensitive for the detection of rotavirus genome segments in polyacrylamide gels than ethidium bromide staining and took only slightly longer to produce a profile, exposure times usually being 12-18h. Herring *et al.* (1982) used silver staining to visualise the genome segments in polyacrylamide gels and reported a 20- to 40-fold increase in sensitivity over ethidium bromide. Therefore 3'-terminal labelling of the rotavirus genome segments is the most sensitive method, to date, for the determination of genome profiles.

Of the 138 calves found excreting rotavirus on the 3 Farms, insufficient faecal sample was available from 6 (4%) calves to perform genome profiles. There were also 8 (6%) faecal samples examined which yielded insufficient RNA to produce a profile, suggesting they contained only small numbers of virus particles. This was probably due either to a low virus excretion rate, or to the peak of virus excretion being missed because samples were collected only 3 times a week. Even with these limitations 90% of the rotaviruses obtained from infected calves on these 3 farms were successfully analysed.

The results clearly show the information that can be obtained about the variation between field isolates of rotavirus by using genome profile analysis. During this work it was assumed that viruses having similar genome profiles and isolated from a single farm were identical. However, a cautionary note comes from experiments, carried out by several groups, on rotavirus genome segments, from different strains, which co-migrate in polyacrylamide gels. Using the techniques of either RNA/RNA (Flores *et al.*, 1982b) or RNA/DNA (Street *et al.*, 1982; Pedley & McCrae, 1984) hybridisation it was shown that co-migration of gene segments should not be used to indicate genetic identity. Further evidence was provided by Clarke and McCrae (1982) who found some evidence of changes in primary structure of co-migrating gene segments by the separation of partial RNase digests of isolated segments in a single dimension. This was corroborated by

Desselberger *et al.* (1986) by the more sensitive method of oligonucleotide mapping of isolated genome segments. The above assumption may still be valid, however, when comparing viruses obtained from farms taken over a short time period, but may not be valid when viruses from different locations are analysed or when study is carried out over a longer time period.

Two techniques for labelling the viral RNA were used and the success rate for producing genome profiles was higher, 96.5%, when 3'-terminal labelling was used as against 87.5% for the *in vitro* labelling method. However, this difference was probably not significant, as relatively low numbers of samples were analysed. Because of the low level of replication of virus, on primary passage in CK/1 cells, large cultures were needed and lengthy procedures were required to extract the small amounts of viral RNA. Coupled with the fact that large amounts of radioactivity ([^{32}P]) were needed, this technique was considered to have limited value and after the examination of samples from Farm A was replaced by the 3'-terminal labelling method.

Comparison of the viruses analysed from the 3 farms showed that although considerable variation was found between isolates from Farm C, those from Farms A and B were a homogeneous population endemic on each farm. Farms A and B were both closed dairy herds which may have resulted in the establishment of a single rotavirus strain that was found in all infected animals. Farm C had a calf rearing unit which accepted calves not only from its own dairy herd but also from neighbouring farms, including the bull calves from Farm A. Therefore the opportunity for introducing new rotavirus strains was greater. The genomic variation observed in isolates from this farm was not confined to any particular genome segment or group of segments, a finding similar to that reported by Rodger and Holmes (1979) for calves infected with rotavirus but showing clinical signs of disease. This farm showed a similar epidemiological profile to that seen in human populations (Rodger *et al.*, 1981; Buitenwerf *et al.*, 1983; Spencer *et al.*, 1983; Follett & Desselberger, 1983; Chiba *et al.*, 1984; Rodriguez *et al.*, 1983). A more extensive study

involving the supply farms may have shown that the spread of rotavirus strains from population to population was similar to that described by Albert *et al.* (1983) for isolated human villages in Papua, New Guinea.

The chronological phasing in and out of the rotavirus variants on Farm C could have been the result of competition between the strains, involving factors such as antigenic variation, competition for the available sites of replication in the gut and the availability of susceptible animals. This chronological phasing of rotavirus strains was first described by Espejo *et al.* (1979 and 1980b) and Croxon and Bellamy (1979) who carried out short term surveys of rotavirus strains from infected human infants confined to hospital because of enteritis. In a longer survey by Rodger *et al.* (1981) covering 7 years, a constantly changing population of rotavirus variants was found, with some dominant types persisting for periods of a year or more with concomitant infections with other types. Since 1981, there have been numerous surveys on similar human infant populations from many parts of the world which describe similar results. It appears that this epidemiologic feature requires the constant introduction of new strains into a constantly changing population in confined accommodation. Although the infections on Farm C were mainly asymptomatic, there were epidemiological features common to the surveys of human rotavirus which could account for the similar findings on this farm. Schnagl *et al.* (1981), by genome profile analysis, conducted a survey over 4 years of rotaviruses from an area of Central Australia where the population was scattered and isolated. They reported little genomic variation, with one virus strain predominating. This result was different from that reported for an isolated community in Papua, New Guinea (Albert *et al.*, 1983) where only one genome type was found in the 33 samples examined whereas in 24 samples from an urban area in the same district, 6 different genome profiles were observed. Although in all these cases the rotaviruses were obtained from the faeces of children with enteric disease, the findings reported above from the closed herds on Farms A and B were similar to those found in isolated human communities, whereas the open Farm C was similar to an urban community.

The mechanisms involved in the generation of rotaviral genome variation have yet to be determined. Analogy with the genomic variation in influenza viruses would suggest that both genomic "shift", with the interchange of genomic RNA segments, and genomic "drift", due to a slow and progressive accumulation of sequential point mutations, may be involved (Laver and Webster, 1979). Evidence for genomic "drift" among rotaviruses has been presented by Follett and Desselberger (1983), who found minor differences in the oligonucleotide maps of reciprocal genome segments from viruses indistinguishable by genome profile and isolated from the same outbreak of infantile gastroenteritis. These minor differences would not account for the genome variation observed here but the accumulation of such mutations may well alter the mobility of genome segments in polyacrylamide gels. There have been many recent papers reporting the nucleotide sequence of rotavirus genes and, in some cases, several reports containing the sequence of the same gene from different virus isolates. For example, the nucleotide sequence of segment 6 from 6 different rotavirus isolates have been described, simian SA11 (Estes *et al.*, 1984), human Wa (Both *et al.*, 1984), bovine RF (Cohen *et al.*, 1984), human 1076, porcine Gottfried and equine H-2 (Gorziglia *et al.*, 1988b) and, with the exception of the H-2 strain which has 6 extra bases contained within the open reading frame (ORF), all the genome segments contained 1356 bases with a single ORF 1181 bases long. A similar finding has been reported for segments 4 (5 virus strains) and 7 (13 virus strains) which were 2362 bases (ORF 2328 bases) and 1062 bases (ORF 978 bases) long respectively. These results would suggest that the variation in electrophoretic migration is not due to segment size differences between isolates. Although individual segments contain the same number of bases there is considerable variation in sequence and hence base composition and this may be the cause of changes in electrophoretic migration. Kantharidis *et al.* (1988) analysed the nucleotide sequences of segment 4 from bovine UK, human RV-5 and simian SA11 strains and reported %A+T values of 57.7%, 68.0% and 63.4% respectively. However, there is as yet no direct evidence to link the variation in electrophoretic mobility and the base composition of the segment.

Evidence of genomic "shift" in rotaviruses was reported by Street *et al.* (1982) who found the same genome segment in isolates with different profiles by RNA hybridisation analysis. Further evidence for the reassortment of rotavirus genes occurring in nature has been reported by Hoshino *et al.* (1987) and Midthun *et al.* (1987) following RNA hybridisation and comparative serological analysis of the porcine isolate SB-1A. Experimental evidence for genome reassortment *in vivo* was provided by Gombold & Ramig (1986) who isolated reassortant viruses from intestinal homogenates of mice infected concurrently with two simian rotaviruses, SA11 and RRV. The interchange of genome segments between rotaviruses *in vitro* has been documented by Matsuno *et al.* (1980) who co-infected cell cultures with two strains of virus and, by genome profile analysis of the cloned progeny, found that genome segments had been reassorted. The prerequisite for segment exchange *in vivo* would be the concurrent infection of animals with two different rotaviruses. In this survey, only one calf was shown to be excreting two rotaviruses concurrently (C4 and C5 types), giving an incidence rate of 0.8%. This value appears low considering the number of different virus strains circulating in Farm C. However, reports from surveys of human rotaviruses quoted similar rates, 0.4 - 1.9% (Buitenwerf *et al.*, 1983; Forster and Pastor, 1983; Dimitrov *et al.*, 1984; Svensson *et al.*, 1986; Tam *et al*, 1986). The exception was the report of Spencer *et al.* (1983) who found a 10% incidence rate of mixed genome profiles in a total of 149 samples examined. The low incidence found in the present study might have reflected the inability of the technique to detect mixed infections. To test this, mixtures of RNA from two viruses with disparate genome profiles were prepared to mimic the type of sample expected from an animal dually infected with 2 rotavirus strains. Analysis of these samples showed that a mixed infection would only be easily detected when the concentration of each virus was within a 10-fold range and suggested that many mixed infections might be missed. The work of Sabara and Babiuk (1983) also showed that progeny rotavirus excreted by infected animals were not genetically homogeneous. They were able to show considerable genetic heterogeneity among bovine rotaviruses, contained in a single sample, by using a series of high percentage polyacrylamide gels, up to 17.5%, this

heterogeneity was not evident when 7.5 or 10% gels were used. Therefore, care should be taken when viruses from field material are used for subsequent experiments involving growth either *in vivo* or *in vitro* without prior cloning.

While most of the isolates from Farm C gave typical genome profiles (C1 to C6 and C8), eight samples produced profile type C7, which differed from the normal rotavirus profile in that segment 11 was replaced by an extra band between segments 6 and 7. This finding was not an artifact as this type of profile was found in samples from 8 different calves over a period of 1 month. The isolates were confirmed as rotaviruses by the fact that they were detected with a rotavirus specific ELISA and they contained 11 segments of double-stranded RNA. It would be impossible to explain the generation of viruses with this type of profile by either genomic "drift" or "shift" and therefore there must be a third mechanism for the occurrence of genomic variants. Further characterisation of these C7 viruses was carried out and is discussed in Section 6.

Table 2.1

Comparison of the infectivity assay and genome profile analysis for the detection of rotavirus in daily faecal samples taken from an experimentally infected calf.

Days p.i.	1	2	3	4	5	6	7	8
Infectivity [1]	ND[2]	0.6	5.6	4.4	3.8	2.3	< 0.5	< 0.5
Total counts [3]	ND	3.5	435.0	320.0	160.0	87.0	18.0	5.8
Complete profile	ND	+	+	+	+	+	+	+

[1] Virus titre $\log_{10}TCID_{50}$ ml^{-1} of 1:10 dilution of faeces

[2] Not done

[3] Total number of ^{32}P counts incorporated x 10^{-3}

Table 2.2

Analysis of a gel containing RNA from two rotavirus isolates C5-175 and C7-176 to determine the number of genome segments in each autoradiography band.

C5-175				C7-176			
Band number	C.p.m.	cpm band n / av. band cpm	No. of segments	Band number	C.p.m.	cpm band n / av. band cpm	No. of segments
1	1,312	1.07	1	1	1,161	1.04	1
2	2,437	1.99	2	2	2,166	1.95	2
3	1,158	0.95	1	3	1,137	1.02	1
4	1,271	1.04	1	4	1,010	0.91	1
5	1,370	1.12	1	5	1,334	1.20	1
6	2,193	1.79	2	6	1,124	1.01	1
7	1,285	1.05	1	7	3,221	2.90	3
8	1,212	0.99	1	8	1,074	0.97	1
9	1,219	1.00	1				
Total	13,457		11		12,227		11
Av. cpm/band	1,223				1,112		



Fig 2.1: Autoradiograph of a polyacrylamide gel containing rotavirus RNA labelled with [^{32}P] during virus growth in cell culture. The RNA was extracted from virus released from cells by freeze/thawing and concentrated by centrifugation. Tracks 1 and 4 were loaded with RNA from the UK rotavirus strain and tracks 2 and 3 with RNA from 2 rotaviruses cultured from faecal samples.

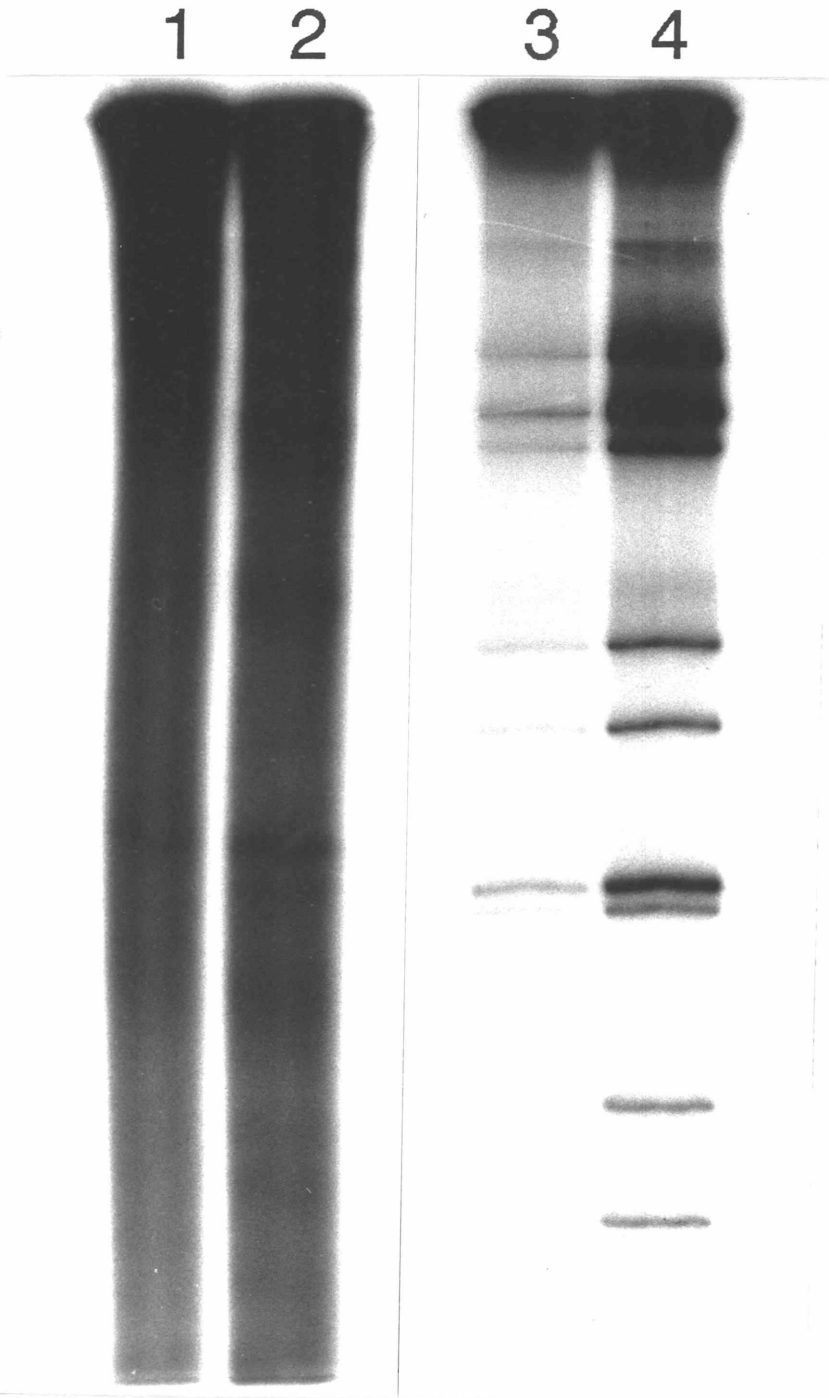


Fig 2.2: Autoradiograph of a polyacrylamide gel containing rotavirus RNA labelled with [^{32}P] during virus growth in cell culture. The RNA was extracted from detergent lysed cells infected with virus from 2 different faecal samples. The viral RNA was separated from cellular RNA by CF11 cellulose chromatography. Tracks 1 and 2 were loaded with RNA which did not absorb to CF11 cellulose in the presence of 15% ethanol and tracks 3 and 4 with the absorbed RNA which eluted with buffer containing 0% ethanol.

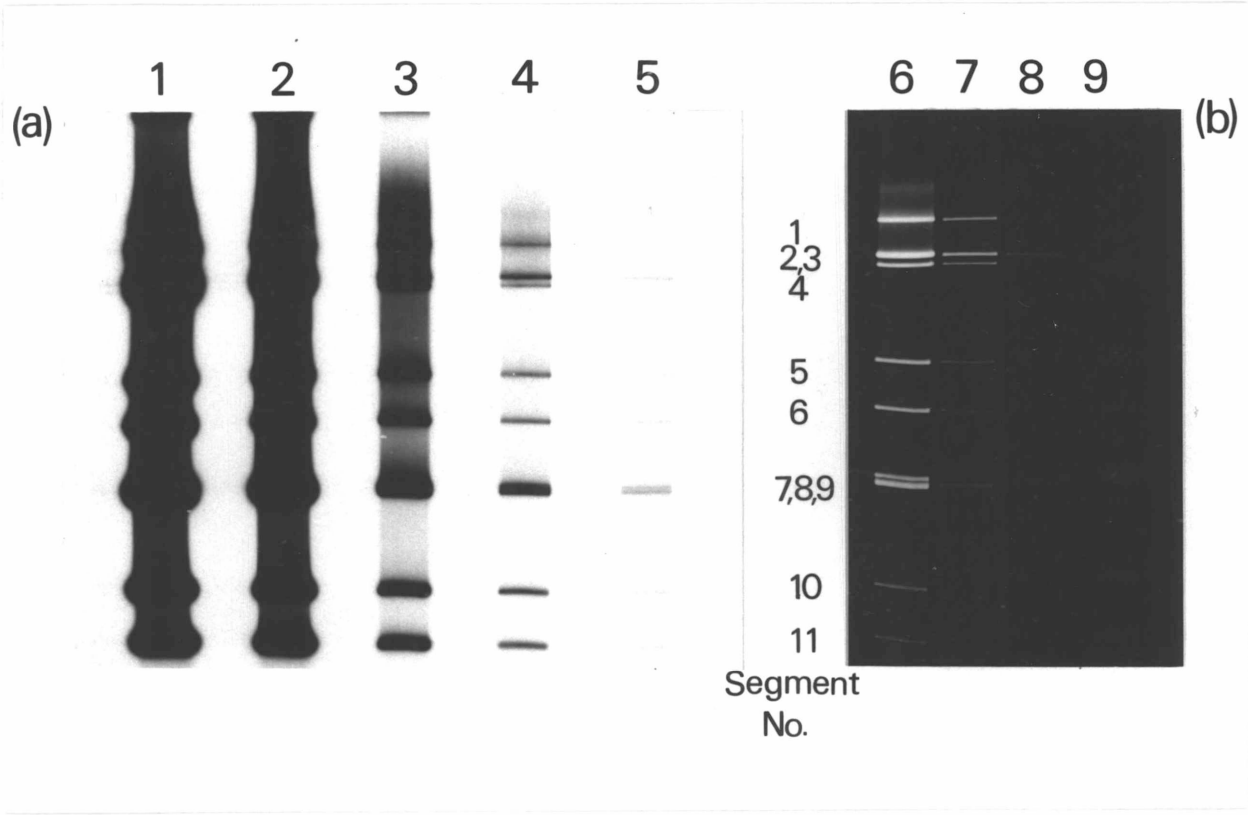


Fig 2.3: Comparison of 3'-terminal labelling of rotavirus RNA with [^{32}P] followed by autoradiography (a) and ethidium bromide staining (b) for the detection of individual genome segments after electrophoresis in polyacrylamide gels. Tracks 1-5 contained 3.125μg, 625ng, 125ng, 25ng and 5ng, and tracks 6-9 contained 5μg, 1μg, 200ng and 40ng rotavirus genomic RNA respectively.

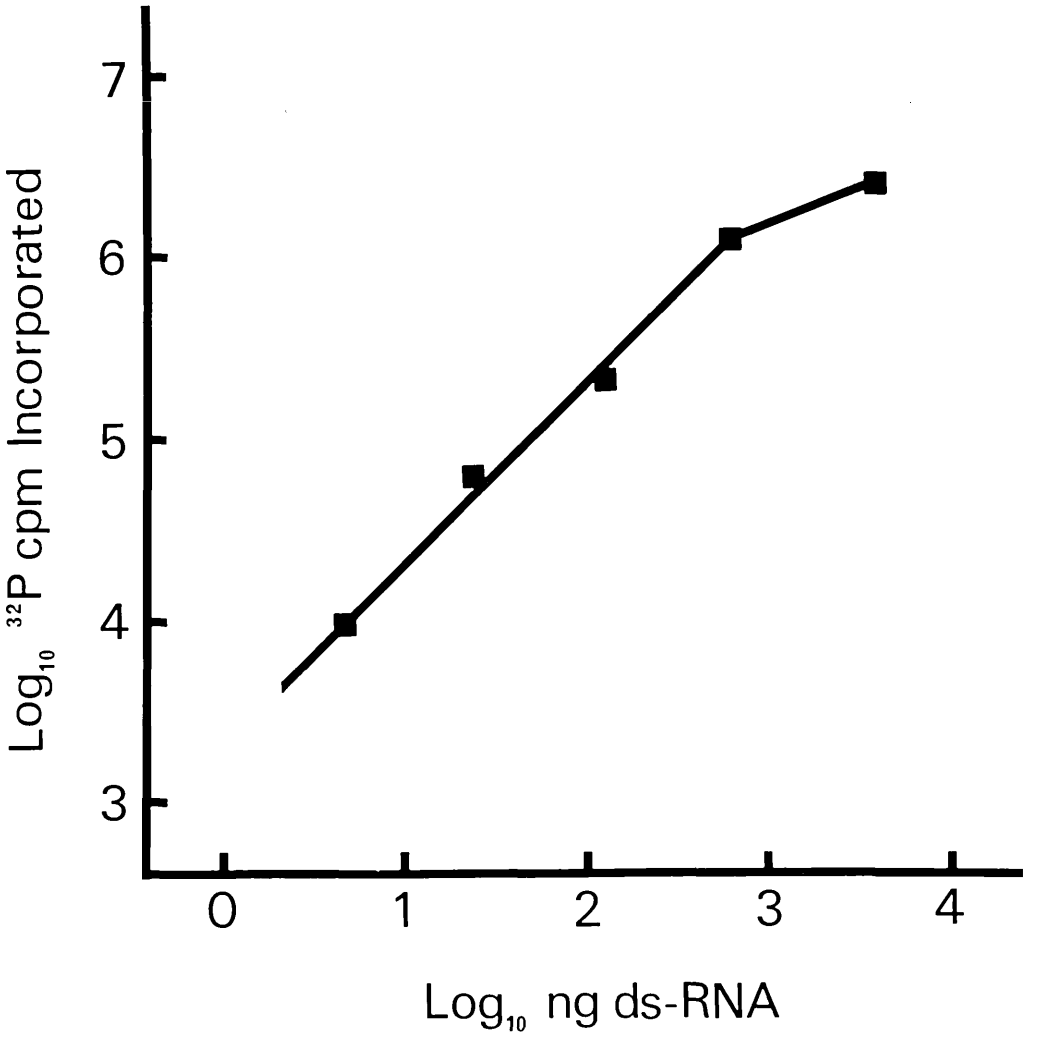


Fig 2.4: Incorporation of [^{32}P] into rotavirus genomic RNA by 3'-terminal labelling as a function of the amount of RNA present in the reaction mixture.

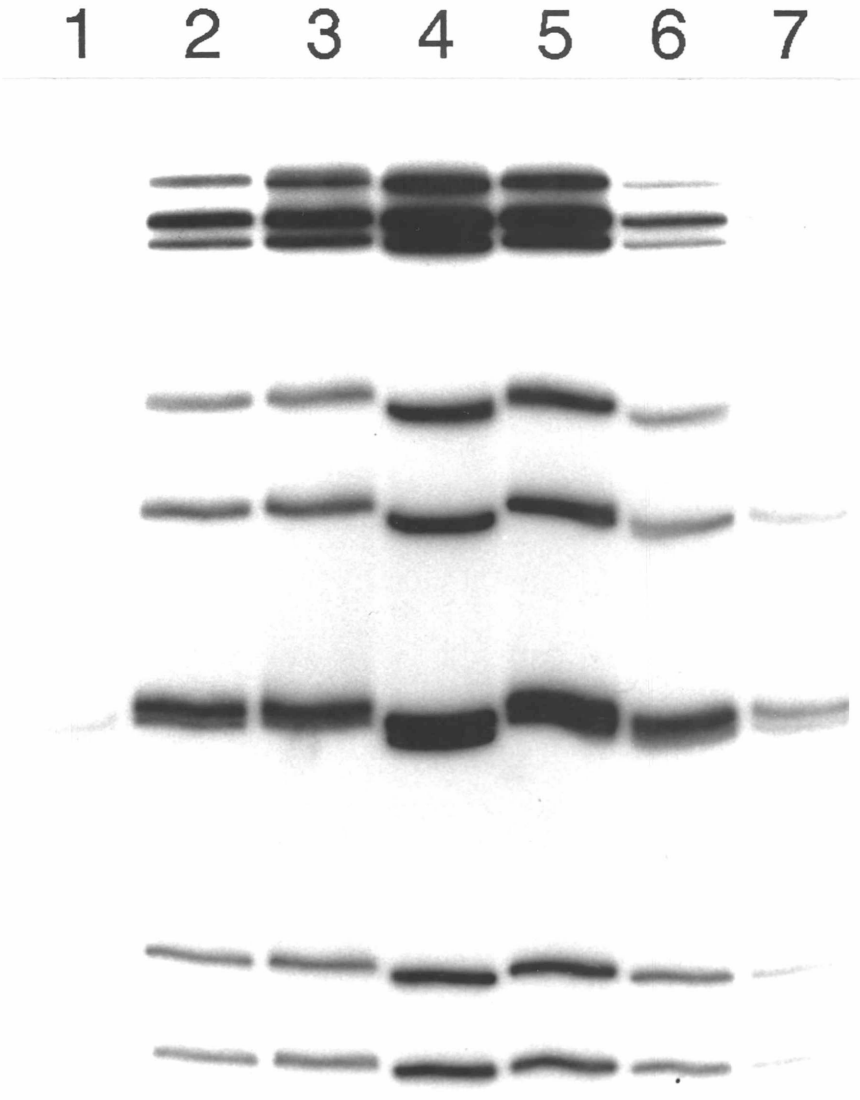


Fig 2.5: Detection of rotavirus genome profiles of virus extracted from daily faecal samples of an experimentally infected calf. The viral RNA was 3'-terminal labelled with [^{32}P] and the genome segments separated by electrophoresis. Tracks 1-7 contained the RNA extracted from faecal samples collected on days 2-8 post infection. For days 2, 6, 7 and 8 the total amount of labelled RNA was loaded, but for days 3-5 an amount containing 20,000 c.p.m. was used.

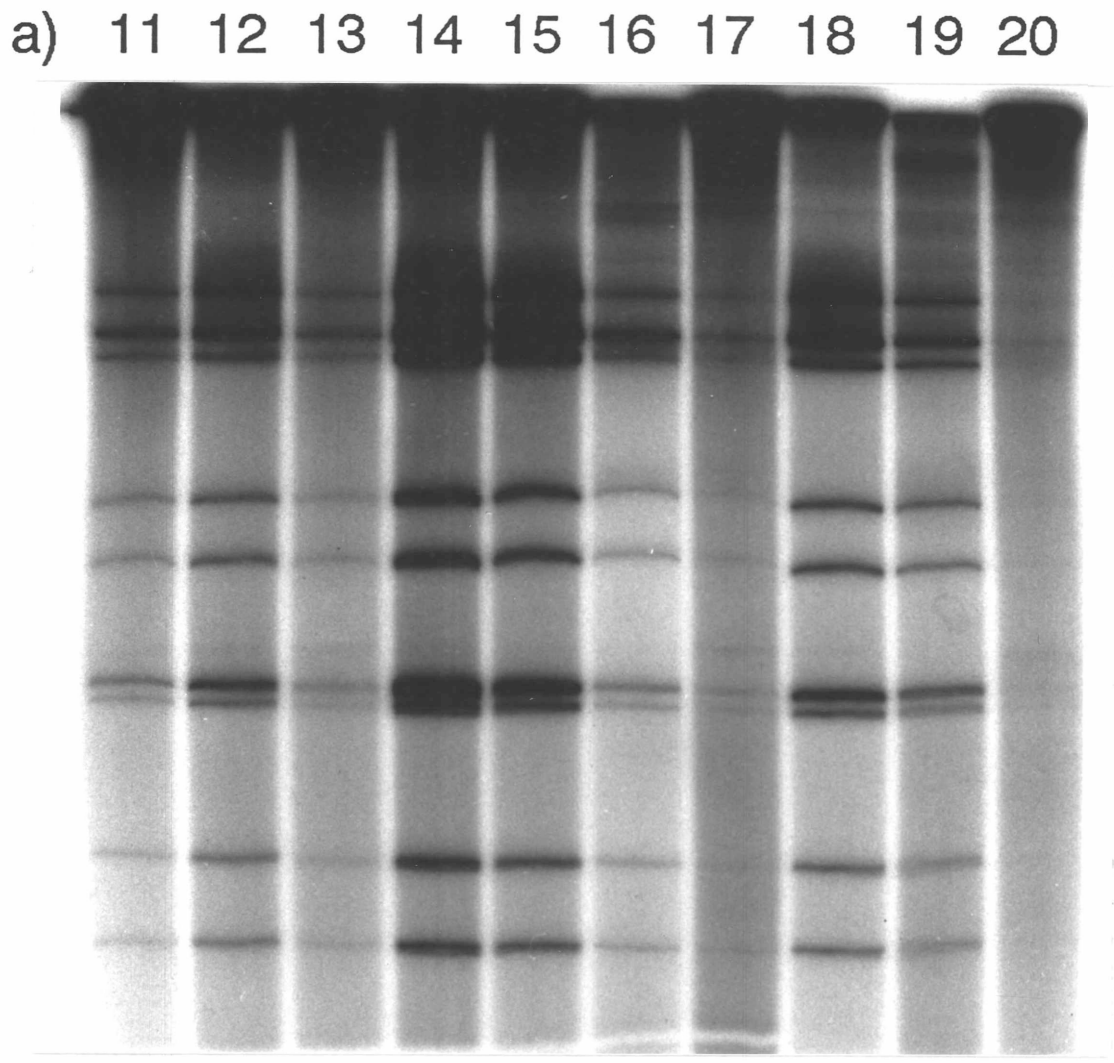


Fig 2.6a: Analysis of the genome profiles of rotaviruses from Farm A. Viral RNA was labelled with [^{32}P] during the growth of the viruses in cell culture. The genome segments were separated by electrophoresis and detected by autoradiography. The profiles for samples 11-20 are shown in (a) and samples 30-39 in (b) - see next page.

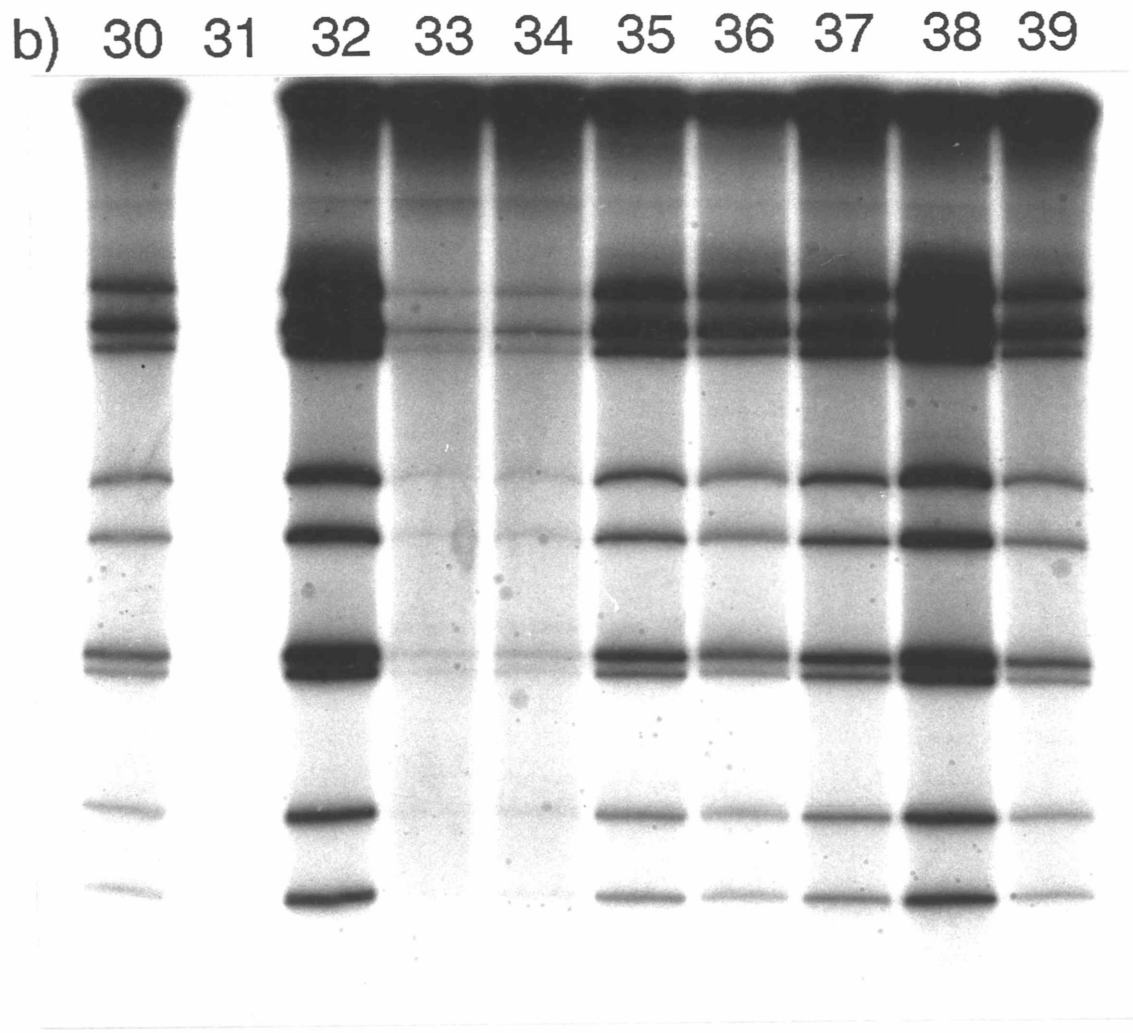


Fig 2.6b: For legend see Fig 2.6a on previous page.

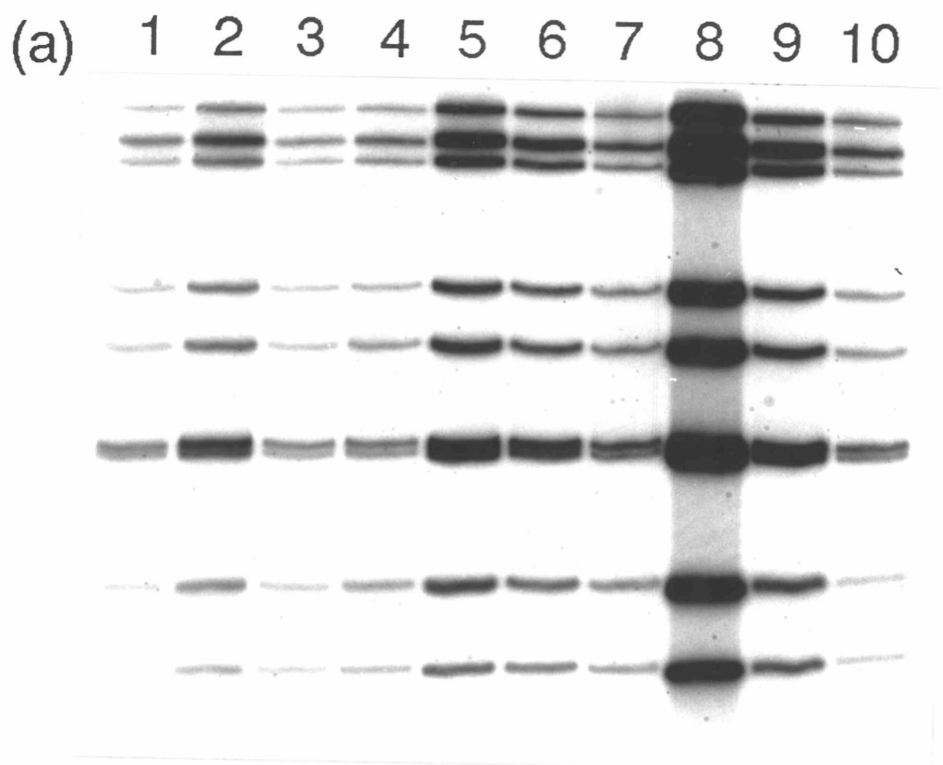


Fig 2.7a: Analysis of the genome profiles of rotaviruses from Farm B. Viral RNA was labelled with [^{32}P]-pCp by 3'-terminal labelling. The genome segments were separated by electrophoresis and detected by autoradiography. The profiles from samples 65-74 are shown in (a) and samples 105-110 and 72 in (b) - see next page.

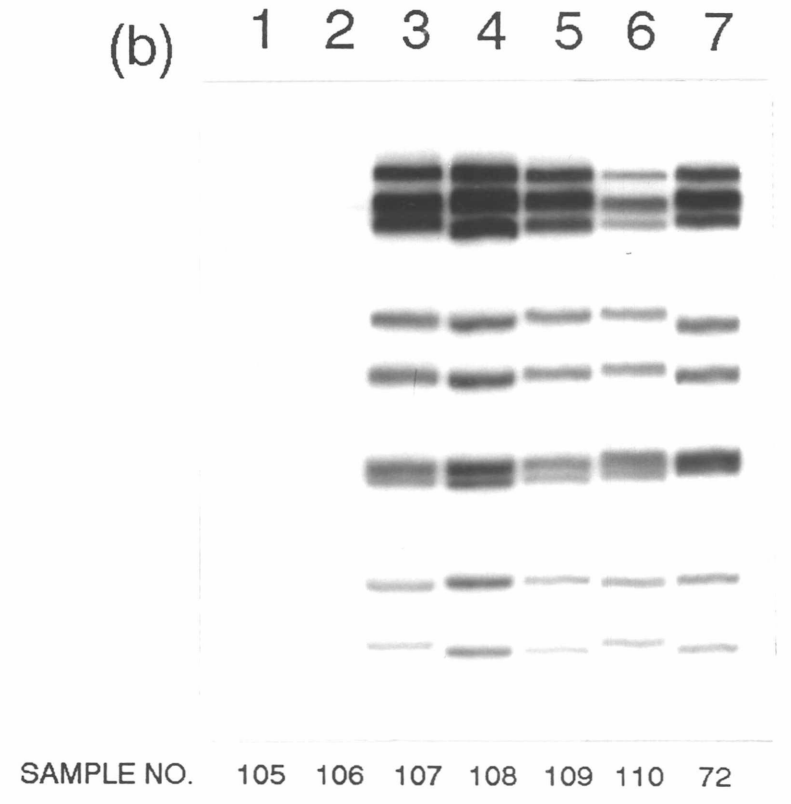


Fig 2.7b: For legend see Fig 2.7a on previous page.

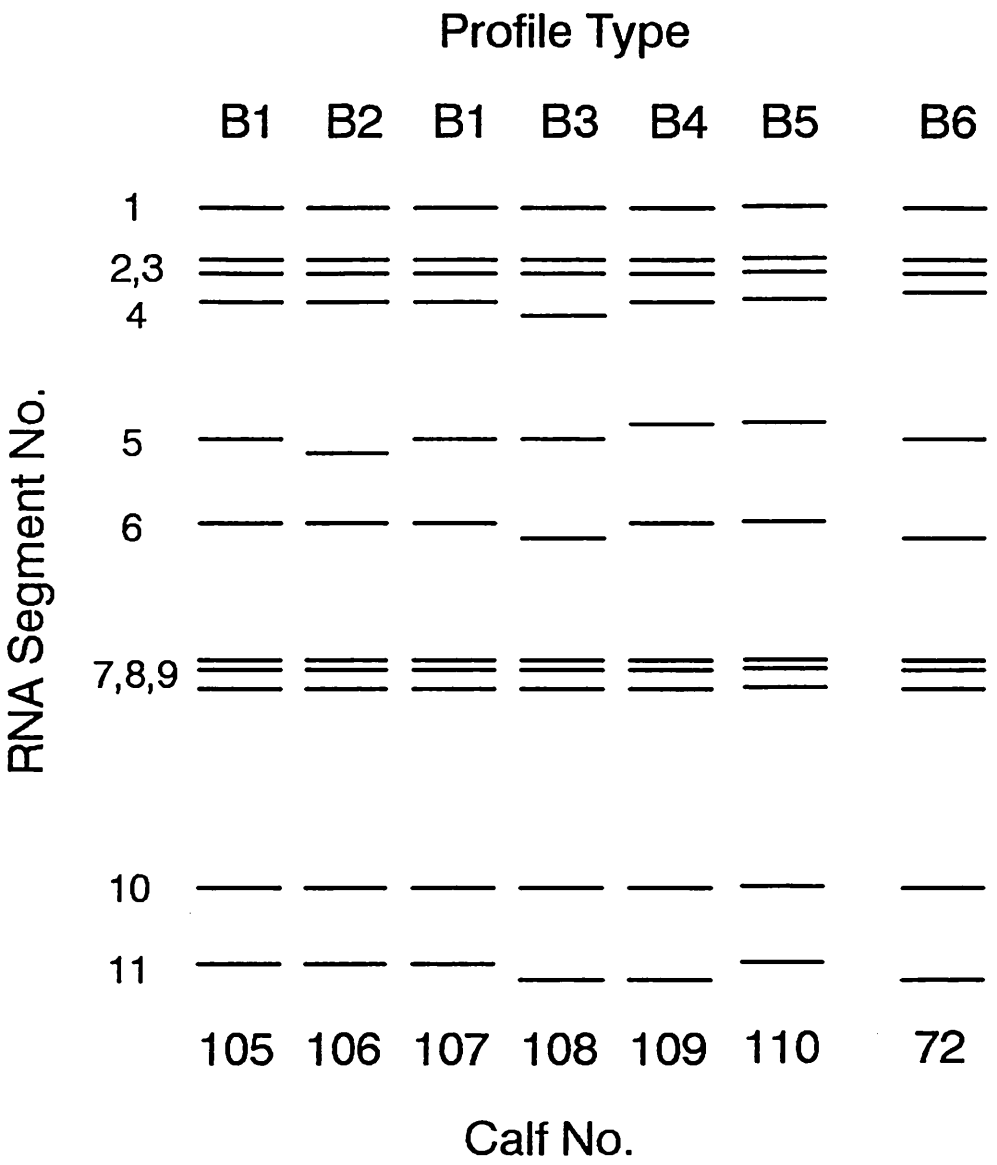


Fig 2.8: Diagramatic representation of the genome profiles for the rotaviruses shown in Fig 2.7(b).

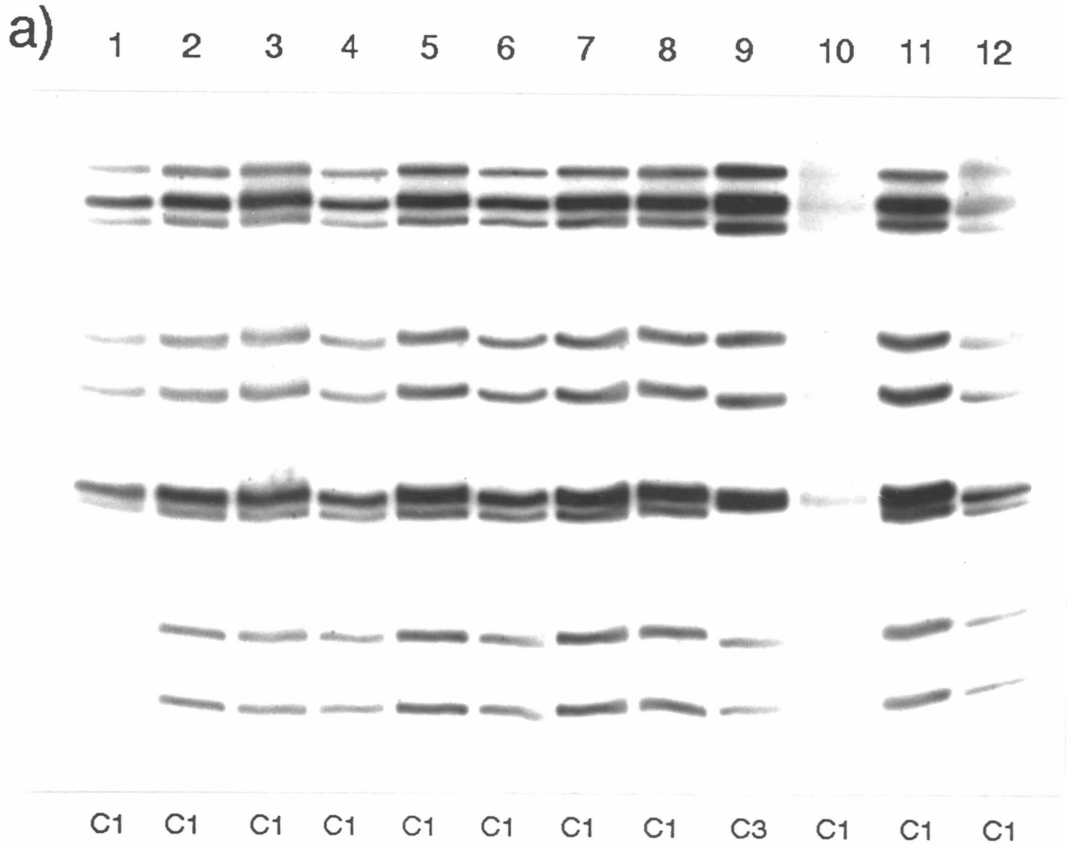


Fig 2.9a: Analysis of the genome profiles of rotaviruses from Farm C. Viral RNA was labelled with [^{32}P] by 3'-terminal labelling. The genome segments were separated by electrophoresis and detected by autoradiography. The profiles from samples 1-12 are shown in (a) and samples 13-23 in (b) - see next page.

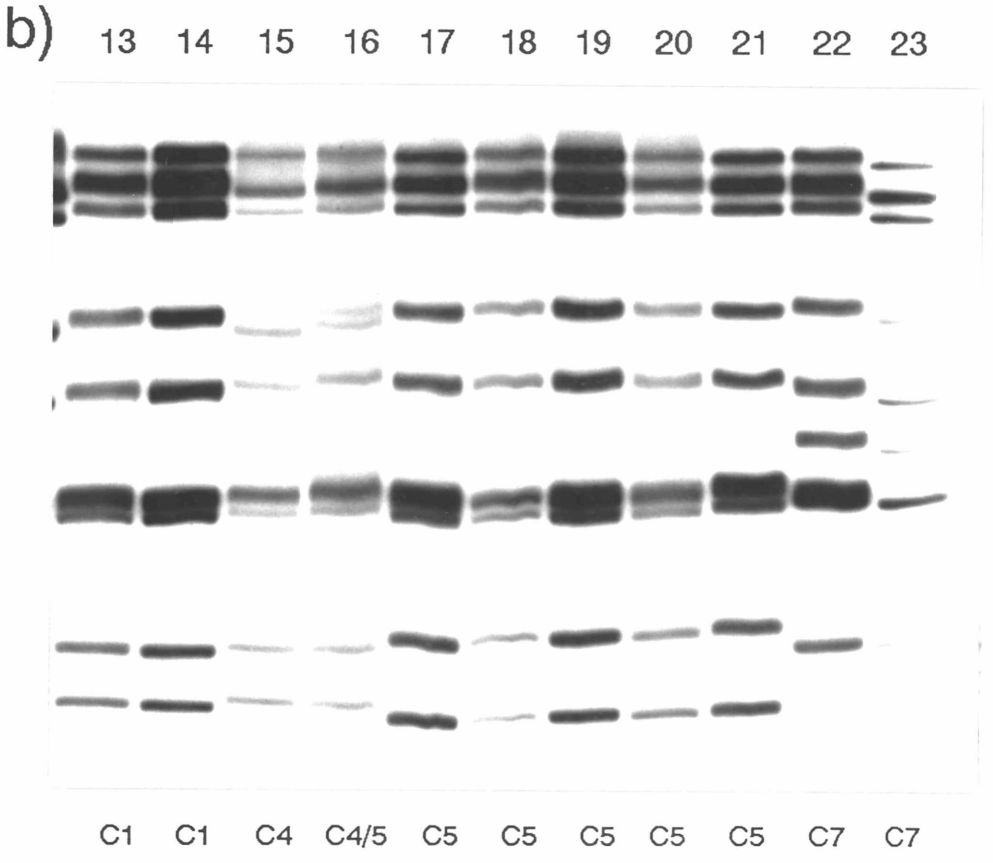


Fig 2.9b: For legend see Fig 2.9a on previous page

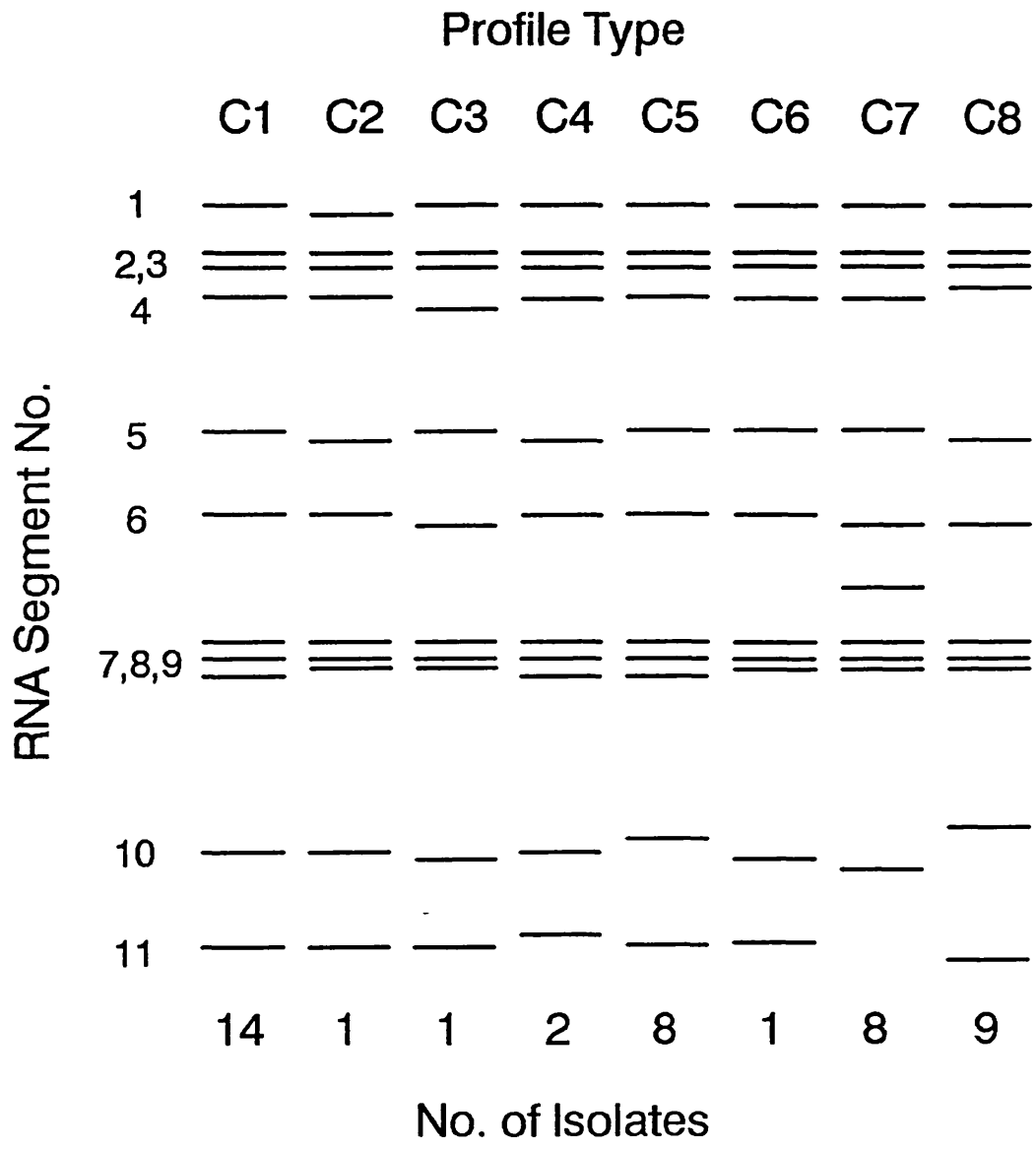


Fig 2.10: Diagramatic representation of the different genome profiles C1 - C8 for the rotaviruses detected on Farm C.

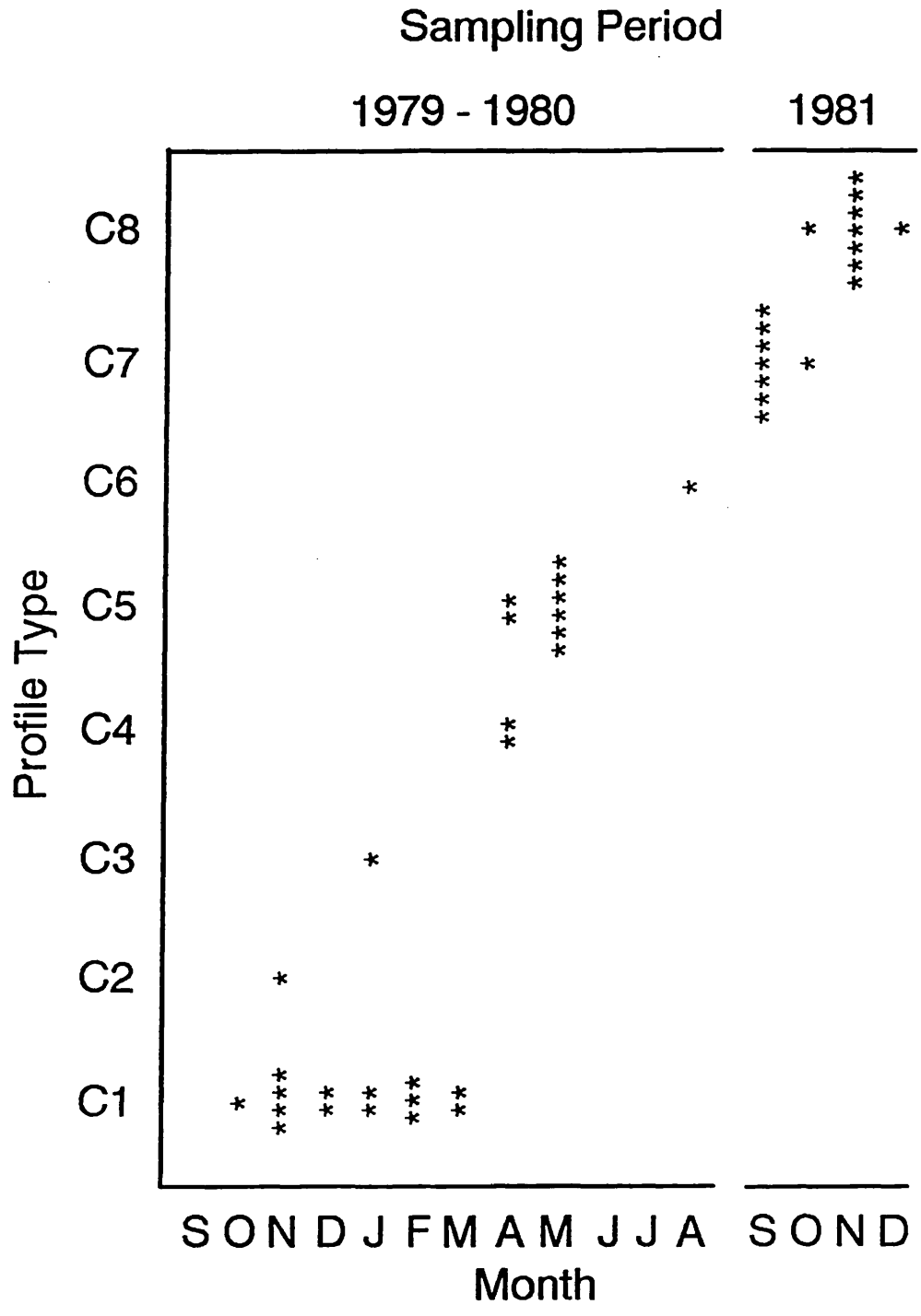


Fig 2.11: The chronological appearance of each genome profile type detected on Farm C. Each * denotes a rotavirus isolation of a given profile type from a faecal sample collected in a given month during the period of study on this Farm.

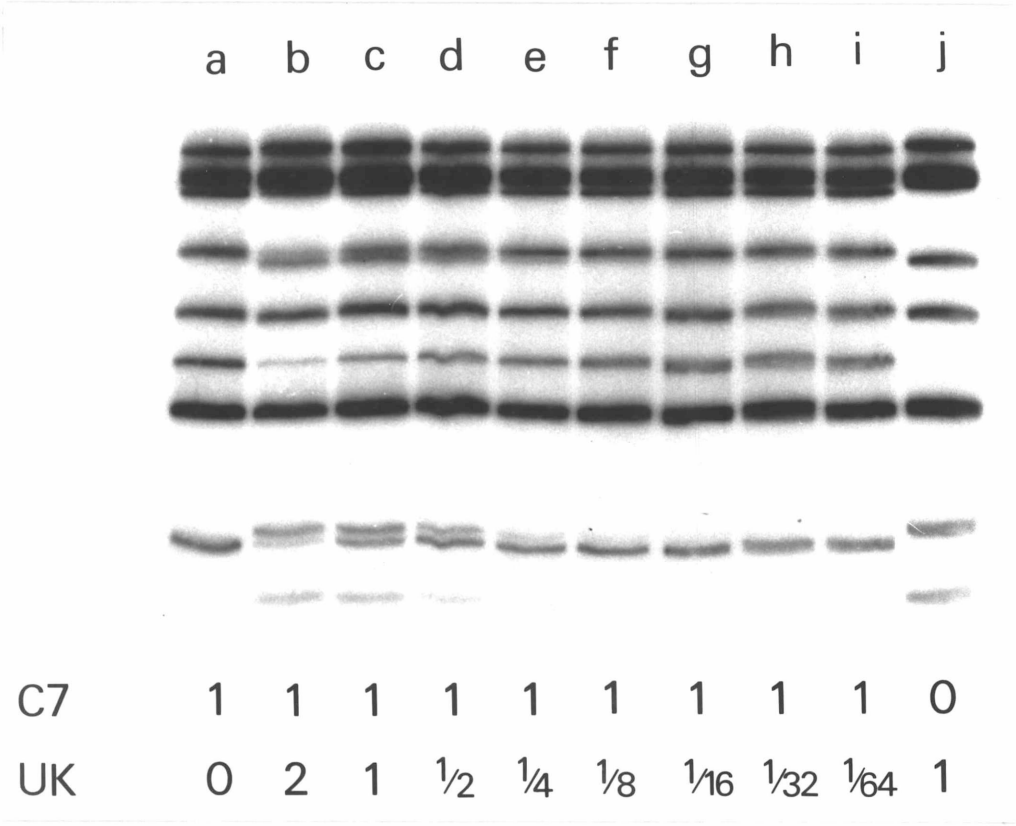


Fig 2.12: Evaluation of genome profile analysis for the detection of heterologous mixtures of rotavirus RNA. Tracks a-j contained mixtures of a C7 profile type and UK strain rotavirus genomic RNAs mixed in the proportions shown. The mixtures were 3'-terminal labelled with [^{32}P]-pCp and aliquots containing equal numbers of counts were electrophoresed.

3.0 CELL CULTURE ADAPTATION OF FIELD ISOLATES OF ROTAVIRUS

3.1 MATERIALS AND METHODS

3.1.1 Cell cultures

The continuous Rhesus monkey kidney cell line MA104 (Microbiological Associates, Los Angeles, U.S.A.) was maintained in Eagle's minimal essential medium (MEM) containing 0.2% sodium bicarbonate, 5% foetal bovine serum, 60μg/ml penicillin, 100μg/ml streptomycin and 50U/ml mycostatin. The cultures were grown in Roux flasks and passed weekly, with a split of 1 Roux flask to 5. Cells were used for virus growth and assay when confluent monolayers had formed, normally 4 days after passing. Prior to inoculation with virus the cell monolayers were incubated for 24h with the above medium without serum. The same serum-free medium containing 0.5μg/ml trypsin (Type XI; Sigma Chemical Co. Ltd., Poole U.K.) was used as maintenance medium for infected cultures.

3.1.2 Viruses

Three bovine virus isolates, labelled UK, PP-1 and CP-1, were all isolated from a single outbreak of diarrhoea in the Institute's herd. Pooled faeces from the outbreak were serially passaged in several gnotobiotic calves and the three viruses were isolated in cell cultures at different times from animals at different passage levels. The UK strain was the original cell culture adapted strain described by Bridger and Woode (1975); the PP-1 strain although derived from bovine faeces was found to be pathogenic in pigs (Hall *et al.*, 1976); the CP-1 strain produced diarrhoea in experimental calves (Woode *et al.*, 1974). A porcine rotavirus, PRV, obtained from a pig herd with a history of diarrhoea has been described by Woode *et al.* (1976). The other viruses used in this study came from Farms B and C, described in Section 2.

3.1.3 Optimisation of UK rotavirus growth in MA104 cells

3.1.3.1 Effect of pH.

Monolayer cultures of MA104 cells in 2oz flat glass bottles were inoculated with UK strain at a m.o.i. of 1. After incubation at 37^{0}C for 1h the inoculum was removed and maintenance medium buffered with 0.1% sodium bicarbonate and 20mM N-2-hydroxyethyl piperizine-N-2-ethane sulphonic acid (HEPES) was added. To achieve the required pH sterile 4N sodium hydroxide was added at the rate 0.375ml/100ml medium to give pH 7.8, 0.25ml for pH 7.5, 0.125ml for pH 7.1 and none to give pH 6.8. The cultures were incubated for 24h at 37^{0}C and then frozen at -20^{0}C, thawed and the virus assayed.

3.1.3.2 Effect of temperature.

MA104 cultures infected as above and maintained with maintenance medium without HEPES buffer were incubated at temperatures ranging from 31^{0}C to 41^{0}C in 2^0 steps. To achieve the correct temperature control the cultures were immersed in water baths adjusted to the required temperature. Cultures were harvested after 24 and 48h incubation and assayed for virus after freezing and thawing.

3.1.4 Assay for virus infectivity

Virus infectivity was assayed by the method of Bridger and Brown (1981). Serial 10-fold dilutions of the virus samples were prepared in maintenance medium and each dilution inoculated into duplicate wells of a 96 well flat bottomed microtitre plate (A/S Nunc, Denmark) containing confluent monolayers of MA104 cells. The plates were then centrifuged at 300g for 1h and incubated at 37^{0}C for 18h. The medium was removed and the cell sheets fixed with cold 80% acetone for 15min and dried. Virus infected cells were identified by indirect immunofluorescence assay. The fixed cells were incubated with bovine anti-UK rotavirus serum, diluted 1:400, for 1h at room temperature, washed 3 times with PBS, incubated for a further 1h with fluorescein-conjugated rabbit anti-bovine serum, diluted 1:80 (Nordic

Laboratories Ltd., Maidenhead, U.K.), washed a further 3 times with PBS and viewed under a Leitz microscope with a UV fluorescence attachment. The number of foci comprising single cells or groups of cells showing fluorescence were counted in each well and the results expressed as $\log_{10}$ fluorescing focus units per ml (ffu/ml).

3.1.5 Plaque assay

The method of Matsuno *et al.* (1977) was used to produce viral plaques in MA104 cell monolayers. Confluent monolayers contained in 50mm petri dishes (Sterilin, Feltham, U.K.) were inoculated with 0.4ml virus sample and incubated at 37^{0}C for 1h. The inoculum was removed and the monolayers overlaid with 5ml maintenence medium containing 0.6% Miles agarose (Miles Scientific, Stoke Poges, U.K.). After 3-5 days incubation 5ml of the same overlay but containing 0.0025% neutral red was added to each dish. Plaques were visible after overnight incubation.

In order to observe the effect of pH on the number and size of the plaques obtained, dishes were overlaid with agarose containing medium buffered to the desired pH with HEPES as described above.

3.1.6 Virus isolation and cloning

The methods described by Fukusho *et al.* (1981) and Sato *et al.* (1981) for the primary isolation of rotavirus were used. The procedure required the treatment of faecal virus with trypsin prior to inoculation onto rolled tube cultures of MA104 cells.

After extraction of 10% faecal suspensions containing virus with an equal volume of fluorocarbon (Arklone-P), the virus was pelleted from the aqueous phase by centrifugation at 75,000g for 90 min, resuspended in 0.02M Tris/HCl buffer pH 7.4 and stored at -70^{0}C. This material was diluted 10-fold with maintenance medium and trypsin added to give a final concentration of 5μg/ml prior to incubation at 37^{0}C for 30 min. Aliquots (100μl) of the trypsinised virus preparations were inoculated

onto confluent tube cultures of MA104 cells. After absorption the inoculum was removed, the cell sheets were washed once with 1ml trypsin-free maintenance medium and incubated with 1ml maintenance medium containing 0.5μg/ml trypsin. The tubes were rolled (10 rev/hr) at 37^{0}C and observed daily for c.p.e. When the c.p.e. affected >50% of the cell sheet the tubes were frozen at -70^{0}C, thawed, the cell debris removed by low speed centrifugation and the supernatant stored at -70^{0}C for further passage.

After 3 serial passages the viruses were cloned three times by plaque purification. Plaques were produced as described above.

3.1.7 Viral RNA analysis

Cell culture grown virus was concentrated by centrifugation at 75,000g and the viral RNA extracted and 3' terminally labelled with [^{32}P]-pCp as described in Section 2.1.1.2. Genome profiles were produced after electrophoresis in 10% polyacrylamide gels followed by autoradiography.

3.2 RESULTS

3.2.1 Effect of pH and temperature on rotavirus growth in MA104 cells

Although the pH of the culture medium, in the range 6.8 to 7.8, did not significantly affect the titre of virus produced after 24h incubation (Table 3.1), the c.p.e. was much more extensive at the higher pH values. After 24h at pH 7.8, however, the control uninfected cells were beginning to show degeneration and therefore in all subsequent experiments the medium was buffered at pH 7.4 - 7.5.

The yields of virus, harvested at 24 and 48h post infection, after incubation at temperatures between 31^{0}C and 41^{0}C are shown in Table 3.2. After 24h incubation titres of virus from cultures incubated at temperatures between 33 and 39^{0}C were similar, whereas titres after incubation at 31 and 41^{0}C were up to 1 $\log_{10}$ lower.

Titres of virus after 48h incubation at 31 to 37^{0}C increased above the levels detected at 24h. However, the virus yields after 48h at 39^{0}C and 41^{0}C were 1 $\log_{10}$ lower than at 24h, suggesting some instability of the virus at these higher temperatures. The level of c.p.e. increased with the increase in incubation temperature; even after 48h incubation at 31^{0}C very little c.p.e. was seen whereas at 41^{0}C c.p.e. was extensive. Uninfected control cultures remained good after 48h incubation at all temperatures except 41^{0}C where cells were beginning to degenerate. Although this experiment showed that incubation at any temperature between 33 and 37^{0}C was optimum for both virus yield and c.p.e., 36-37^{0}C was used for further experiments.

3.2.2 Effect of pH on rotavirus plaque size and number

As the pH of the culture was shown to affect the extent of the c.p.e. but not necessarily the yield of the UK strain, it was perhaps not surprising to find that as the pH was increased the size of the plaques increased whereas the number of plaques remained constant (Table 3.3). After 5 days incubation at pH 7.8, however, the cell monolayers showed diminished uptake of the neutral red suggesting both a lowering of the cellular metabolic rate and cell death. Subsequent plaque assays were performed at pH 7.4 - 7.5.

In this experiment HEPES was used in the culture medium in order to give good buffering at the desired pH. As an alternative buffer system to maintain the culture medium at pH 7.4, the bicarbonate/CO_2 was also tested and found to give improved results. No difference in the number of plaques was seen but the length of incubation required to give 2mm diameter plaques was reduced from 5 days to 2 days. The cells appeared to be more metabolically active as judged by both the appearance of the cell sheets and the increased uptake of neutral red. This buffer was used in all subsequent experiments.

3.2.3 Isolation and cloning of rotavirus from faecal samples

Faecal samples containing rotavirus were selected from Farms B and C. From Farm B only those samples from the period Sept. 1980 to Jan. 1981 were available for virus isolation. As all the rotaviruses analysed during this period had similar genome profiles, two samples were selected which contained the largest amount of viral RNA as judged by the number of counts detected after 3'-terminal labelling. From Farm C samples containing viruses with different genome profiles were selected. Again when more than one sample was available for a given profile type two samples were selected as described above.

All the samples containing the selected virus types, except those with C2 and C8 type profiles, gave c.p.e. on the first passage in rolled tube cultures of MA104 cells, in the presence of trypsin. The failure to cultivate the C2 type virus was probably due to the low level of virus present in the single faecal sample available. The reason for the lack of growth of the C8 type virus was unclear, but after repeated attempts with different samples the virus still failed to propagate. In the successful isolations the viruses all gave c.p.e. within 7 days post inoculation, but the incubation time was reduced during the subsequent two passes with c.p.e. evident 2-3 days post inoculation during the third pass. The titre of virus was either maintained after each cell culture pass (e.g. C3-160; titre of faecal sample: $\log_{10}$ 6.5; titre after 3 passes: $\log_{10}$ 6.5) or increased (e.g. C7-183; titre of faecal sample: $\log_{10}$ 4.5; titre after 3 passes: $\log_{10}$ 6.5).

During cloning all the virus isolates produced cytopathic plaques at the first attempt, taking 3-4 days to give plaques 1-2mm diameter. Well separated plaques were selected for further cloning.

3.2.4 Genome profile analysis of cloned rotavirus isolates.

Following plaque purification, the genome profiles of all the isolates were checked against the profiles determined for the viruses in the original faecal samples. Fig 3.1a shows the profiles of isolate C1-165, which revealed that the pattern obtained from both the faecal sample (track 1) and the final cloned virus (track 2) were identical. Fig 3.1b shows a similar result for isolate C3-160, with the profiles for the faecal virus (track 1) and virus from each of the three plaque purifications (tracks 2 to 4) being identical. This result was found for each of the isolates, except for isolate C6-167. With this isolate the profile of the finally cloned virus was clearly different from the original faecal virus (Fig 3.2; tracks 5 and 1 respectively). Samples of virus from the initial three cell culture passes were then analysed (Fig 3.2; tracks 2 to 4) and revealed that on the first pass extra feint bands were detected in the region of segments 1, 4, 6, 9 and 10 suggesting a mixed infection of the cell culture. This mixed infection could have arisen from the contamination of the sample during the processing of the faecal sample prior to cell culture inoculation. The procedure was therefore repeated with fresh material again with the same result suggesting that the calf had been infected with more than one rotavirus and the less dominant virus in the faeces grew more readily in MA104 cells. In the profile from cell culture pass 2 (Fig 3.2; track 3) the second virus became dominant and no trace of RNA bands in the position expected for the faecal virus were observed. This was also true for the virus from the third pass (track 4) and the subsequent cloning stages. Attempts were then made to produce plaques from the faecal material, but these failed. Plaques were produced after inoculation of the virus from the first cell culture pass. A total of thirty virus plaques were picked and their genome profiles analysed. All thirty isolates gave profiles similar to the final C6-167 cloned virus and not the faecal virus. The profiles of ten of these viruses are shown in Fig 3.3 and although at first glance all ten looked identical, the profile in track 1 differed in the spacing of segments 7 and 8 from the other 9 viruses. Further attempts to isolate the C6 profile type isolate were abandoned.

An autoradiograph showing the genome profiles of all the cloned rotavirus isolates (except C4-169) is shown in Fig 3.4. Variation was clearly seen in the profiles of these viruses and was not confined to any particular segment or group of segments. It was interesting that the separation of segments 1 and 4 for the two pig pathogenic isolates, PP-1 and PRV, was greater than seen with any of the other viruses. However, bearing in mind the considerable variation seen in just a few viruses it would be dangerous to link aspects of genome profile variation with any variation in the biological behaviour of these isolates.

3.3 DISCUSSION

This Section describes the adaptation for growth in cell culture and subsequent cloning of the different genome profile types of rotavirus from Farm C and a representative of the viruses from Farm B.

Initial experiments showed that rotavirus grew to optimum titre in MA104 cells over a wide range of temperature, 33-39^{0}C, and pH, 6.8-7.8. Although c.p.e. was more evident at the higher temperatures and pH values, these conditions did not favour the maintenance of the cell cultures and so more physiological values of 37^{0}C and pH 7.4-7.5 were used. These same values were also found to be optimum for the production of virus plaques. To maintain the culture medium at pH 7.4-7.5, the addition of HEPES was unnecessary and replaced with the carbonate/bicarbonate buffer system.

Trypsin was used throughout all the isolation and cloning rotavirus in MA104 cells as suggested by Fukusho *et al.* (1981), Sato *et al.* (1981), Ramia and Sattar (1980), Urasawa *et al.* (1981) and Matsuno *et al.* (1977). Recently Agliano *et al.* (1985) reported the isolation of 8 human rotavirus strains directly from faeces without the aid of trypsin. It is possible that many more of the virus isolations reported in the literature may have been successful in the absence of trypsin, but as there is no evidence that trypsin treatment inhibits virus isolation most workers have

preferred to include it. It has also been reported that some rotavirus isolates produce plaques in the absence of trypsin (McCrae and Faulkner-Valle, 1981) but again most workers include it in the overlay medium in case it is required.

Under these conditions it was possible to isolate rotavirus from all the faecal samples attempted, except one, and to further serially propagate and clone the viruses. The one failure was probably due to the low level of virus in the faecal sample and as this was the only faecal sample found to contain this genome profile type, C2, it could not be analysed further. The cell culture grown viruses were checked by genome profile analysis to be certain that the isolates were similar to the original faecal viruses. This proved to be the case with all the virus strains except one, that isolated as C6-167. After primary isolation RNA bands corresponding to the C6 type profile were clearly visible but genome segments belonging to a different profile type were also detected. As has been mentioned rotaviruses have been difficult to culture and even with the various growth enhancing techniques they may still not be optimal for all strains. So if this animal was doubly infected with more than one strain of rotavirus which, as described in Section 2, is possible even though the original genome profile analysis of the faecal sample suggested only one virus was present, it is conceivable that a second virus may have adapted more readily to growth and passage in MA104 cells. Contamination of the faecal sample with another strain of virus appeared unlikely as repetition of both genome profile analysis and growth of virus in cell culture gave identical results. Unfortunately attempts to produce plaques directly from the faecal material failed, possibly because of the difficulty of the major virus present adapting to cell culture growth. Also attempts to isolate the C6 virus by plaquing the virus after one tube pass in MA104 cells also failed as the only viruses isolated were of the different RNA profile type. Despite not having the C6 profile the finally cloned virus from this faecal sample was included in further analyses. It remains however a warning that any rotavirus isolated in cell culture must always be checked by genome profile with the virus in origial sample.

It was interesting to note that the virus with the C7 type profile maintained its abnormal genome structure after isolation and cloning in cell culture. This supported the fact that the abnormal genomes of these viruses were not artefacts. Further analysis of these viruses is described in Section 6.

The genome profiles of the two porcine pathogenic rotavirus strains, PP-1 and PRV, could be distinguished from the profiles of the bovine strains by the increased separation of segments 1 and 4. This was due to both an increase in the size of segment 1 and a marked decrease in the size of segment 4. Genome segment 4 has been shown to code for a viral protein which determines the tissue specificity (Greenberg *et al.*, 1983a) and may also, therefore, be involved in the species specificity of rotaviruses.

Table 3.1

Effect of varying the pH of the cell culture medium on the titre of the UK strain of rotavirus grown in MA104 cells.

pH of medium	Virus titre 24h p.i.	Extent of c.p.e.
6.8	7.5 [1]	+ [2]
7.1	7.0	++
7.5	7.5	++
7.8	7.5	+++

[1] Virus titres were expressed as $\log_{10} TCID_{50} ml^{-1}$

[2] C.p.e. was recorded as + when <20% of the cell sheet was affected, ++ 20-80% and +++ >80%.

Table 3.2

Effect of temperature on the growth of UK rotavirus in MA104 cells.

Temperature (°C)	24h p.i.		48h p.i.	
	C.p.e.	Titre	C.p.e	Titre
31.5	-[1]	5.75[2]	+	6.5
33.1	+	6.50	++	7.5
35.2	+	6.00	++	7.0
37.3	+	6.50	++	7.5
39.1	++	6.50	+++	5.5
41.2	++	5.50	+++	4.5

[1] C.p.e. was recorded as + when <20% of the cell sheet was affected, ++ 20-80%, +++ >80% and - when no c.p.e. was evident

[2] Virus titres were expressed as $\log_{10} TCID_{50} ml^{-1}$

Table 3.3

Effect of varying the pH of the overlay medium on the size and number of plaques produced with a standard inoculum of UK rotavirus in MA104 cells

pH of medium	No. of plaques at 10^{-5} diln.	No. of plaques at 10^{-6} diln.	Plaque diam. (mm)
6.8	78 [1]	13	0.5-1.0
7.1	87	11	0.5-1.0
7.5	80	13	1.0-2.0
7.8	47	7	1.0-2.0

[1] Mean of the number of plaques from 3 replicate petri dishes

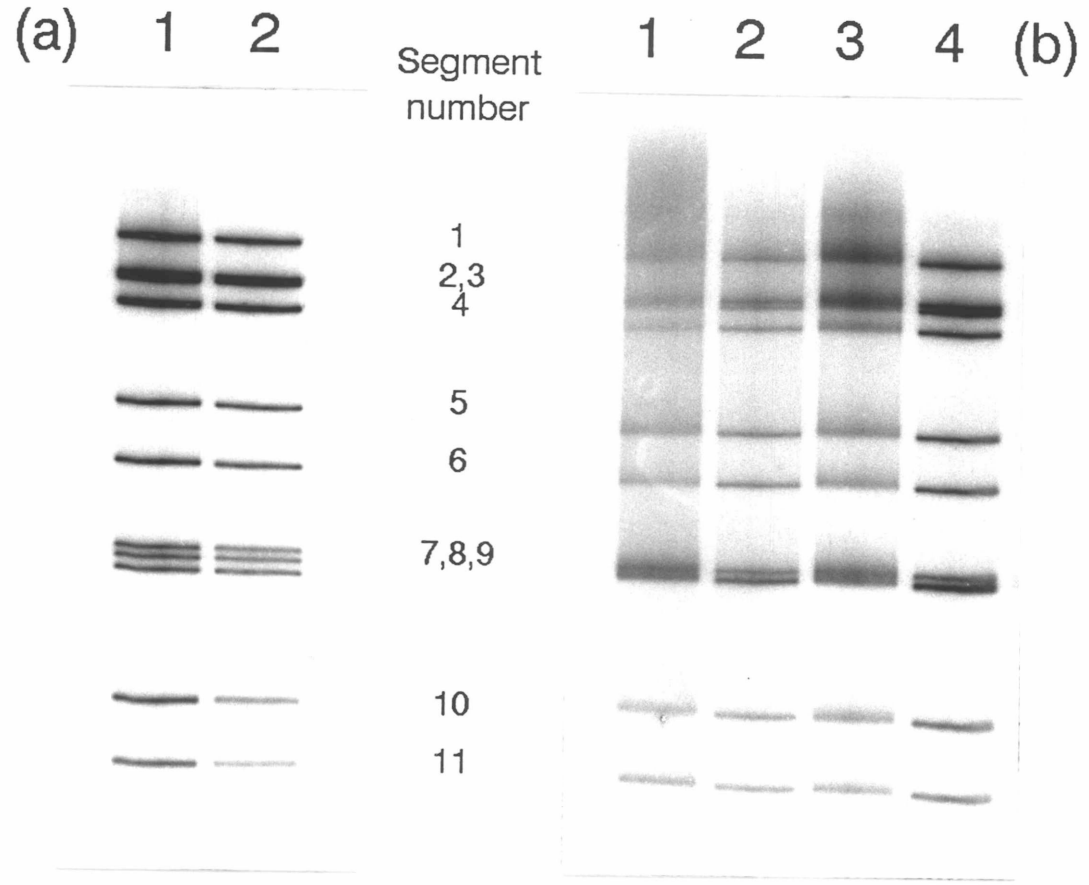


Fig 3.1: Genome profile analysis of cloned field isolates of rotavirus. The viral RNA was extracted from the original faecal sample and from samples taken during virus isolation and cloning. The RNA was 3'-terminal labelled and the genome segments separated by electrophoresis. The profiles of the faecal virus and the cloned C1-165 isolate are shown in (a) tracks 1 and 2 respectively. Profiles for the faecal virus and virus from the 3 cloning stages for C3-160 are shown in (b) tracks 1-4 respectively.

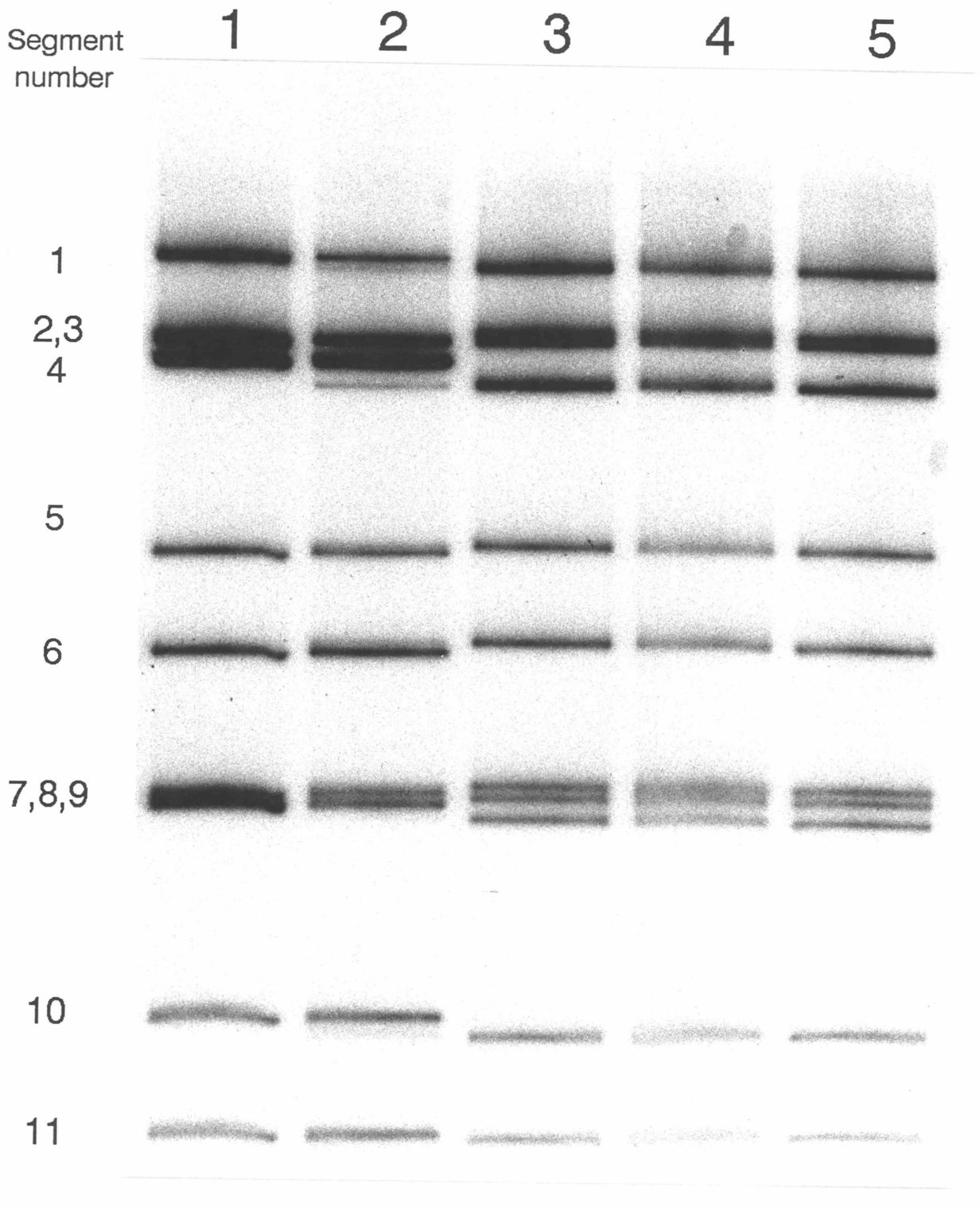


Fig 3.2: Genome profile analysis of virus samples taken during the cloning of C6-167. Track 1 contains RNA from faecal virus, tracks 2-4 virus from the initial 3 cell culture passes and track 5 virus cloned by 3 plaque purifications of cell culture pass 3. Viral RNA was 3'-terminal labelled and the genome segments separated by electrophoresis.

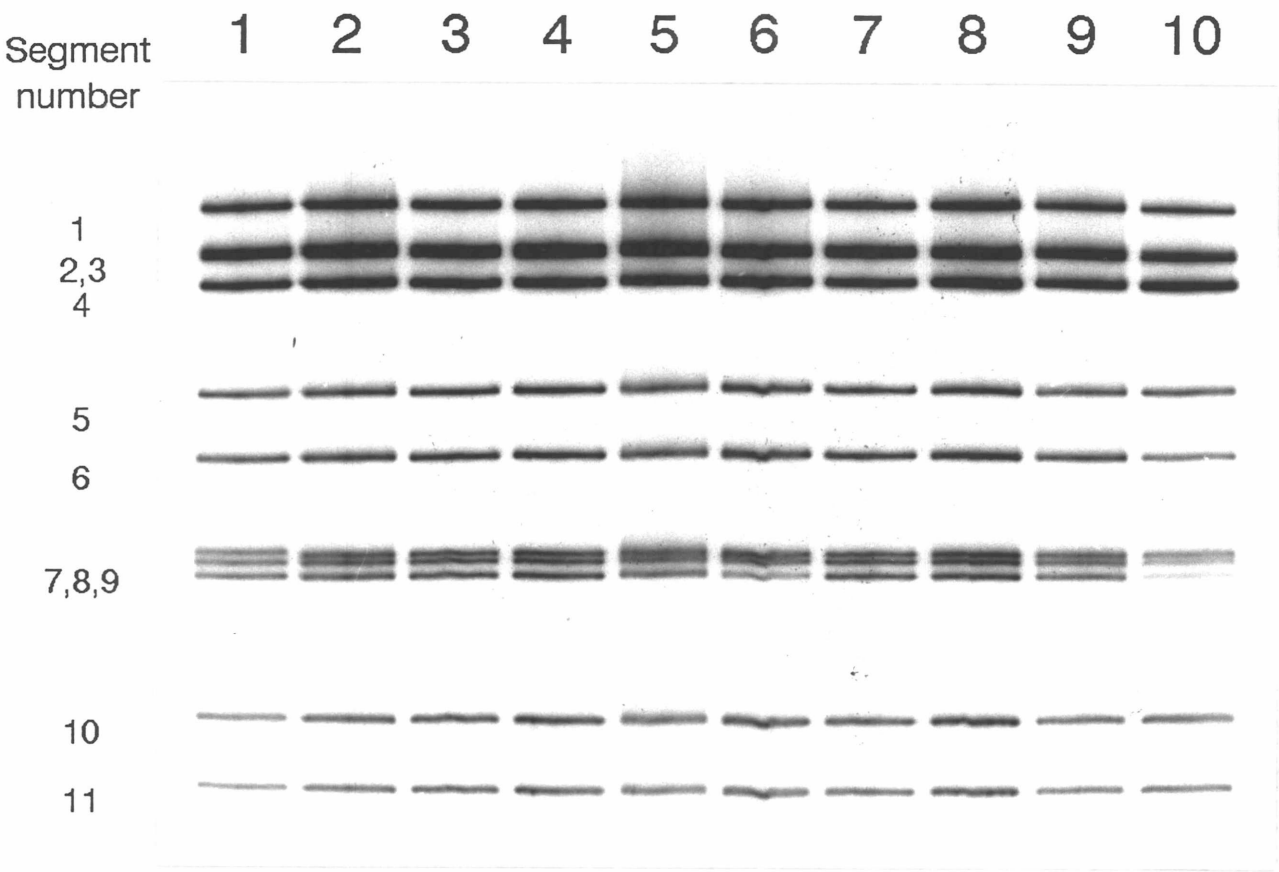


Fig 3.3: Genome profiles of the virus grown from 10 plaques, tracks 1-10, picked from plates infected with C6-167 cell culture pass 1.

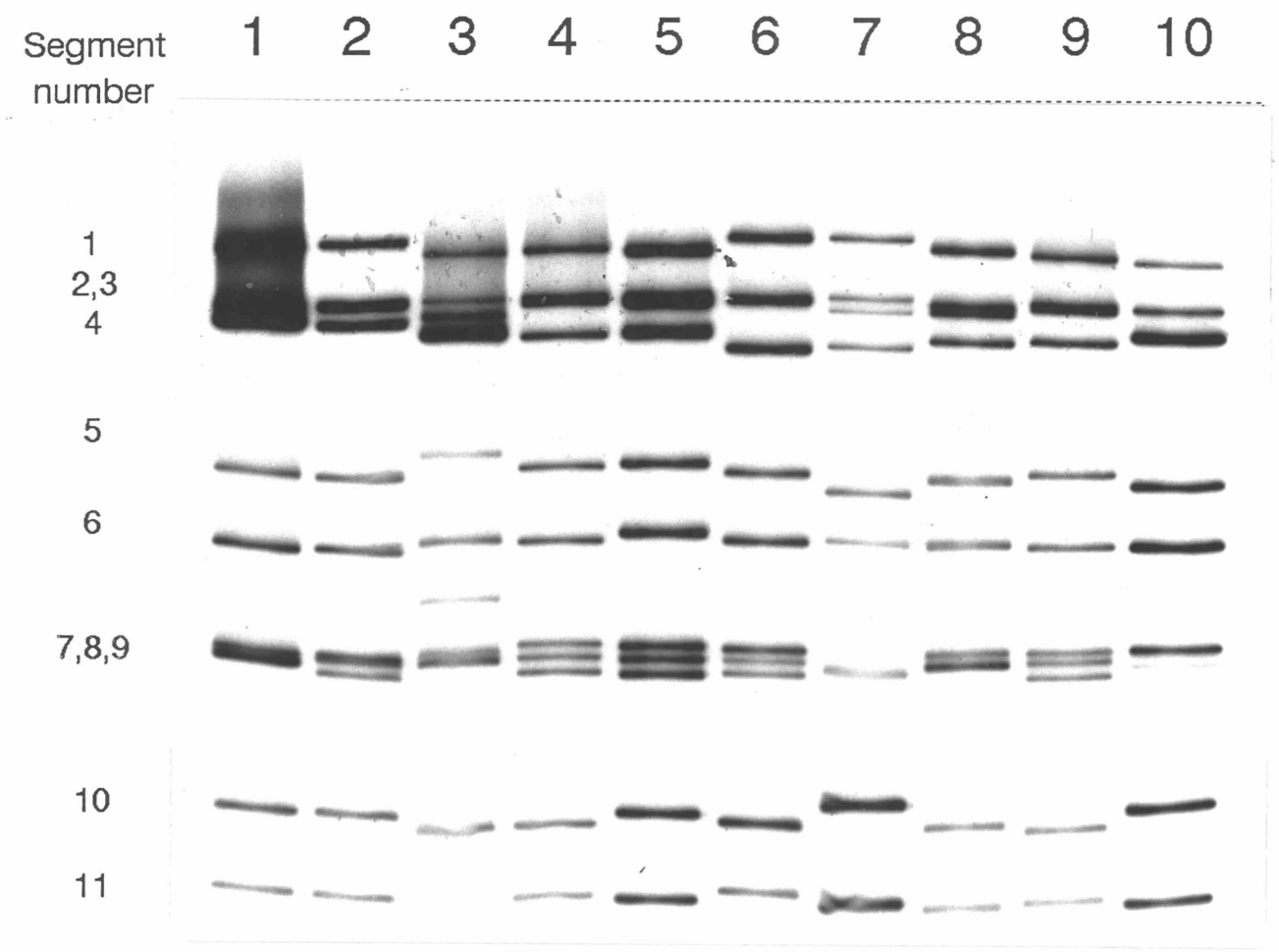


Fig 3.4: Genome profiles of the cloned bovine and porcine rotavirus isolates. Tracks 1-10 contained RNA from UK, CP-1, C7-183, C1-165, C5-175, PP-1, PRV, C3-160, C6-167 and B6-62 rotaviruses respectively.

4.0 ANALYSIS OF THE INTRACELLULAR POLYPEPTIDES ENCODED BY THE CLONED ROTAVIRUS ISOLATES FROM FARM C

4.1 MATERIALS AND METHODS

4.1.1 Analysis of virus induced intracellular polypeptides

Confluent monolayers of MA104 cells, contained in 25cm^2 tissue culture flasks (Falcon, Beckton Dickinson (UK) Ltd., Oxford U.K.) were inoculated with the virus isolates at a m.o.i. between 1 and 10 and incubated at 37^{0}C for 16h. After incubation for 1h in methionine-free medium the cells were pulse-labelled for 3h in methionine-free medium containing 25μCi/ml L-[^{35}S]-methionine ([^{35}S]-met; sp. act. >800 Ci/mmol, Amersham International plc, Amersham, U.K.). At the end of the labelling period the medium was removed, the cell sheets washed with cold isotonic (6%) glucose and the cells lysed in Laemmli sample buffer (62.5mM Tris/HCl pH 6.8; 2% SDS; 2% 2-mercaptoethanol;10% glycerol containing bromophenol blue (BPB) as marker). The samples were boiled for 2 min and, to aid pipetting of the samples, the cellular DNA sheared by passage through a 25 gauge needle to improve sample handling. Electrophoresis was performed in 10% polyacrylamide gels (Laemmli, 1970) at 100V constant voltage until the BPB dye band was 1cm from the bottom of the gel. The gels were treated with En^{3}Hance (NEN, Du Pont (UK) Ltd., Stevenage, U.K.), following the manufacturer's instructions, before drying and fluorography at -70^{0}C with preflashed Fuji RX X-ray film.

4.1.2 Analysis of rotavirus glycoproteins

To determine which of the rotavirus polypeptides were glycosylated, cells infected for 16h with rotavirus isolates were first incubated in medium in which the glucose was replaced with galactose and then pulse-labelled for 3h with the same medium containing 25μCi D-[2-^{3}H]- mannose (sp act. 16Ci/mmol; Amersham International plc). At the end of the labelling period cell lysates were prepared as described above. Electrophoresis was performed in 10-20% polyacrylamide gradient gels.

4.1.3 Polypeptide molecular weight estimation

Apparent molecular weights for the viral polypeptides were determined by comparison with standard protein molecular weight markers electrophoresed on the same gel. The protein standards used were the high molecular weight standard mixture (Sigma Chemical Co. Ltd., Poole, U.K.) containing myosin (molecular weight 205,000), β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) with the addition of lysozyme (14,300).

4.2 RESULTS

4.2.1 Analysis of the intracellular polypeptides induced by the rotavirus isolates

The intracellular polypeptide patterns induced in MA104 cells by UK, CP-1, PP-1 and PRV strains and the cloned isolates from Farm C are shown in Figs 4.1 and 4.2. Twelve viral polypeptides, labelled vp1 to vp12, were identified in cells infected with the UK strain (Fig 4.1; track 4 and Fig 4.2; track 1). Other protein bands seen in the profiles corresponded to bands found in the tracks containing uninfected cell controls. The amount of cellular protein background varied between isolates which may reflect either, variation in the ability of different rotaviruses to switch off host protein synthesis or, failure to infect all the cells. The molecular weights of the UK strain polypeptides were estimated by comparing their electrophoretic mobilities with those of proteins of known molecular weight. A standard curve was constructed by plotting the $\log_{10}$ molecular weight of the standard proteins against their electrophoretic mobility relative to the bromophenol blue marker dye (Fig 4.3). After calculating the relative electrophoretic mobilities of the viral polypeptides their molecular weights could then be estimated from the standard curve. The comparison of the molecular weights of the polypeptides for the UK strain and those published for the simian SA11 strain (Dyall-Smith and Holmes, 1981a; Ericson *et al.*, 1982) is shown in Table 4.1. Although the intracellular

polypeptides of the UK strain have been described previously (McCrae & Faulkner-Valle, 1981) no estimation of molecular weights was given, however the pattern of polypeptides shown here was similar to that reported in their publication.

Comparison of the intracellular polypeptide patterns of all the strains, with the exception of the pig pathogenic strains PRV and PP-1, showed that there was less inter-strain variation than seen when comparing the RNA genome profiles of the same viruses. In fact the molecular weights of vp1, vp2, vp3, vp5, vp6, vp8 and vp9 appeared to be identical for all these strains. The inter-strain variation seen with vp4 could be used to separate the strains into three groups: UK and CP-1; C1-165, C4-169, C7-176 and C7-183; and C3-160, C5-175 and C6-167. Comparisons of the other bovine strains with the UK strain, with respect to vp10, showed that in three isolates C5-175, C7-176 and C7-183, the molecular weight of this polypeptide was slightly reduced, whereas with C6-167 it was slightly higher. The variation seen in vp10 was mirrored by the variation in vp12. Some inter-strain variation was also seen with vp11.

Perhaps the most interesting observation was the similarity of the polypeptide profiles of C7-176 and C7-183 to the other strains. Although the C7 genome profile type was atypical, the pattern of intracellular polypeptides encoded by these viruses were similar to those of Group A rotaviruses, suggesting that the abnormally large RNA segment seen between segments 6 and 7 produced a viral polypeptide of typical size.

Comparisons of the polypeptide profiles of PP-1 and PRV revealed similarities in their patterns, although variations in the molecular weights of vp9, vp10 and vp12 distinguished the two strains. When comparing these two viruses with the other strains tested it was found that the two pig pathogenic strains could be grouped separately due to the striking difference in mobility of vp6 and also to variations in vp1 and vp2.

4.2.2 Analysis of the rotavirus glycoproteins

Under the conditions used to label the intracellular polypeptides with [^{35}S]-met the detection of the major glycoprotein vp7 in the profiles was variable. In an attempt to resolve this cells infected with the various strains were pulse labelled with [^{3}H]-mannose. Considerable variation in the mobility of vp7 was then observed (Fig 4.4). These profiles also distinctly show the presence of the second rotavirus induced glycoprotein vp10. The variation seen with the [^{3}H]-mannose labelled vp10 were, as expected, consistent with that seen when this polypeptide was labelled with [^{35}S]-met. The labelled bands which migrated in front of vp10 were assumed to be various glycosylation states formed during the processing of vp12 to give the fully glycosylated vp10. No intermediate glycosylated forms were detected for vp7.

4.3 DISCUSSION

Analysis of the intracellular polypeptides produced in rotavirus infected cells revealed the presence of 12 separate bands. This was in agreement with McCrae and Faulkner-Valle (1981), Dyall-Smith and Holmes (1981a) and Sato *et al.* (1986). However, although Ericson *et al.* (1982) detected 12 intracellular polypeptides in cells infected with simian rotavirus SA11 they only described 3 polypeptides of molecular weight >80,000 whereas the other reports found 4. This has led to some confusion in the nomenclature of the rotavirus polypeptides but recently Liu *et al.* (1988) found that SA11 vp3 and vp4 co-migrated in polyacrylamide gels thus confirming the nomenclature of McCrae and Faulkner-Valle (1981). As can be seen in Figs 4.1 and 4.2 with some isolates vp3 and vp4 are quite distinct whereas in others the two polypeptides have similar molecular weights.

Comparative analysis of the intracellular polypeptides encoded by the different rotavirus isolates revealed that whereas the isolates showed marked variation in genome profiles there appeared much less variation in the polypeptide profiles. From *in vitro* translation studies of the genome segments (McCrae and McCorquodale, 1982; Arias *et al.*, 1982; Ericson *et al.*, 1982) each segment was

thought to produce a monocistronic message. Subsequent sequence data has confirmed that each of the rotavirus genes contains at least one long open reading frame following the first initiation codon. Although genes 7, 9, 10 and 11 contain potential second open reading frames, only the gene encoding vp7 is thought to be bicistronic (Chan *et al.*, 1986). Therefore, RNA segment size variation between two viruses without an accompanying change in polypeptide molecular weight suggests that the insertion/deletion causing the genome segment variation must occur outside the coding area. This would fit the data presented here since the majority of the genome variations seen between the isolates used in this study did not result in a corresponding variation in the size of the viral protein. A good illustration of this is that although genome segment 4 of the two porcine viruses was smaller than the corresponding segments of the bovine viruses, their gene products, vp4, were similar or even larger in size. However, sequence data has shown that electrophoretic migration variation may not be as a result of a change in the size of the segment but may be linked to the base composition of the RNA (see Section 1.2.4).

It is, however, significant that most variation was seen in the molecular weights of vp4 and vp7 as both these polypeptides are located on the surface of rotavirus particles (McCrae and Faulkner-Valle, 1981). One of these, vp4, is cleaved during trypsin treatment of virus leading to the enhancement of infectivity (Clark *et al.*, 1981; Espejo *et al.*, 1981 and Estes *et al.*, 1981). The other major surface viral polypeptide, vp7, is one of the two glycosylated proteins of rotavirus, the other being vp10. Vp7 has been described as the major antigen involved in virus neutralisation and determines the serotype of rotaviruses (Kalica *et al.*, 1981). In the present study vp7 showed remarkable heterogeneity in size between the isolates tested. This could be due the variation in the size of either the protein or carbohydrate moieties. Variation in the carbohydrate content of vp7 of the human Wa and the simian SA11 strains of rotavirus was found to be due to the presence of two glycosylation sites on the vp7 of Wa but only one on the vp7 of SA11 (Kouvelos *et al.*, 1984).

In some isolates, vp7 was detected as a double band on autoradiographs. It has been suggested that this is due to the synthesis of two separate precursors (Estes *et al.*, 1982 and Ericson *et al.*, 1983), however, on cloning their stocks they found that each precursor was associated with a different clone of the same virus. Another explanation was put forward by Chan *et al.* (1986) that the two precursors are translated from the same gene from two in-phase initiation codons. They were able to show the production of two polypeptides following *in vitro* translation of the gene but when the translation was performed in the presence of microsomes only a single glycosylated band was seen. The simpler explanation for the doublet of bands seen here is that the lower molecular weight band comprises an intermediate, formed during the glycosylation process. This is the probable reason for the two bands located between the fully glycosylated vp10 and its precursor vp12 (McCrae & Faulkner-Valle, 1981).

The two porcine isolates show another interesting feature of the polypeptide profiles. Although the molecular weights of vp6 from the bovine isolates were similar in all cases, the vp6 of both the porcine viruses were smaller in size. However, it would require the analysis of a larger group of strains to determine if this was a characteristic of porcine rotaviruses.

Table 4.1

Comparison of the molecular weights of the intracellular polypeptides encoded by the UK strain of bovine rotavirus with those published for simian rotavirus SA11

Viral polypeptide	UK strain	SA11 [1]	SA11 [2]
vp1	125 [3]	125	113
vp2	96	94	96
vp3	90	-	91
vp4	88	88	84
vp5	53	53	57
vp6	41	41	42
vp7	37	35.5	34
vp8	33	35	33
vp9	32	34	31
vp10	26	26	26
vp11	23	-	25
vp12	20	20	21

[1] Data from Ericson et al (1982)

[2] Data from Dyall-Smith & Holmes (1981)

[3] Molecular weight x 10^{-3}

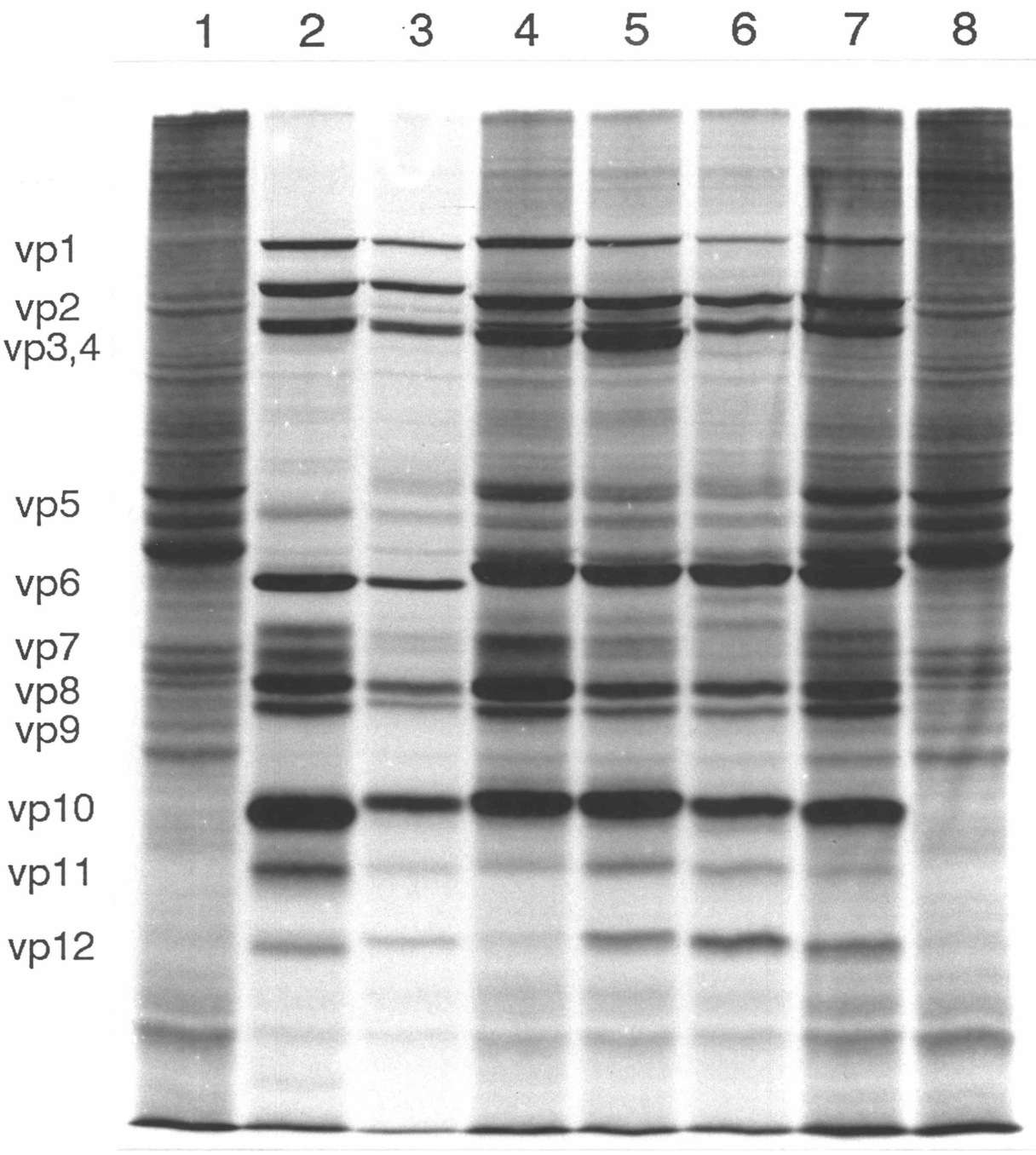


Fig 4.1: Analysis of the intracellular polypeptides encoded by rotavirus isolates. MA104 cells were infected with the virus isolates, incubated for 16h when the polypeptides were labelled with [^{35}S]-met for 3h. The cells were disrupted with electrophoresis sample buffer and the polypeptides separated on a 10% polyacrylamide gel. Tracks 1 and 8 contained lysates of uninfected cells; tracks 2-7 lysates of cells infected with PRV, PP-1, UK, CP-1, C3-160 and C5-175 respectively. Virus encoded polypeptides are labelled vp1-vp12.

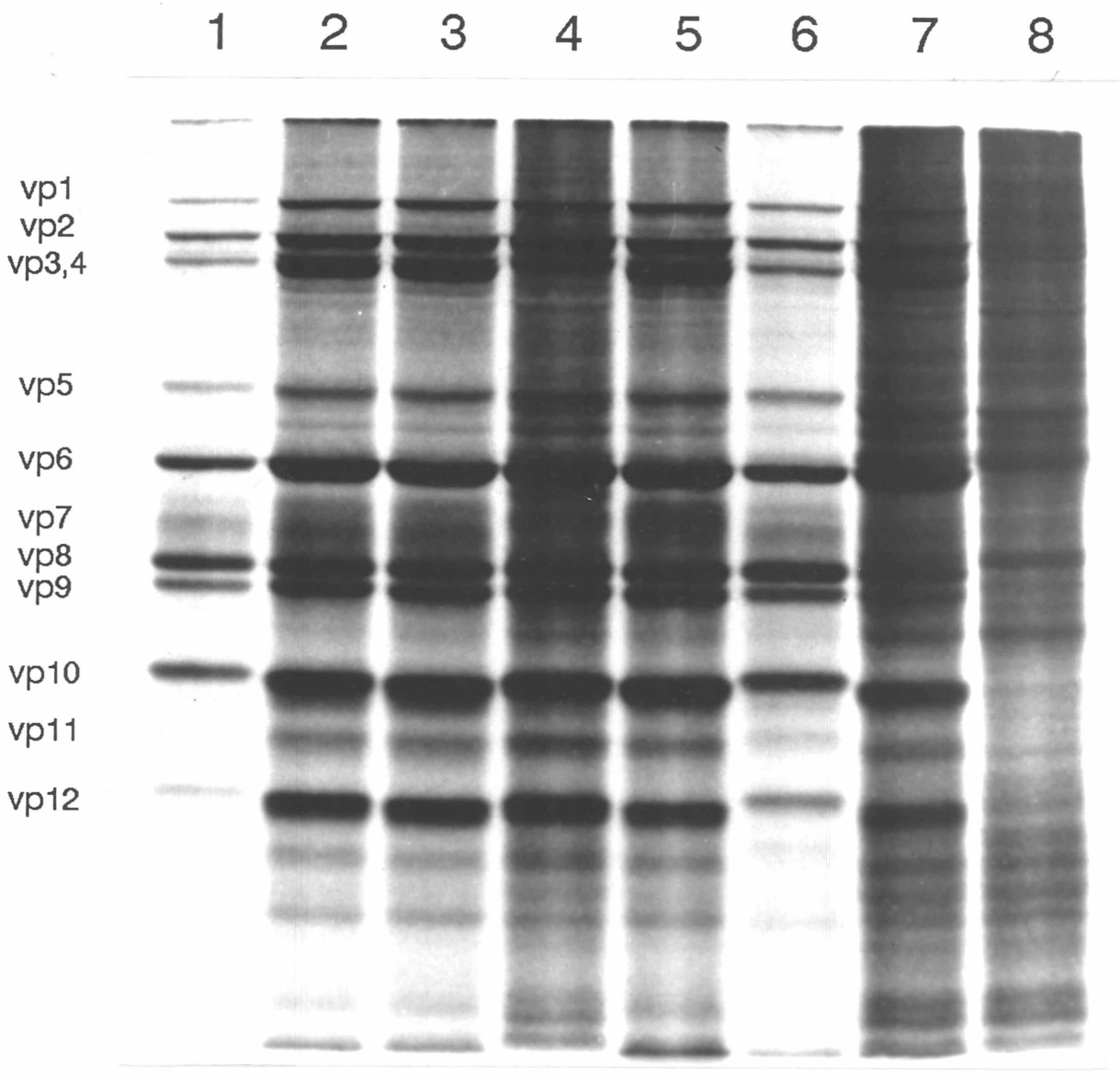


Fig 4.2: Analysis of the intracellular polypeptides encoded by rotavirus isolates. Viral polypeptides were labelled and separated as described in the legend of Fig 5.1. Tracks 1-8 contained lysates of cells infected with UK, C7-176, C7-183, C3-160, C1,165, C6-167, C4-169 and control uninfected cells respectively.

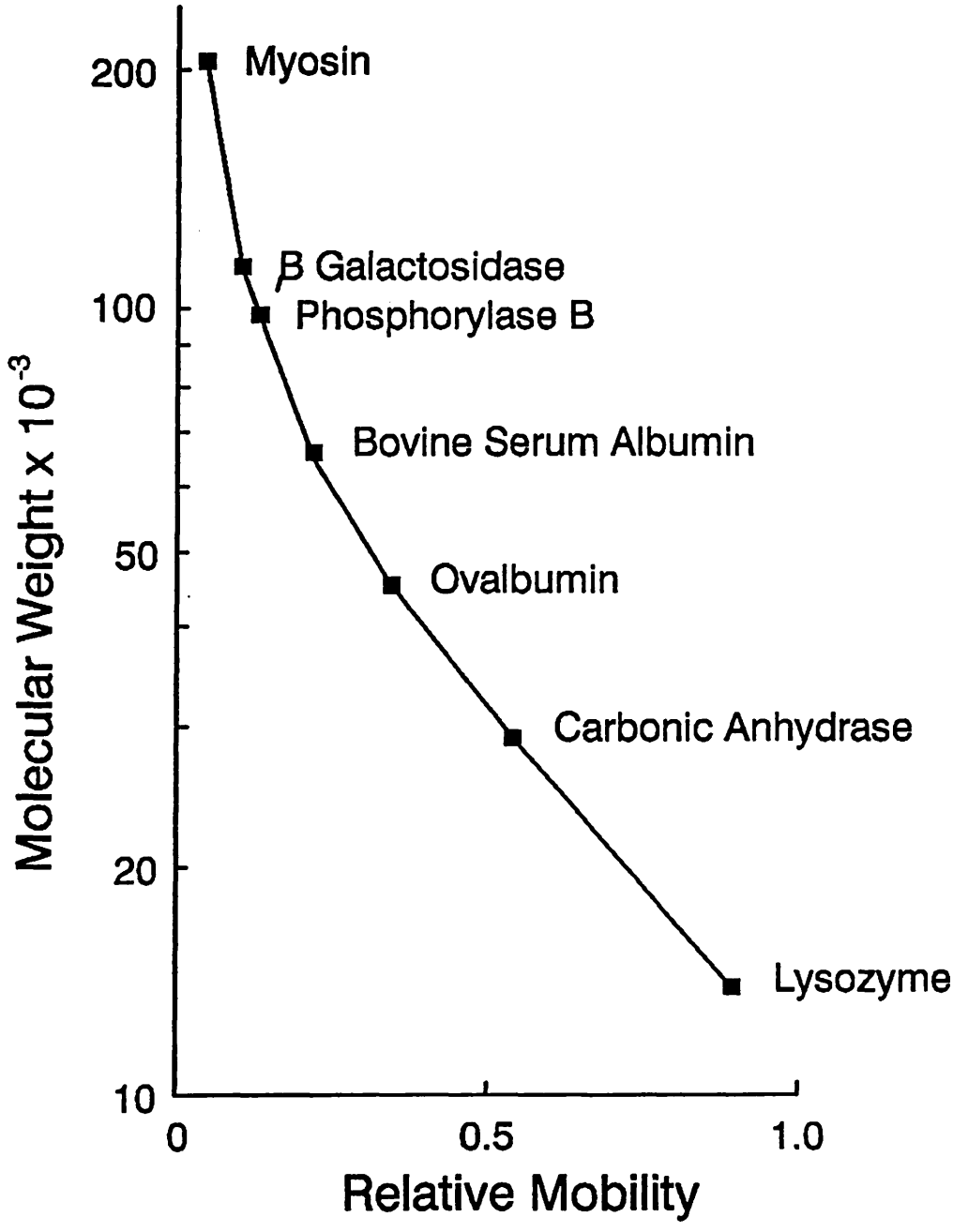


Fig 4.3: Relationship between the migration and molecular weight of standard proteins electrophoresed in 10% polyacrylamide gels. The migration was calculated as the distance moved by the standard protein relative to the marker dye bromophenol blue.

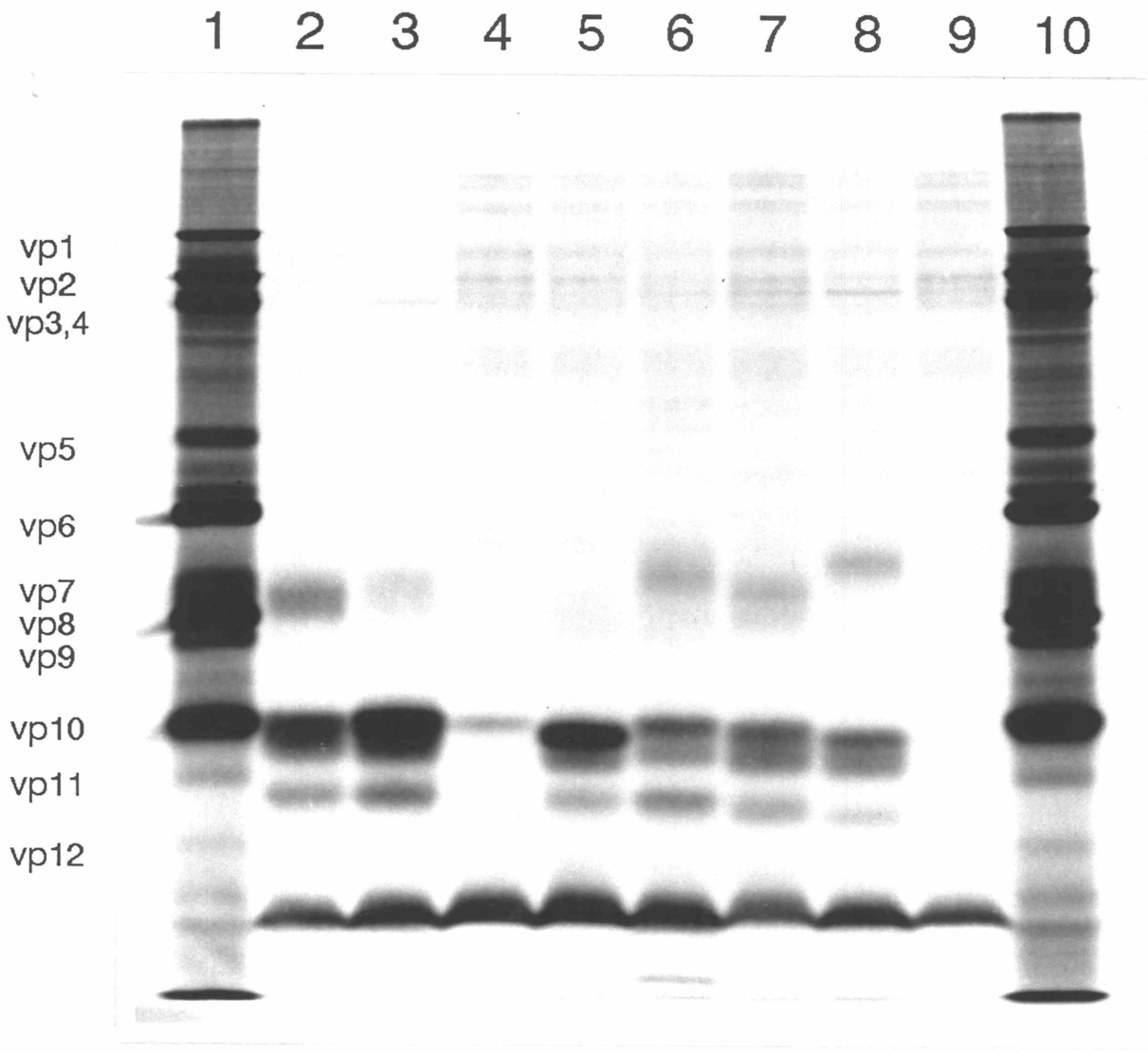


Fig 4.4: Analysis of the glycoproteins of the rotavirus isolates. MA104 cells infected for 16h with the isolates were incubated with D-[^{3}H]-mannose for 3h to label the viral glycoproteins. At the end of the labelling period the cells were lysed with electrophoresis sample buffer and the polypeptides separated on 10-20% polyacrylamide gradient gels for 16h. Tracks 1 and 10 contained [^{35}S]-met labelled UK isolate polypeptides and tracks 2-9 contained [^{3}H]-mannose labelled lysates of cells infected with PRV, UK, CP-1, C3-160, C6-167, C1-165, PP-1 and control uninfected cells respectively.

5.0 ANTIGENIC ANALYSIS OF ROTAVIRUS FIELD ISOLATES

5.1 MATERIALS AND METHODS

5.1.1 Antisera.

Antisera were raised to the UK, CP-1 and PP-1 strains of rotavirus by oral (convalescent) or oral plus parenteral (hyperimmune) inoculation of gnotobiotic animals as described previously by Bridger and Brown (1984). All the antisera used in this study were collected from a single animal for each virus strain. An early convalescent antiserum (a) was taken 18-21 days after oral inoculation of animals with 10^7-10^8 $TCID_{50}$ of each virus strain. A second oral inoculation was given at this time and a second convalescent serum (b) taken 14-21 days later. The animals were then given an intra-muscular inoculation of partially purified virus in Freund's incomplete adjuvant and a hyperimmune serum (c) taken 6-7 weeks later. Two antisera were prepared against C3-160 and C7-183; the first (a) was taken from calves 3 weeks after a single oral inoculation of virus. The animals then received an intra muscular inoculation of virus in adjuvant and serum, labelled (c) to be consistent with the above sera, was removed 3 weeks later.

5.1.2 Virus neutralisation test.

A fluorescent focus reduction test was used with microtitre plate cultures of MA104 cells as described by Bridger and Brown (1981). Samples of virus, diluted to give 100-200 fluorescent foci per well after overnight incubation, were mixed with an equal volume of serial twofold dilutions of the antiserum and incubated at 37^0C for 1h. Aliquots (100μl) of the mixtures were added to the cultures, centrifuged at 1,000 rpm for 1h, and incubated for 18h. The foci of virus infected cells were detected by the indirect immunofluorescence procedure described above. End points were expressed as the reciprocal of the highest dilution of antiserum which gave a 50% reduction in the number of fluorescent foci found in control cultures.

5.1.3 Virus neutralisation screening assay.

In order to test the serological relationship of the field isolates of rotavirus to the well adapted cell culture strains a neutralising screening assay was developed. This assay was performed in the same as the normal neutralisation test except that 50-100 fluorescing foci of the test virus were incubated with a single dilution of the antisera directed against the UK, CP-1, PP-1 and PRV strains of rotavirus. Each antiserum was diluted 20 fold less than that required to give a 50% reduction of fluorescing foci when tested against homologous virus.

5.1.4 Experimental inoculation of calves.

5.1.4.1 Primary inoculation.

Gnotobiotic calves were derived and maintained as described previously (Dennis *et al.*, 1976; Hoare *et al.*, 1976). Calves were infected when 6-10 days of age by oral inoculation of 2ml tissue culture fluid containing virus. The daily faecal output of male calves was determined by the collection of all faeces with a specially designed harness (Bridger and Brown, 1984). Faecal samples from female calves were collected daily. Any change in the colour of the faeces on consecutive days was recorded. Anorexia was assessed by a comparison of the feed records before and after inoculation and was recorded as positive if calves failed to consume all the food offered within 1h when the normal time was 5-10 min.

5.1.4.2 Challenge inoculation.

All calves were challenged with a virulent strain of rotavirus (CP-1) 3 weeks after the primary inoculation. Faecal samples were again collected daily to assess virus excretion, faecal output and changes in faecal colour.

5.2 RESULTS

5.2.1 Serology

The antigenic relationships between the 3 laboratory strains UK, CP-1 and PP-1 were first investigated with a cross neutralisation test. The results are shown in Table 5.1. The antibody titres of the 3 sera a, b and c against the homologous virus strains showed that the 3 animals seroconverted after the primary oral inoculation. A 2-8 fold increase in antibody titre was observed after the repeated oral inoculation. It could not be determined whether this rise in titre was due to a response to the second challenge or a continuation of the primary response. These antibody levels were further boosted >15 fold after an intramuscular inoculation of partially purified virus in adjuvant. Neutralisation tests carried out with these sera against heterologous virus revealed that the UK and CP-1 strains cross reacted but in one direction only; the UK convalescent sera a and b neutralised CP-1. The UK and PP-1 strains showed no cross reactivity in either direction. However, antisera against CP-1 and PP-1 neutralised both viruses to similar levels indicating that these 2 strains were antigenically very similar. The UK, CP-1 and PP-1 sera were also tested against the C3-160 field isolate but all failed to show any antigenic relationships in the one direction tested.

This type of neutralisation assay was laborious because for each serum dilution used, the number of fluorescent foci had to be counted in 3 replicate microtitre plate wells and this restricted the number of virus isolates that could be analysed. The screening assay overcame this problem by the use of only one serum dilution. To test the accuracy of this assay for detecting antigenic relationships between virus strains, the assay was applied to the UK, CP-1 and PP-1 strains. The results are given in Table 5.2 and confirm that the antigenic cross reactions shown by the 2 tests were identical. This test was further applied to the field isolates from Farms B and C (Table 5.3). C1-165, C3-160, C5-175 and C6-167 isolates showed no antigenic relationship with UK, CP-1 and PP-1 strains. The other field isolates were neutralised by antisera to the UK strain but, as with CP-1, C7-176 and the 2

C8 isolates, reacted only with the convalescent sera. Only C4-169 and the 2 C7 isolates were neutralised by CP-1 antisera. None of the field isolates were neutralised by any of the antisera to PP-1.

Antisera against C3-160 and C7-183 were tested for their ability to neutralise UK, CP-1, PP-1, PRV and the cloned field isolates. The results are shown in Table 5.4. Two antisera were used for each strain which had homologous neutralisation titres of 1:1,700 and 1:28,500 for C3-160 a and c and 1:44,000 and 1:200,000 for C7-183 a and c. C7-183 was the only virus, besides the homologous strain, that was neutralised by the 2 C3-160 antisera. Both C7-183 antisera neutralised UK and CP-1 isolates as well as the homologous virus and 1 antiserum, the hyperimmune serum c, neutralised C3-160. These results showed that C7-183 cross neutralised, in both directions, with UK and CP-1; a 2 way cross was also observed between C7-183 and C3-160, although only the hyperimmune and not the convalescent C7-183 antiserum neutralised C3-160. So, C7-183 was shown to be serologically related to UK, CP-1 and C3-160 but interestingly C3-160 did not cross react with UK and CP-1, only C7-183.

5.2.2 Calf inoculations

All calves used in these experiments were 7-10 days of age and shown to be free of rotavirus antibody prior to oral inoculation with cloned tissue culture grown virus. After inoculation the calves were monitored daily for faecal output, excretion of virus, changes in faecal colour and signs of anorexia. These parameters were determined for a calf infected with a well characterised virulent rotavirus, CP-1, and are shown in Fig 5.1. Virus was first detected in the faeces of this animal 2-3 days after inoculation, reached a peak titre of 6.3 $\log_{10}$ $TCID_{50}$/gm of faeces at 4 days and virus could still be detected up to day 8, a total excretion period of 6 days. Faecal output started to increase between days 3 and 4, reached a peak on day 6 and then declined to normal levels on day 10. A significant change in faecal colour, from dark brown to yellow, was first detected on day 4 and continued until day 10. This animal did not suffer from anorexia during the period of virus infection.

The results from calves inoculated with 5 of the cloned isolates from Farm C are shown in Fig 5.2 (a-e). All the calves became infected and started to excrete virus 2-4 days after inoculation and the excretion periods were similar for all the isolates, 4-5 days. Increases were seen in faecal output for 3 of the isolates, C1-165, C5-175 and C7-183 and although this parameter could not be quantified for the female calf infected with C6-167, from daily observations it was clear that faecal output had been increased. Interestingly the calf infected with C3-160 (Fig 5.2b) showed no elevation of faecal output above normal. Faecal colour changes were observed in 4 of the 5 animals starting on days 4 or 5 and lasting for for 5-7 days. However, no change in faecal colour was shown by the animal inoculated with C3-160. Anorexia was seen in the 2 animals inoculated with C5-175 and C6-167 which lasted for 3 days.

Three weeks after the primary inoculation all the animals were orally challenged with virulent rotavirus to determine the level of protection afforded by the initial infection. Firstly the animal inoculated with CP-1 was re-challenged with the same virus (Fig 5.3 a and a'). No excretion of virus was detected and there was no change in either faecal colour or output. Another animal which served as a challenge control did not receive an initial inoculation but was infected with CP-1 at 4 weeks of age (Fig 5.3 b and b'). Virus excretion was observed in this animal after inoculation and an increase in faecal output as well as a change in faecal colour were seen.

The results of the challenge of calves initially infected with C3-160 and C7-183 are shown in Fig 5.4 (a and a') and Fig 5.4 (b and b') respectively. The challenge control calf is shown again in Fig 5.4 (c and c') for comparison. Both calves resisted the challenge with the virulent CP-1 virus. No virus was detected in the faeces of either animal and daily faecal outputs remained normal. The other 3 calves inoculated with C1-165, C5-175 and C6-167 were also challenged and all 3 calves were protected.

5.3 DISCUSSION

There have been several attempts to separate rotavirus isolates from many species into distinct serotypes on the basis of cross neutralisation data. An initial report suggested that rotaviruses from each species constituted a single serotype (Yolken *et al.*, 1978), but it was soon realised that this was not the case as in the same year Zissis and Lambert (1978) and Thouless *et al.* (1978) independantly described the detection of two serotypes of human rotaviruses. Since that time there have been reports of five serotypes of human rotaviruses (Sato *et al.*, 1982; Matsuno *et al.*, 1985; Clark *et al.*, 1987), three serotypes of bovine rotavirus (Murakami *et al.*, 1981 & 1983; Bridger *et al.*, 1983; Ihara *et al.*, 1983; Woode *et al.*, 1983; Ojeh *et al.*, 1984) and at least two serotypes of porcine rotavirus (Bohl *et al.*, 1984). A rotavirus serotype definition based on cross neutralisation tests with 20-fold differences of serum titres in both directions was suggested by Wyatt *et al.* (1982) to be consistent with the serotype parameters established for enteroviruses. However, it was soon discovered that not all rotaviruses conformed to this definition with some isolates reacting with a one way cross reaction (Murakami *et al.*, 1983; Bridger and Brown, 1984 - see Table 5.1) and some reacting with viruses assigned to different serotypes (Hoshino *et al.*, 1985b). Initially it had been assumed that only vp7, the surface glycoprotein, was involved in virus neutralisation but it has since become clear that the other surface protein vp4 is also involved (Greenberg *et al.*, 1983b; Hoshino *et al.*, 1985a; Taniguchi *et al.*, 1985; Offit & Blavat, 1986). Recently Offit *et al.* (1986b) confirmed this by showing that monoclonal antibody against either vp4 or vp7 was capable of protecting passively against rotavirus disease. It would therefore be expected that with two proteins involved rotavirus serology would not be simple, especially as these two proteins are capable of independant segregation in different virus strains (Hoshino *et al.*, 1985a). It has been suggested that rotaviruses should be classified in a similar way to influenza viruses which would take into account the neutralisation moieties of both proteins. At the present time it is not known how many vp4 and vp7 variants are in circulation and along with the absence of suitable reagents this type of classification will have to wait.

In this study UK and PP-1 clearly belonged to different serotypes whereas CP-1 and PP-1 belonged to the same serotype. CP-1 was therefore intermediate between two serotypes showing both a two way cross reaction with PP-1 and a one way cross reaction with UK. Analysis of the cloned field isolates revealed that three viruses C4-169, C7-176 and C7-183 were neutralised by 20 antibody units of both UK and CP-1 sera, but were not neutralised by PP-1 antisera so these viruses would appear to be intermediate between serotypes. C8-185, C8-186, B3-62 and B3-76 reacted only with UK antisera and could be said to be antigenically very similar to UK virus. C1-165, C3-160, C5-175 and C6-167 isolates appeared antigenically distinct from UK, CP-1 and PP-1 viruses. C3-160 and C7-183 were shown to be related but only C7-183 was related to UK and CP-1.

It seems quite probable that rotaviruses consist of a spectrum of antigenic types and therefore the more isolates that are typed the more complicated the picture will become. This may be explained by the fact that rotaviruses have two major surface polypeptides, vp4 and vp7, and antibody against either will neutralise the virus (Hoshino *et al.*, 1985a) and each of these contain multiple neutralisation epitopes defined by monoclonal antibodies (Shaw *et al.*, 1986). Because of the segmented nature of the rotavirus genome, reassortment of genes can take place in cells infected with two distinct strains and the resulting reassortant viruses could contain a mixture of the antigenic features of each parent. This type of experiment was performed by Midthun *et al.* (1986) with the serotypically distinct UK bovine and a simian rotavirus. Antisera to reassortants containing only vp7 from the simian virus were capable of neutralising both parental strains.

Comparison of the serological and genome profile data of the rotavirus strains tested revealed that the antigenic composition of a particular strain could not be predicted from its genome profile e.g. both C3-160 and C7-183 were antigenically very close but had very distinct genome profiles. This observation confirms the findings of Beards (1982) who demonstrated significant polymorphism in the genome profiles of human rotaviruses within three serotypes.

Inoculation of gnotobiotic calves with the cloned isolates resulted in infection in all cases, as determined by virus shedding. Clinical signs such as faecal colour change and increased faecal output have been shown to be good indicators of rotavirus infection (Bridger and Pocock, 1986), whereas anorexia and changes in the dry matter content of faeces occur only in infections with some viruses. Clinical signs of disease were seen in calves infected with all the cloned field strains except C3-160. In this calf, faecal output remained normal and there was no change in faecal colour even though infection was confirmed by virus excretion. With the exception of CP-1, all the viruses tested were isolated from calves sub-clinically infected with rotavirus, showing no clinical signs. However, only C3-160 caused the same type of infection experimentally while all the other strains induced disease. This indicated that sub-clinical infections could be due to infections either with avirulent rotavirus strains such as C3-160, or with virulent strains with suppression of clinical signs. Rotaviruses of differing virulence have been described (Carpio *et al.*, 1981) and implicated in sub-clinical infections. Supression of clinical signs has been shown to occur after feeding rotavirus antibody whilst infecting animals with virulent viruses (Snodgrass & Wells, 1976; Bridger and Brown, 1981). This situation probably occurs in the field with neonates receiving rotavirus antibody in mammary secretions and has been described in studies of human babies (Bishop *et al.*, 1983). The transfer of antibody contained in colostrum and milk from dam to neonate, is very important since in the main rotavirus causes disease in the neonate. The passive transfer of antibody was thought to be all that was necessary for the protection of calves from disease because it was assumed that rotaviruses caused disease only in the first 2 weeks of life. However, the finding of Bridger & Pocock (1986) of rotavirus strains capable of causing disease in older animals means that protection is required for a longer period. Immunisation of the dam to prolong lactogenic immunity has proved successful in cattle (Snodgrass *et al.*, 1980b; Saif *et al.*, 1983) but until widespread vaccination is achieved it will not be known how effective the protection will be against the strains which infect the older animal. Also passive protection relies on the effective neutralisation of the virus in the gut lumen and antigenic variation could be a problem.

A primary rotavirus infection has been shown to protect animals against subsequent challenge by the same or different rotaviruses, including isolates from different species (Woode *et al.*, 1978, 1983; Wyatt *et al.*, 1979; Tzipori *et al.*, 1980b; Gaul *et al.*, 1982; Zissis *et al.*, 1983; Bohl *et al.*, 1984). In some cases these workers found no cross protection and it was concluded that for protection to occur the challenge virus had to be antigenically similar to the virus causing the primary infection. However, several groups (Woode *et al.*, 1983; Wyatt *et al.*, 1983b; Bishop *et al.*, 1986; Torres *et al.*, 1986) have shown that cross protection could be demonstrated between rotaviruses of different serotypes and in other cases viruses which were antigenically similar, failed to cross protect.

In the present study, infection of calves with either C3-160 or C7-183 gave protection against challenge with the virulent CP-1 strain. C3-160 was shown, by *in vitro* neutralisation tests, to be antigenically dissimilar to CP-1 whereas C7-183 was similar, even though C3-160 and C7-183 themselves were serologically close. These studies therefore confirm that cross protection can occur between antigenically similar or distinct strains of rotavirus. It therefore seems that viral antigens other than the surface proteins may play a role in active immunity. Recently Offit & Dudzik (1988) showed that rotavirus specific cytotoxic T lymphocytes were capable of lysing syngeneic cells infected with either homologous virus or viruses from different serotypes. Cross protection has been described between different strains of influenza A viruses and this also has been linked to the presence of cross reactive cytotoxic T cell populations (Effros *et al.*, 1977; McMichael & Askonas, 1978; Zweerink *et al.*, 1977). The internal viral nucleoprotein was found to be the important target antigen for these cytotoxic T cells (Townsend *et al.*, 1984; Yewdell *et al.*, 1985), and immunisation of mice with this antigen confers protection against disease (Wraith *et al.*, 1987). Monoclonal and polyclonal antibody directed against the major capsid protein of rotavirus, vp6, show only low level neutralising activity (Sabara *et al.*, 1987), but whether this group A rotavirus antigen is involved in protection is not known.

Protection against disease, in the absence of neutralising antibody, has been reported for two flaviviruses, yellow fever (Schlesinger *et al.*, 1986) and dengue virus (Schlesinger *et al.*, 1987), following immunisation with a viral non-structural glycoprotein, NS1. In this case stimulation of complement-fixing antibody to NS1 provided protection by complement-mediated lysis of infected cells. It is interesting to note that rotaviruses also produce a non-structural glycoprotein, vp10, which by analogy may be important in cross protection.

The finding in this study that an infection with the avirulent strain C3-160 protected against challenge with an antigenically distinct but virulent strain CP-1 was significant. There have been several attempts to use live rotavirus vaccines to elicit active immunity. The vaccine strains used have been either strains from a heterologous species e.g. a bovine virus for use in humans (Delem *et al.*, 1984; Clark *et al.*, 1986b) or reassortant viruses, usually between homologous and heterologous parental strains (Midthun *et al.*, 1985; Clark *et al.*, 1986b). These vaccines have met with mixed success with the lack of results probably due to the level of attenuation of the vaccine strains in the heterologous host leading to poor immune responses. The possibility of using an avirulent strain in the homologous species which is capable of providing wide protection would seem advantageous. More work on this aspect is required to investigate the feasibility of this approach.

Table 5.1

Cross neutralisation titres of antisera produced against 3 bovine rotaviruses.

		Virus			
Antiserum		UK	CP-1	PP-1	C3-160
UK	a	800 (1)	200 (2)	10	40
	b	6,000	2,600	20	120
	c	400,000	8,800	600	3,600
CP-1	a	30	3,200	5,000	20
	b	200	8,000	60,000	50
	c	400	200,000	200,000	800
PP-1	a	<10	3,000	5,000	70
	b	<10	30,000	20,000	70
	c	800	800,000	300,000	3,400

(1) Antibody titres against homologous virus

(2) Antibody titres against heterologous virus which differ from the homologous titres by less than 20-fold are underlined

Table 5.2

Antigenic relationships between 3 bovine rotaviruses determined by a neutralisation screening assay.

		Virus		
Antiserum		UK	CP-1	PP-1
UK	a	⊕[1]	+	
	b	⊕	+	
	c	⊕		
CP-1	a		⊕	+
	b		⊕	+
	c		⊕	+
PP-1	a		+	⊕
	b		+	⊕
	c		+	⊕

(1) ⊕ >50% homologous neutralisation.

\+ >50% heterologous neutralisation.

Table 5.3

Antigenic relationships between UK, CP-1 and PP-1 and the field isolates of bovine rotavirus determined by a neutralisation screening assay.

	Virus										
Antiserum	C1-165	C3-160	C4-169	C5-175	C6-167	C7-176	C7-183	C8-185 [1]	C8-186 [1]	B6-62	B6-76
UK a			+[2]			+	+	+	+	+	
b			+			+	+	+	+	+	+
c			+				+			+	+
CP-1 a			+			+	+				
b			+			+	+				
c											
PP-1 a											
b											
c											

[1] Uncloned faecal virus.

[2] + >50% neutralisation.

Table 5.4

Antigenic relationships between rotaviruses determined by a neutralisation screening assay using antisera prepared against C3-160 and C7-183 isolates

	Virus									
Antiserum	UK	CP-1	PP-1	PRV	C1-165	C3-160	C5-175	C6-167	C7-183	B6-62
C3-160 a						⊕[1]			+	
c						⊕			+	
C7-183 a	+	+							⊕	
c	+	+				+			⊕	

[1] ⊕ >50% homologous neutralisation

\+ >50% heterologous neutralisation

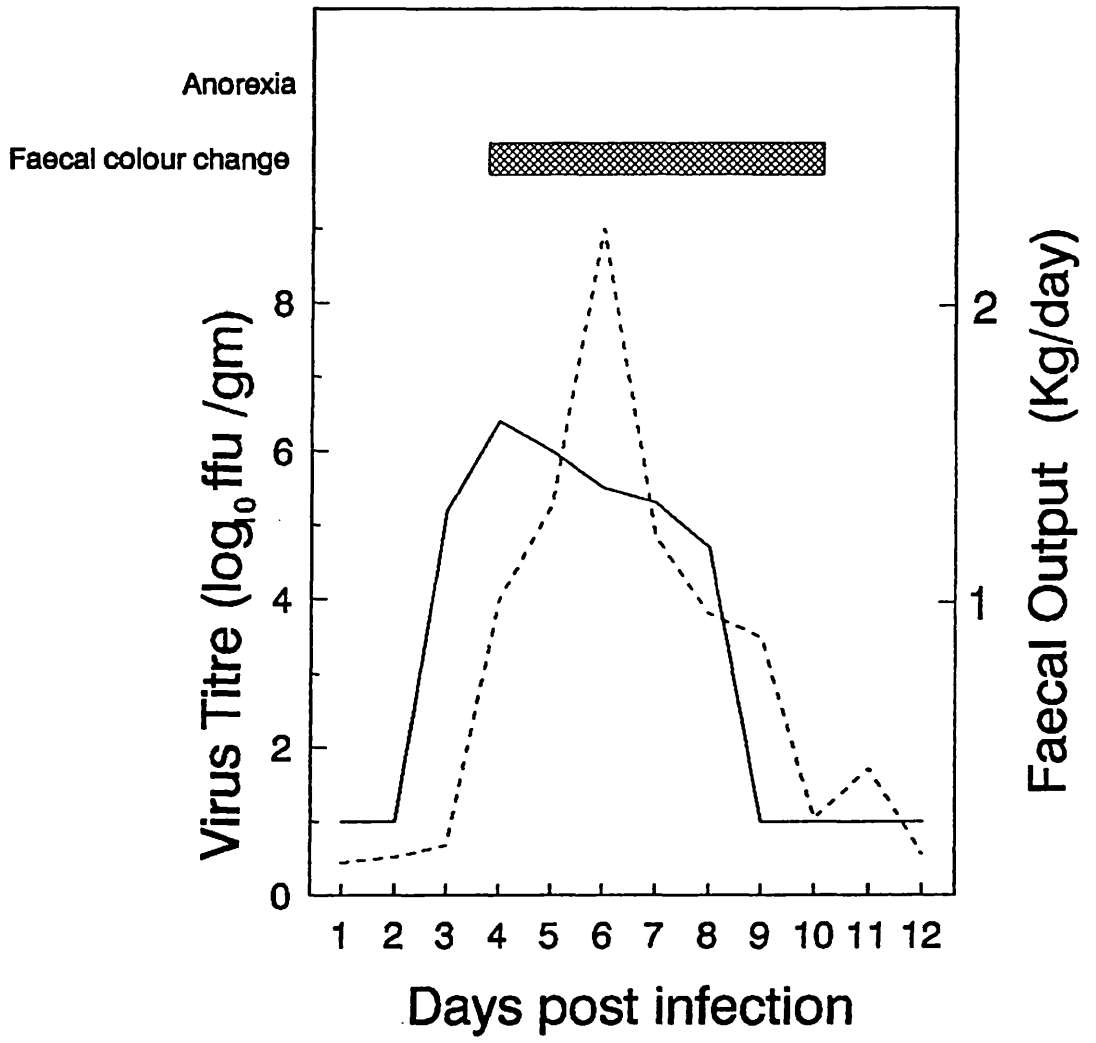


Fig 5.1: Experimental infection of a 7 day old gnotobiotic calf with CP-1 strain of rotavirus. Daily faecal samples were collected and analysed for total daily weight (- - -) infectious virus content (——) and changes in faecal colour (▩). Anorexia (▨) was recorded if the calf failed to consume all the food offered within 1 h.

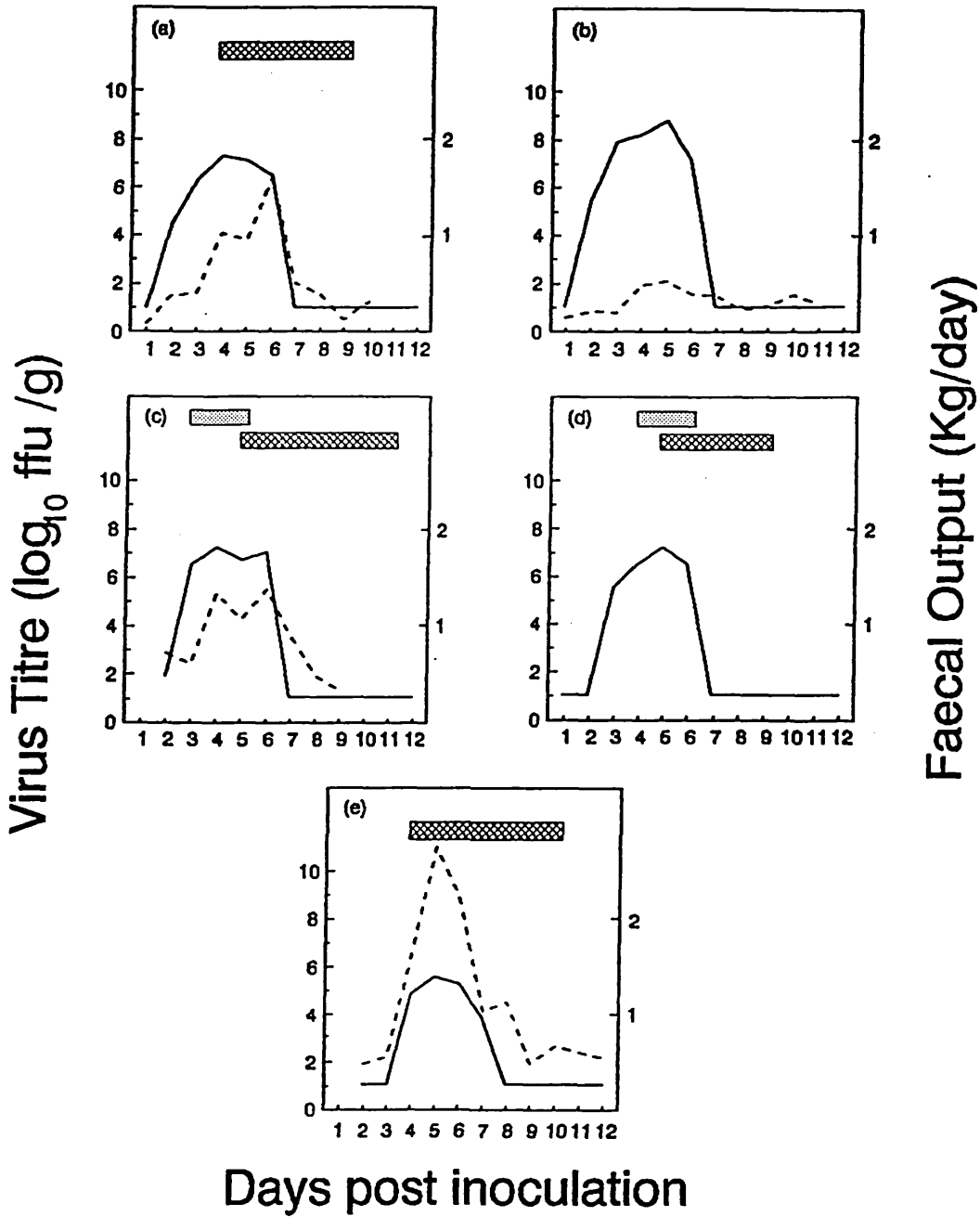


Fig 5.2: Results from the experimental infection of 7 to 10 day old calves with the cloned rotavirus isolates, (a) C1-165, (b) C3-160, (c) C5-165, (d) C6-167 and (e) C7-183. Daily recorded parameters are described in Fig 5.1. The calf infected with C6-167 (d) was a female so daily faecal output could not be detected.

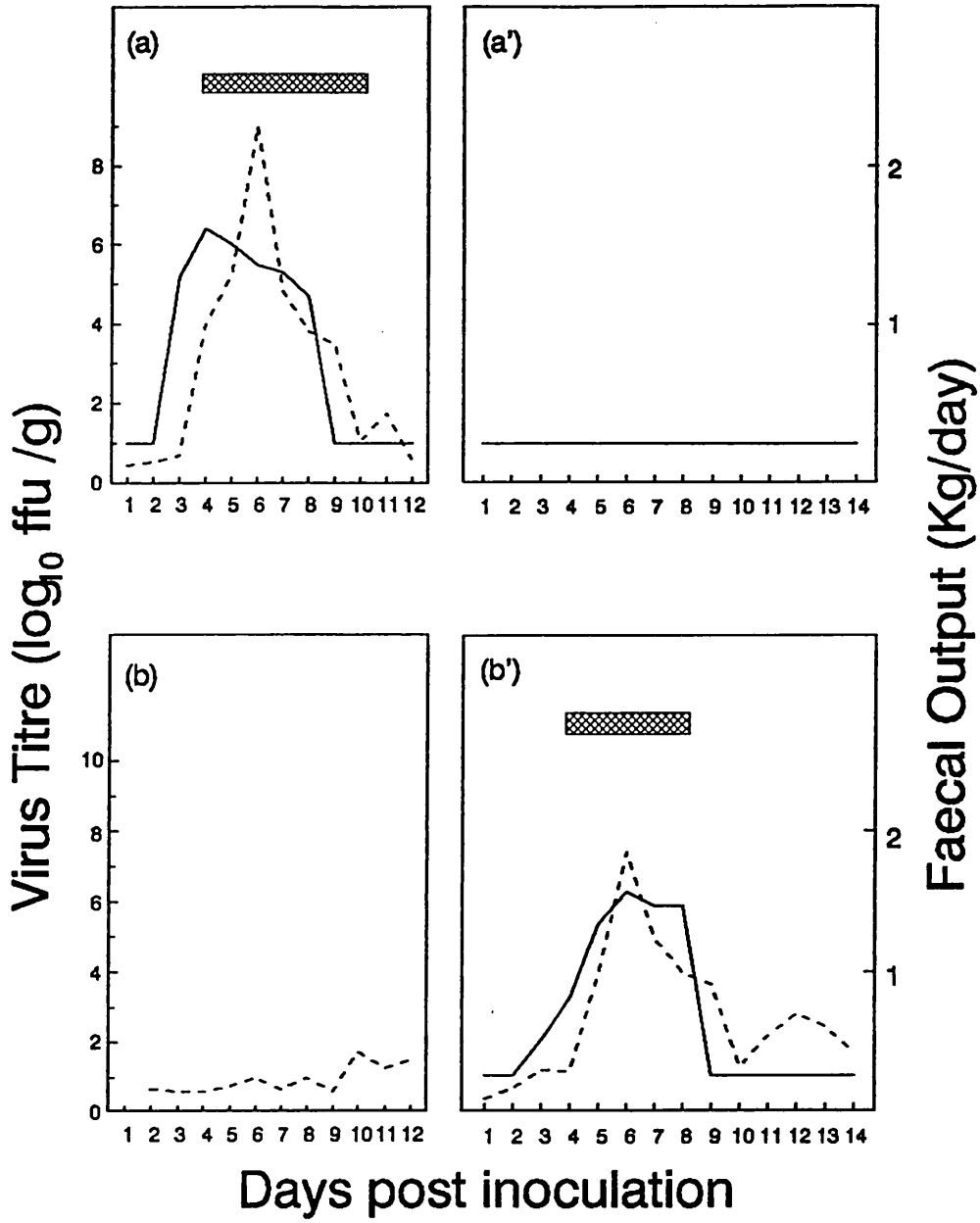


Fig 5.3: Results of challenging calves with CP-1 rotavirus 3 weeks after primary inoculation with (a) CP-1 and (b) uninfected control. Panels (a) and (b) show the results of the primary inoculations and (a') and (b') after challenge. Daily recorded parameters are described in Fig 5.1.

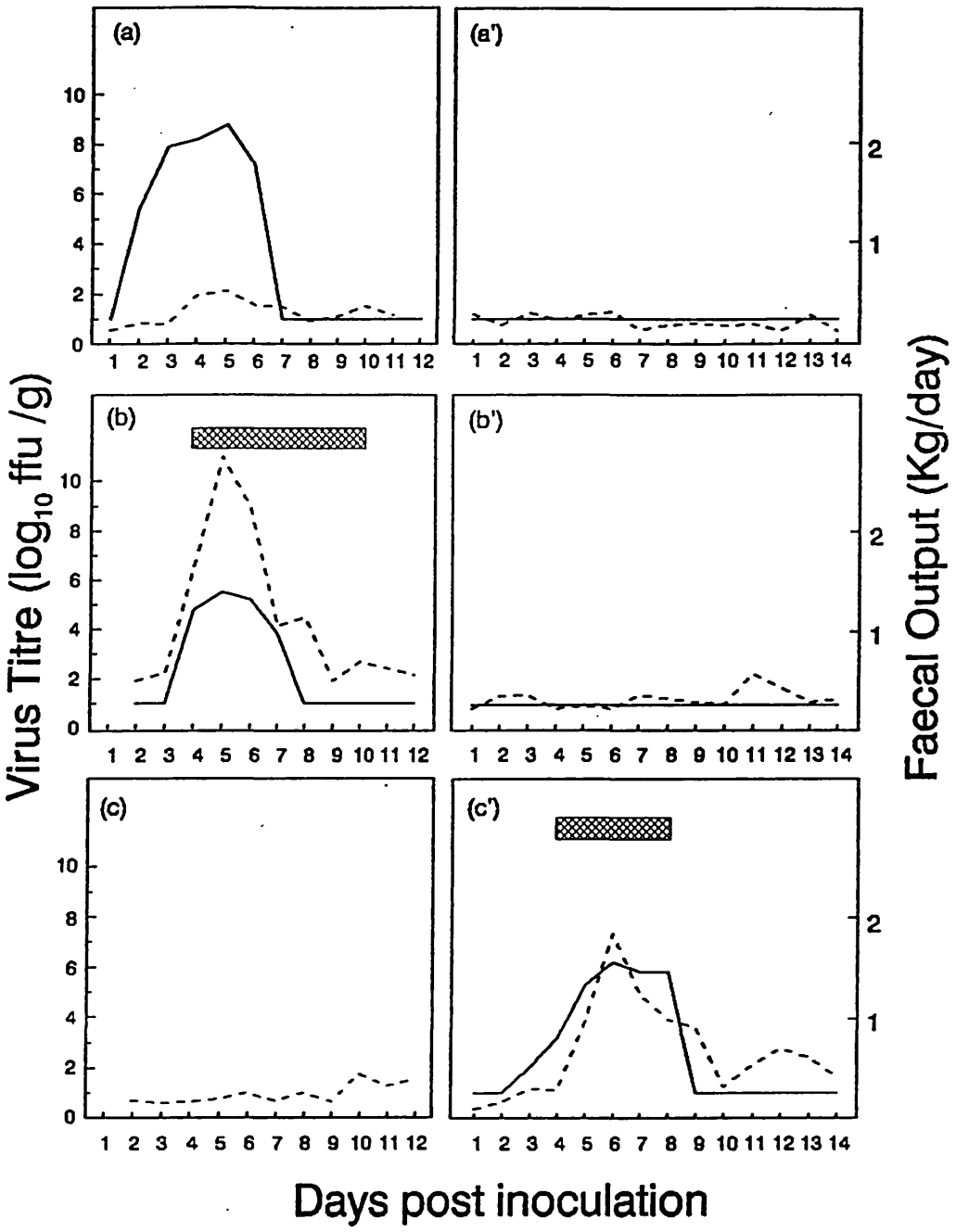


Fig 5.4: Results of challenging calves with CP-1 strain 3 weeks after primary inoculation with (a) C3-160, (b) C7-183 and (c) uninfected control. Panels (a), (b) and (c) show the results of the primary inoculations and (a'), (b') and (c') the results after challenge.
Daily recorded parameters are described in Fig 5.1.

6.0 ANALYSIS OF ROTAVIRUS ISOLATES WITH ABERRANT GENOME PROFILES

6.1 MATERIALS AND METHODS

6.1.1 Growth and purification of viruses.

Cloned UK, C7-175 and C7-183 rotavirus isolates were grown in monolayers of MA104 cells and purified by CsCl gradient centrifugation as described earlier.

6.1.2 In vitro transcription.

Cohen (1977; 1979) found that the endogenous RNA-dependant RNA polymerase contained within rotavirus particles could be activated by the removal of the outer viral capsid with EDTA. Mason *et al.* (1980), however showed that substituting bentonite for EDTA gave better activation. Also, Flores *et al.* (1982a) found that the ATP regenerating system of phosphoenolpyruvate and pyruvate kinase used by Mason *et al.* (1980) could be omitted by increasing the initial concentration of ATP. Finally Spencer and Garcia (1984) showed that the rate of transcription was increased when S-adenosyl methionine (SAM) was added to the reaction mixture.

The reaction mixture contained 100µl purified virus and 150µl containing 100mM Tris/HCl pH 8.0, 2.5mM CTP and GTP, 10mM ATP, 10mM $MgCl_2$, 0.5mM SAM, 0.1% Macaloid (purified bentonite; NL Industries Inc., New Jersey, USA), 0.625mM UTP and either 10µCi [^{3}H]-UTP or 50µCi α-[^{32}P]-UTP and was incubated at 43^{0}C.

To evaluate the rate of production of viral RNA transcripts labelled with [^{3}H]-UTP , 25µl aliquots were removed from the reaction mixtures, containing various concentrations of purified virus, at 1h intervals and added to 100µl yeast RNA (100µg/ml). The mixture was cooled on ice and 3ml ice-cold 5% trichloracetic acid (TCA) containing 10mg/ml sodium pyrophosphate added. After 15min incubation the precipitate was removed by filtration through glass fibre membranes

(GF/A; Whatman LabSales Ltd.) which were subsequently washed x2 with 3ml cold 5% TCA and x2 with 3ml cold ethanol and finally dried. The filter were added to 3ml toluene-based scintillation fluid and the bound radioactivity was measured.

For the analysis and isolation of individual viral RNA transcripts the reaction mixtures contained α-[^{32}P]-UTP and were incubated at 43^{0}C for 5h. The virus and Macaloid was then removed by centrifugation at 100,000g for 1h, the supernatant extracted with phenol/chloroform and the RNA precipitated from the aqueous phase with $2^1/_2$ vol. ethanol at -20^{0}C.

6.1.3 In vitro translation of viral RNA.

6.1.3.1 Virion ds-RNA.

Genomic RNA was extracted from purified virus preparations by proteinase K digestion and phenol/chloroform extraction as described earlier. After ethanol precipitation the pelleted RNA was dissolved in sterile distilled water to give a concentration of 200μg/ml, determined spectrophotometrically assuming that 1 OD_{260} was equivalent to 50μg/ml ds-RNA (Gaillard and Joklik, 1982). Prior to translation the ds-RNA was denatured by the addition of methylmercury hydroxide (Ventron GMBH., W. Germany) to give a final concentration of 10mM (Payvar and Schimke, 1979).

6.1.3.2 Virus transcripts.

The precipitated RNA from the *in vitro* transcription reaction was dissolved in sterile distilled water to give 200μg/ml. These RNA preparations were treated with 10mM methylmercury hydroxide prior to translation.

Samples (5μl) of either denatured ds-RNA or viral transcripts were added to the translation mixture (35μl rabbit reticulocyte lysate [Anglian Biotechnology Ltd.,

Colchester]; 1μl amino acid mixture lacking methionine - supplied with the lysate; 7μl [^{35}S]-met [10mCi/ml]; 2μl distilled water) contained in 0.5ml stoppered plastic microfuge tubes and incubated at 30^{0}C for 1h.

To measure the incorporation of [^{35}S]-met into protein 2μl aliquots of the reaction mixture were removed at various times during the incubation and added to 0.5ml 1M NaOH in 5% hydrogen peroxide. When all the samples had been taken the tubes were incubated at 37^{0}C for 5min and 2ml ice-cold 2% lactalbumin hydrolysate in 25% TCA added. After incubation on ice for 30min the precipitates were removed by filtration through glass fibre membranes (GF/C; Whatman LabSales Ltd.). The filters were washed with 20ml 8% TCA then 10ml methanol and dried. The filters were added to 10ml toluene based scintillation fluid containing 10% tissue solubiliser (Soluene 350; Packard) and the acid precipitable radioactivity was measured.

6.1.4 Analysis of translation products.

After incubation the translation mixture was frozen at -70^{0}C. Aliquots (5μl) of the mixture were mixed with 50μl Laemmli sample buffer and applied to a 10% polyacrylamide gel and electrophoresed at 100V constant voltage until the bromophenol blue marker dye reached 1cm from the bottom of the gel. The gels were fixed with a solution containing 10% (w/v) TCA; 10% (v/v) acetic acid; 30% (v/v) methanol, then impregnated with En^{3}Hance, dried and autoradiographed with preflashed Fuji RX X-ray film at -70^{0}C.

6.1.5 Analysis of transcription products.

Viral RNA transcripts, labelled with α-[^{32}P]-UTP, were separated by agarose gel electrophoresis in the presence of 5mM methylmercuric hydroxide by the method of Bailey and Davison (1976) with the modifications described by Chandler *et al.* (1979).

Gels containing 1% low melting temperature agarose (BRL UK Ltd., Cambridge) and 5mM methylmercuric hydroxide in electrophoresis (E) buffer (50mM boric acid; 5mM sodium borate; 10mM sodium sulphate; pH 8.2) were cast in 0.6cm diameter glass tubes 18cm long. RNA samples were made up in E buffer containing 5mM methlymercuric hydroxide and 10% glycerol and layered onto the gels. Electrophoresis was carried out at 4^0C for 16h with 1.5mA/gel constant current. The gels were removed from the tubes and fractionated into 2mm slices. The positions of the RNA bands were determined by estimating the [^{32}P] content of each slice with Cerenkov counting on the preset [^{3}H] channel of a β-scintillation counter.

6.1.6 Recovery of isolated viral RNA transcripts.

Viral RNA transcripts were separated by agarose gel electrophoresis and the gel slices containing the individual transcripts identified. The RNA was electroeluted from the slices by the method developed by McDonnel *et al.* (1977). The gel pieces were placed in a dialysis bag filled with tris-borate buffer (45mM tris; 45mM boric acid; 1mM EDTA pH 8.0 containing 0.1% SDS) and the bag sealed taking care to remove all air bubbles. The bags were placed in a flat-bed electrophoresis tank containing the same buffer and subjected to 100V for 3h. At the end of the run the current was reversed for 10min to remove any RNA adhering to the bag. The gel pieces were discarded, the bag contents extracted once with phenol/chloroform and the RNA precipitated by the addition of $2^1/_2$ vol ethanol and storage at -20^0C.

6.1.7 Translation of isolated viral RNA transcripts.

The ethanol precipitated transcripts were pelleted at 10,000g for 30min and dissolved in 20μl sterile distilled water. Prior to translation the RNA samples were heated at 60^0C for 10min. 5μl of each RNA sample was added to 20μl translation mixture (350μl rabbit reticulocyte lysate; 10μl amino acid mixture without methionine; 70μl [^{35}S]-met, sp. act. 10mCi/ml) and incubated at 30^0C for $1^1/_2$h. Translation was stopped by freezing the samples at -20^0C. Analysis of the translation

products was performed by electrophoresis on 10% polyacrylamide gels and detection of the protein bands by fluorography as described above. The identity of the protein bands was determined by comparison with the the proteins produced by the translation of a mixture of all the viral transcripts.

6.2 RESULTS

6.2.1 Comparison of UK, C7-176 and C7-183 genome profiles

RNA, terminally labelled with [^{32}P], from the three strains was electrophoresed either separately or as mixtures of RNA from pairs of viruses to show any differences in segment migration more clearly (Figs 6.1 and 6.2). Comparison of the UK genome profile with those of the C7 isolates showed clear differences in the migration of all the segments with the exception of segments 2 and the 7,8,9 triplet. Co-electrophoresis of the RNAs from the two C7 isolates showed that their genome profiles were similar with a slight variation in segment 3 which differentiated the two strains. This difference was readily seen when the electrophoresis time was extended such that segment 10 migrated to the bottom of the gel (Fig 6.2; tracks 3-5).

6.2.2 Production of ss-RNA transcripts of the UK strain virus genome

To determine the concentration of purified virus required for optimum production of ss-RNA transcripts, the rate of incorporation of [^{3}H]-UTP into TCA precipitable material was analysed after the addition of various amounts of UK virus. When measured with respect to time over a 7h period (Fig 6.3) the incorporation rates appeared linear for virus concentrations of 1 - 11 μg. The rate for the highest virus concentration used, 22μg, was linear over the first 3h but then dropped away during the next 3h. This effect became clearer when these same data were plotted as incorporation against virus concentration (Fig 6.4) with the points derived from the highest virus concentration failing to fall on the straight line drawn through the points from the other virus concentrations. This effect was probably due to the

limiting concentration of one or more of the constituents of the reaction mixture. However, as the level of incorporation of radioactivity was adequate, it was decided to standardise the input of virus at 15-18μg.

6.2.3 Production of ss-RNA transcripts from UK and C7-183 viruses

In order to produce a sufficient amount of transcribed ss-RNA from these viruses for further analysis 0.1ml aliquots containing 15-18μg (0.8-1.0 OD_{260}) of purified virus were added to 0.3ml of reaction mixture and α-[^{32}P]-UTP used to label the transcripts. The incorporation of radioactivity with respect to incubation time (Fig 6.5) indicated little difference in the rates of incorporation between the two viruses. For the preparation of sufficient quantities of labelled transcripts the incubation time was restricted to 5h.

6.2.4 Analysis of SS-RNA transcripts from UK and C7-183 by agarose gel electrophoresis.

To determine the optimum electrophoresis conditions for the separation of UK rotavirus ss-RNA transcripts in methylmercuric hydroxide containing agarose gels, samples were electrophoresed at either 2.0, 1.5 or 1.25mA per gel for 16h. After fractionating the gel into 2mm slices the positions of the [^{32}P]-labelled transcripts were determined by Cerenkov counting. The results are shown in Fig 6.6. Eight peaks of radioactivity were seen in gels electrophoresed at either 1.5 or 1.25mA, with a pattern similar to the ds-RNA genome profile. Electrophoresis at 2mA resulted in the peaks containing the two smallest transcripts being lost from the bottom of the gel. The failure to resolve transcripts 2 and 3 and transcripts 7, 8 and 9, even at 2mA, was expected because the genome segments 2 and 3 were shown to be of similar size, as were segments 7, 8 and 9. As this method would uniformly label the ss-RNA it was expected that the peaks containing the larger transcripts, 1 to 4, would contain more radioactivity than the smaller transcripts. However, the

larger transcripts had fewer associated counts than expected, probably due to the increased failure rate of the viral polymerase during the transcription of the larger genome segments, as shown for reovirus (Skehel & Joklik, 1969).

The best separation of the viral transcripts was therefore achieved by electrophoresis with a current of 1.5mA and this was used for subsequent agarose gels.

Analysis of the transcripts of UK and C7-183 by agarose gel electrophoresis is shown in Fig 6.7. As described above, eight peaks were separated for the UK strain. Eight peaks were also found for C7-183 but instead of a peak in the position of UK transcript 11 a peak of radioactivity was seen between the expected positions of transcript 6 and transcripts 7,8 and 9. This peak, labelled 6a, gave the C7-183 transcript pattern an appearance similar to the genome profile of the virus.

6.2.5 In vitro translation of rotavirus ds-RNA

In order to determine the optimum incubation time for the translation of denatured UK and C7-183 genomic ds-RNA, samples were removed from primed rabbit reticulocyte lysates after 0, 20, 40 and 60min incubation and the incorporation of [^{35}S]-met into acid precipitable material assessed. The results of this experiment are shown in Fig 6.8 and compared with an unprimed lysate. The incorporation was essentially linear for 40min and although the rate had dropped by 60min this incubation time was used in all subsequent translations.

The polypeptides translated from denatured genomic RNA from UK and C7-183 isolates of rotavirus and separated by electrophoresis are shown in Fig 6.9. Eleven polypeptides were detected for both viruses, ten of which corresponded in size to the polypeptides produced during replication of the viruses in cell culture. Polypeptide vp7, present in infected cells, was not detected following translation. However, as vp7 is a glycoprotein its primary translation product would be expected

to be smaller and a polypeptide migrating in the region of vp9, labelled *, was detected. Two other bands were consistently seen but these were also seen after incubation of unprimed translation mixtures. Some minor bands were also seen after translation of the primed mixture but these bands were not consistently seen and their origin remained obscure.

6.2.6 In vitro translation of rotavirus ss-RNA transcripts

The polypeptides detected after the translation of UK and C7-183 ss-RNA transcripts are shown in Fig 6.10. The same array of polypeptides were found as described for the translation of denatured genomic RNA. However, the amount of lower molecular weight polypeptides was greater when ss-RNA was used. One major band which did not correspond to a viral protein was also present in control unprimed translation mixtures. Therefore, in order to determine the coding assignment of the aberrant C7-183 genome segment, translation of its ss-RNA transcript was attempted.

6.2.7 Coding assignments of UK and C7-183 rotavirus ss-RNA transcripts

UK and C7-183 ss-RNA transcripts were separated by agarose gel electrophoresis and the individual bands recovered by electroelution. With the method used, elution was effective irrespective of the molecular weight of the transcript. Typical recovery rates for both viruses are shown in Table 6.2. No attempt was made to separate further the transcripts of similar size, i.e. transcripts 2 and 3, and transcripts 7,8 and 9. Comparison of the translation products of the total transcript mixture with those from the individual ss-RNA transcripts of UK rotavirus is shown in Fig 6.11. No products were detected in translations primed with transcripts 1 and the 2,3 mixture. Transcripts 4,5,6, the 7,8,9 mixture, 10 and 11 produced translation products corresponding to the viral polypeptides vp4, vp5, vp6, a mixture of vp8, vp9 and vpr7, vp12 and vp11 respectively. These coding assignments were in agreement with those published by McCrae and McCorquodale (1982). The translation products of the ss-RNA transcripts of C7-183 are shown in

Fig 6.12. No products were detected for transcripts 1 and 5 for this virus but transcripts 2 and 3, 4, 6, the 7,8,9 mixture and 10 encoded vp2, vp4, vp6, a mixture of vp8, vp9 and vpr7, and vp12 respectively. Only one polypeptide vp2 was found after the translation of the mixture of transcripts 2 and 3 which was unexpected and the reason obscure. Transcript 6a produced a single polypeptide corresponding in size to vp11. This suggested that the normal sized group A rotavirus genome segment 11 had been replaced by segment 6a in isolate C7-183.

6.3 DISCUSSION

This section describes the characterisation of two bovine group A rotaviruses, C7-176 and C7-183, having unusual genome profiles with no segment 11 but an extra segment between segments 6 and 7. As described earlier, the number and sizes of the intracellular polypeptides induced by these strains were similar to those produced by the UK strain suggesting that the aberrant genome segment coded for a normal sized viral protein. Analysis of the *in vitro* transcription products of C7-183 revealed an abnormally large ss-RNA transcript which when translated produced a single viral polypeptide, vp11. This polypeptide has been shown to be the product of UK segment 11 (McCrae and McCorquodale, 1982) and therefore the unusual genome profiles of C7-176 and C7-183 resulted from an increase in size of the normal rotavirus segment 11.

The detection of the C7 type genome profile was interesting because these strains might shed some light on the observed genome profile variation between rotavirus isolates. Until recently all rotaviruses were thought to contain a common group-specific antigen but then several viruses were described which did not possess this antigen and were called atypical. Pedley *et al.* (1983) defined three rotavirus groups, group A containing the original viruses and groups B and C the atypical viruses. Since then two further groups have been described (Pedley *et al.*, 1986). Besides serological differences between the group A viruses and viruses from other groups, Pedley *et al.* (1986) found that the genome profiles were distinct. The genome of the group A viruses was defined as comprising four high molecular

weight ds-RNA segments (1 to 4), five middle-sized segments (5 to 9) including a distinctive triplet of segments (7 to 9) and two smaller segments (10 and 11). As described earlier, variation in genome profiles of group A rotaviruses is common, however, in most cases this basic pattern has been maintained with the triplet of segments 7, 8 and 9 being diagnostic for this rotavirus serogroup. Even though C7-176 and C7-183 showed unusual genome profiles for group A viruses, the characteristic triplet was still evident. So far few viruses from other serogroups have been analysed and therefore assignment of a new rotavirus isolate to a particular serogroup should not be on genome profile evidence alone.

Variation in the size of genome segment 11 has been seen before in group A rotaviruses but not to such a large extent. In investigations of human rotaviruses, Espejo *et al.*, (1980b) and Rodger *et al.*, (1981) showed that the viruses could be divided into two groups, termed short and long electropherotypes, according to their genome profiles. By gene coding assignment, the short electropherotype was shown to be due to an increase in the size of segment 11 but not segment 10 (Dyall-Smith and Holmes, 1981b). This could also explain the "super short" RNA patterns described for other human rotavirus strains (Hasegawa *et al.*, 1984; Albert,1985) in which segment 10 migrated just below the segments 7, 8 and 9 triplet. However, the increase in size of segment 11 of both the short and "super short" viruses was not as great as seen with C7-176 and C7-183.

Other group A rotaviruses with abnormal genome profiles were isolated from chronically infected immunodeficient children (Pedley *et al.*, 1984). The RNA segments of increased size were derived from the segments of normal size by concatemer formation and are referred to as genome rearrangements. When rearranged segments were introduced into UK bovine rotavirus by gene reassortment, Allen and Desselberger (1985) found that they performed the normal function of the replaced bovine rotavirus gene. A further study with rotaviruses from a chronically infected immunodeficient child (Hundley *et al.*, 1987) showed the existence of mulitple subpopulations of viruses differing in genotype and containing rearrangements involving segments 8, 10 and 11. Viruses with genome

rearrangements were also produced by cell culture passage of UK rotavirus at high m.o.i. (Hundley *et al.*, 1985), in this case genome segment 5 was lost and replaced distinct additional bands between segments 1 and 6. They also showed that, in common with C7-183, these rearranged segments were transcribed *in vitro* to give enlarged transcripts. However, whereas the aberrant gene 11 of C7-183 encoded a normal sized polypeptide, the protein product of rearranged segment 5 of these viruses could either not be detected or was present as an abnormal protein of corresponding size to the increased coding capacity of the rearranged segment.

There have been several other reports describing the presence of group A rotaviruses, having atypical genome profiles, in faeces. Besselaar *et al.* (1986) reported the detection of such viruses in the faeces of six healthy human neonates. These viruses had a similar profile abnormality to the bovine viruses described here in that the 11th genome segment was missing but another segment was detected between segments 6 and 7. Thouless *et al.* (1986) described two lapine rotaviruses, one with segment 11 being replaced with a segment between 6 and 7, but the other lacked a segment 10 which was again replaced with a larger segment between segments 6 and 7. Finally, Bellinzoni *et al.* (1987) reported the genome profiles of swine rotavirus isolates in which genome segment 11 was replaced by a segment of higher molecular weight. With all the reports of genome rearrangements published to date the greatest number of rearrangements have involved segment 11. At present it is unknown whether gene 11 is more prone to rearrangement or whether some other factor or positive selective advantage is involved.

Recently published sequence data on rearranged rotavirus genes has confirmed that these enlarged segments are indeed formed by concatemer formation. All the reports have recorded sequence data of rearranged gene 11 segments and all have found that the increase in size of the genome segment has involved head to tail concatemerisation of the normal gene 11 with partial deletions in both monomers, leading to an enlarged segment but with normal 3' and 5' ends. In all cases the upstream copy of the gene has lost its 3' end and the downstream copy its 5' end, however, the truncation of the upstream copy does not disrupt the open reading

frame and therefore the normal gene product is produced. The variation in the size of the rearranged gene is generated by the variation in the length of the truncated downstream copy. Three groups have published sequences of the rearranged segments from "supershort" genome profile viruses, Gorziglia *et al.* (1989) from the Alabama strain (ala) of rabbit rotavirus, Matsui *et al.* (1990) 69M human and VMRI bovine strains and Mattion *et al.* (1990) CC86 porcine strain. Their results show an increase of 369 (ala), 281 (69M), 300 (VMRI) and 400 nucleotides (CC86) over the normal length of segment 11 (667 nucleotides for UK bovine and SA11). Matsui *et al.* (1990) also sequenced the rearranged gene 11 of a short profile virus, human rotavirus DS-1, and found an increase of 154 nucleotides. The sequence of the rearranged segment of C7-183 has been reported (Scott *et al.*, 1989) and again shown to be a head to tail concatemer of gene 11 with an increased size of 575 nucleotides. In this strain the truncation of the upstream copy occurs within the termination codon of the open reading frame, however, the addition of the downstream copy restores the stop codon converting it from a UAG to a UGA thereby maintaining the coding capacity of the gene. Although a similar increase in the size of the rearranged gene of the porcine strain C60, by 564 nucleotides, has been reported (Gonzalez *et al.*, 1989) the addition of the downstream copy occurs in the 3' non-coding region of the upstream copy.

As all the rearranged genes so far sequenced have a similar structure it would suggest that they arose by a common mechanism. A copy choice mechanism involving the "jumping" of the viral polymerase from one template strand to another has been reported for several ss-RNA viruses (Bujarski and Kaesberg, 1986; Makino *et al.*, 1986; Kirkegaard and Baltimore, 1986). For this mechanism to produce rotavirus gene rearrangements would require explanations as to why the polymerase retains template specificity and does not "jump" to other rotavirus genes and the polymerase resumes transcription within the gene and not at the beginning. A second mechanism was described by Scott *et al.* (1989) called the "transcriptional slippage" model which suggests that the polymerase transcribes normally to within the 3' non-coding region and then it slips back along the template from where it

completes the transcription terminating at the normal 3' end. This model does not require the polymerase to detach from the template and could explain why the rearrangements are not of uniform size.

Table 6.1

Recovery by electroelution of UK and C7-183 rotavirus ss-RNA transcripts separated by agarose gel electrophoresis.

UK		C7-183	
Transcript number	Counts recovered (%)	Transcript number	Counts recovered (%)
1	75	1	87
2,3	89	2,3	72
4	74	4	99
5	83	5	74
6	94	6	83
7,8,9	92	6a	90
10	77	7,8,9	87
11	90	10	89

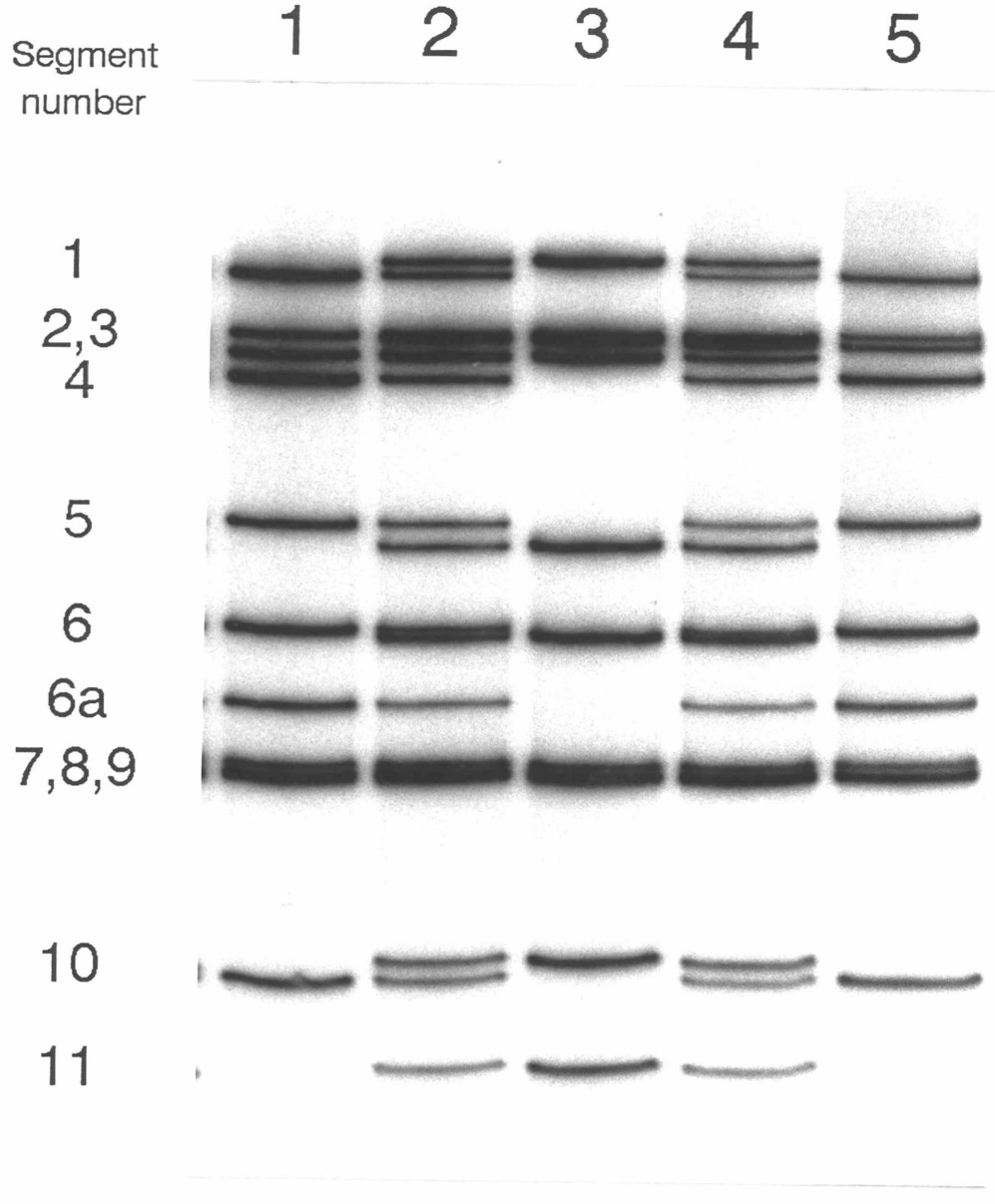


Fig 6.1: Comparison of the genome profiles of cloned C7-176, C7-183 and UK rotaviruses. [^{32}P] labelled genome segments from C7-183 (track 1), UK (track 3) and C7-176 (track 5) with mixtures of C7-183 and UK (track 2) and C7-176 and UK (track 4) were separated by electrophoresis in 10% polyacrylamide gels for 16h at 20mA. The positions of the genome segments are numbered on the left of the figure. 6a refers to the position of the aberrant segment of C7-176 and C7-183.

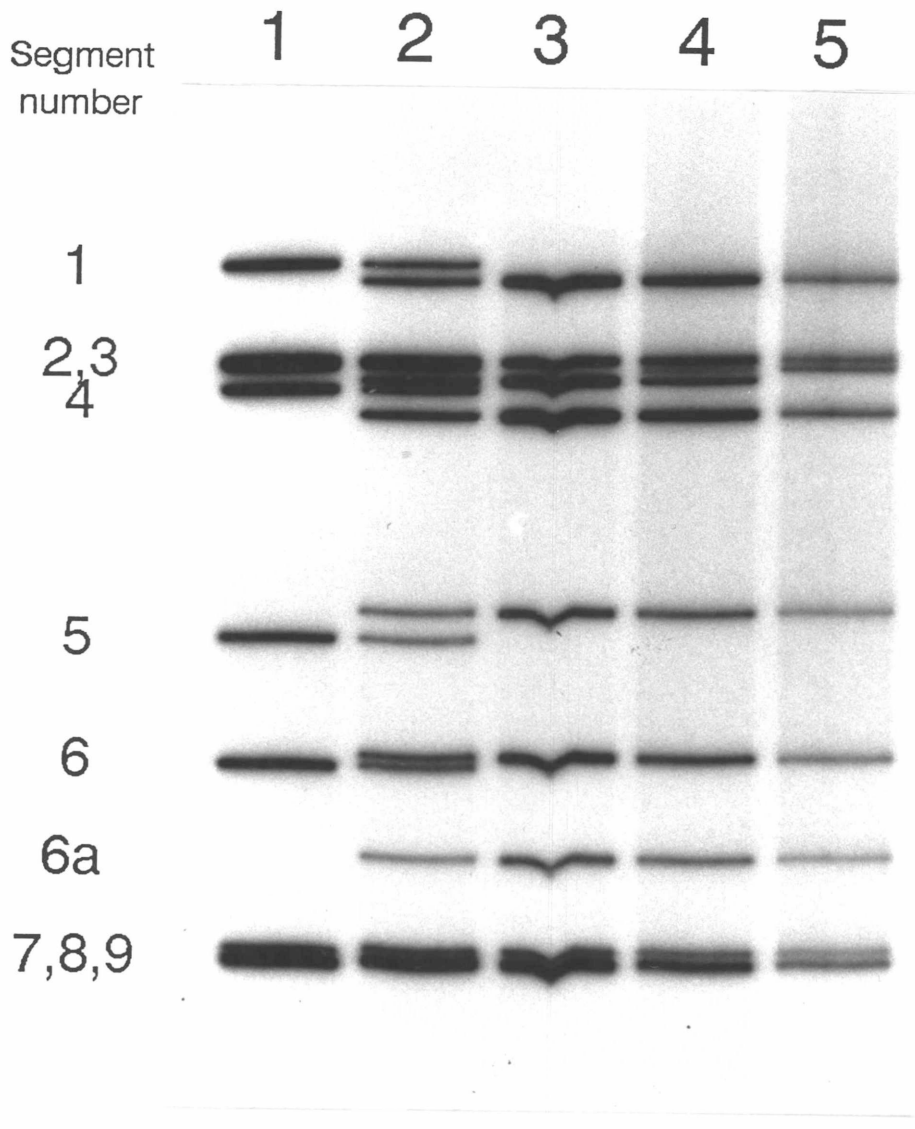


Fig 6.2: Comparison of the genome profiles of cloned C7-176, C7-183 and UK rotaviruses. [^{32}P] labelled RNA from UK (track 1), C7-183 (track 3) and C7-176 (track 5) with mixtures of UK and C7-183 (track 2) and C7-183 and C7-176 (track 4) were electrophoresed for 24h at 20mA in a 10% polyacrylamide gel. The numbering of the genome segments is described in Fig 7.1.

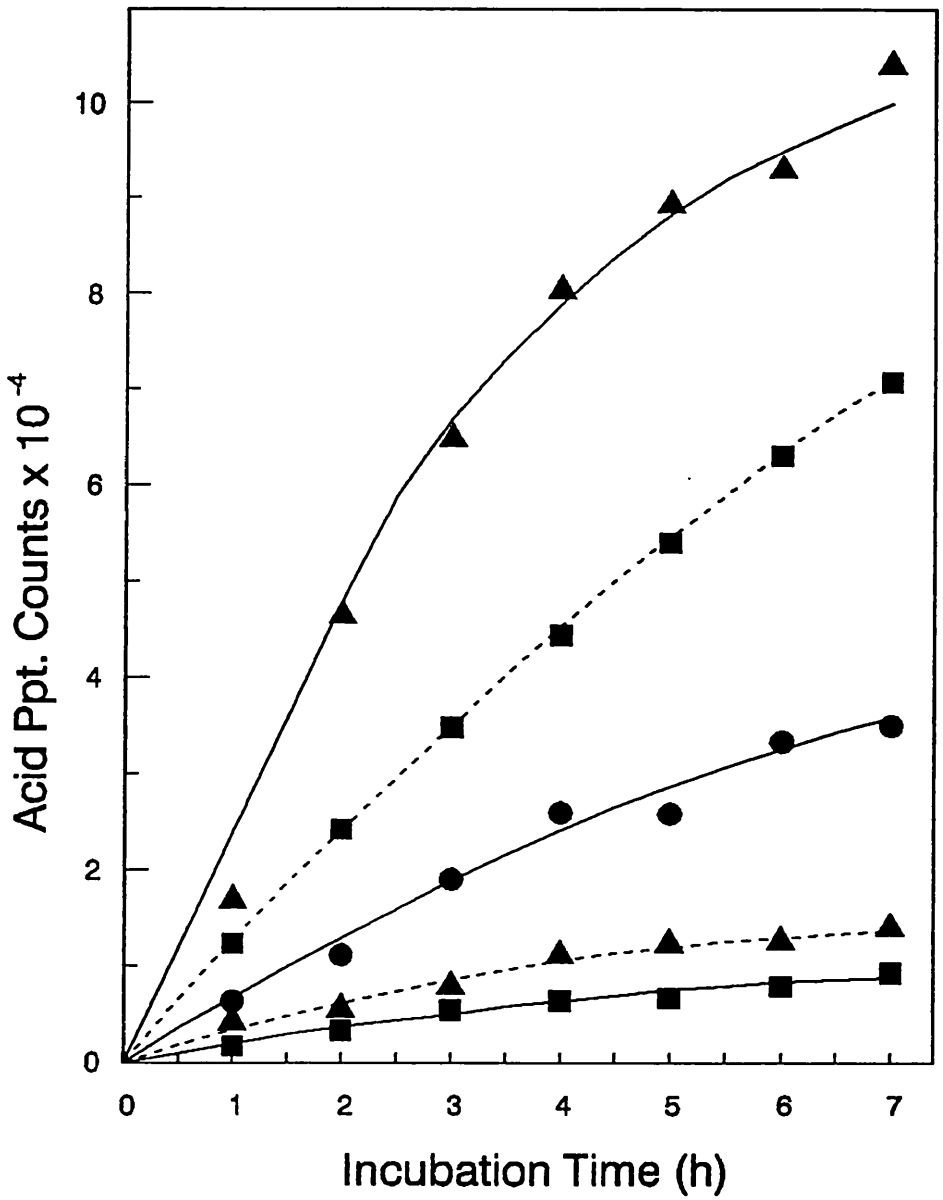


Fig 6.3: Effect of length of incubation on rotavirus in vitro transcription. Samples were removed at hourly intervals to determine the incorporation of [^{3}H]-UTP into acid precipitable material from transcription mixtures containing 21.5µg (▲—▲) 10.75µg (■-■), 5.38µg (●—●), 2.2µg (▲-▲) and 1.1µg (■—■) purified UK rotavirus.

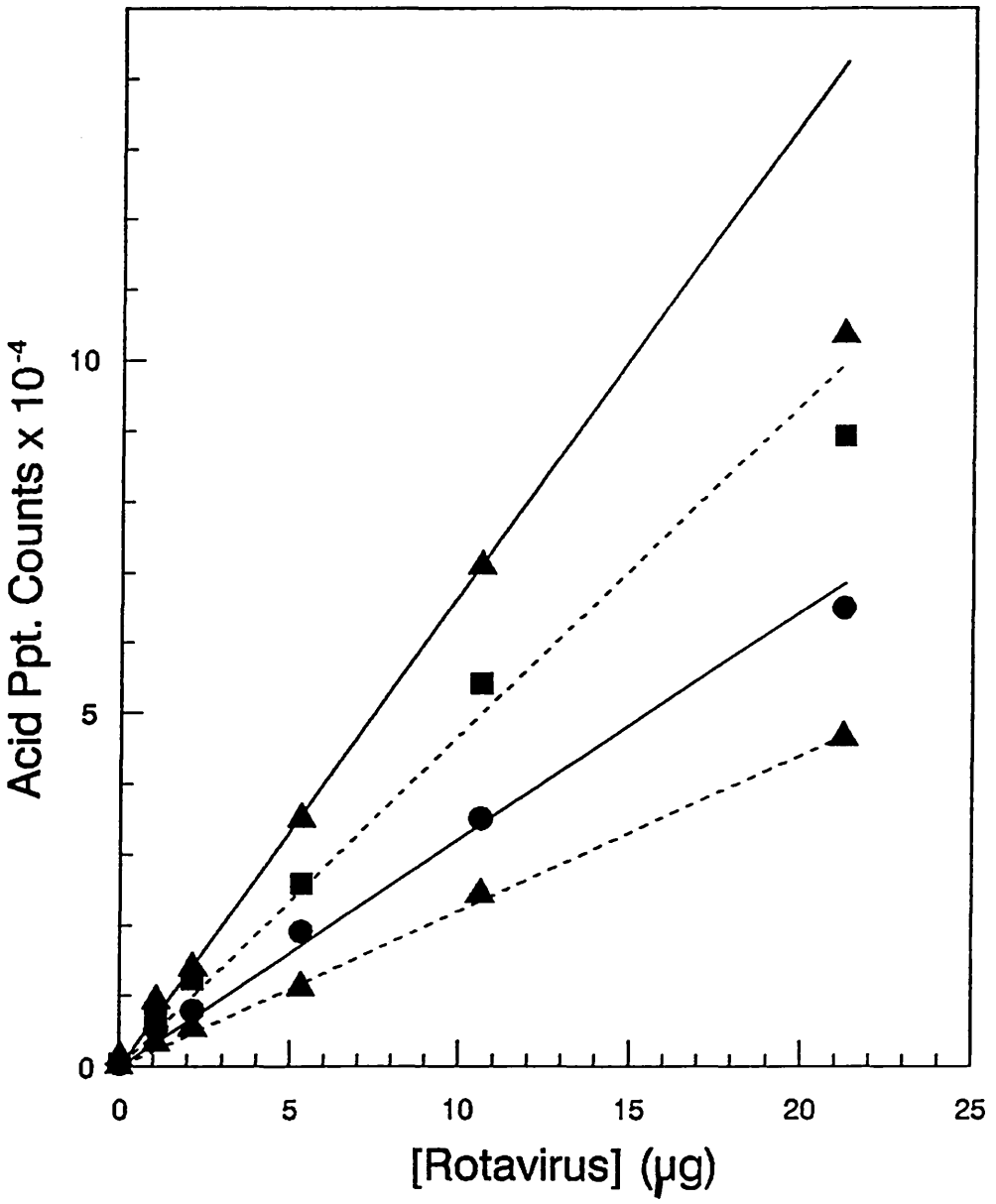


Fig 6.4: Effect of varying the concentration of purified UK rotavirus on in vitro transcription. Samples were removed from transcription mixtures containing different amounts of purified virus at 1h (▲-▲), 3h (●—●), 5h (■-■) and 7h (▲—▲) to determine the incorporation of [^{3}H]-UTP into acid precipitable material.

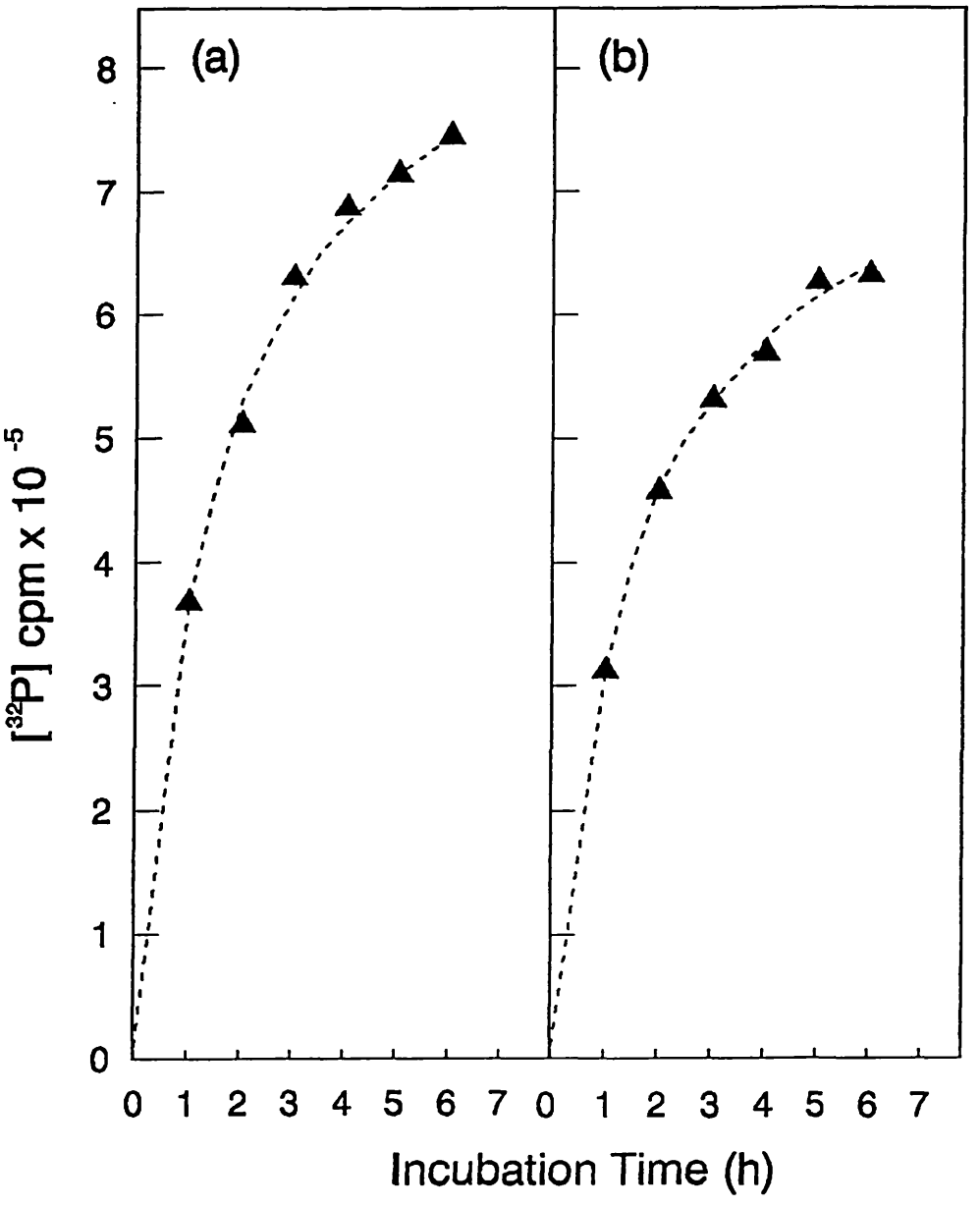


Fig 6.5: Comparison of the *in vitro* transcription rates of (a) UK and (b) C7-183 rotaviruses. Samples were removed from transcription mixtures at hourly intervals to determine the level of incorporation of α-[^{32}P]-UTP into acid precipitable material.

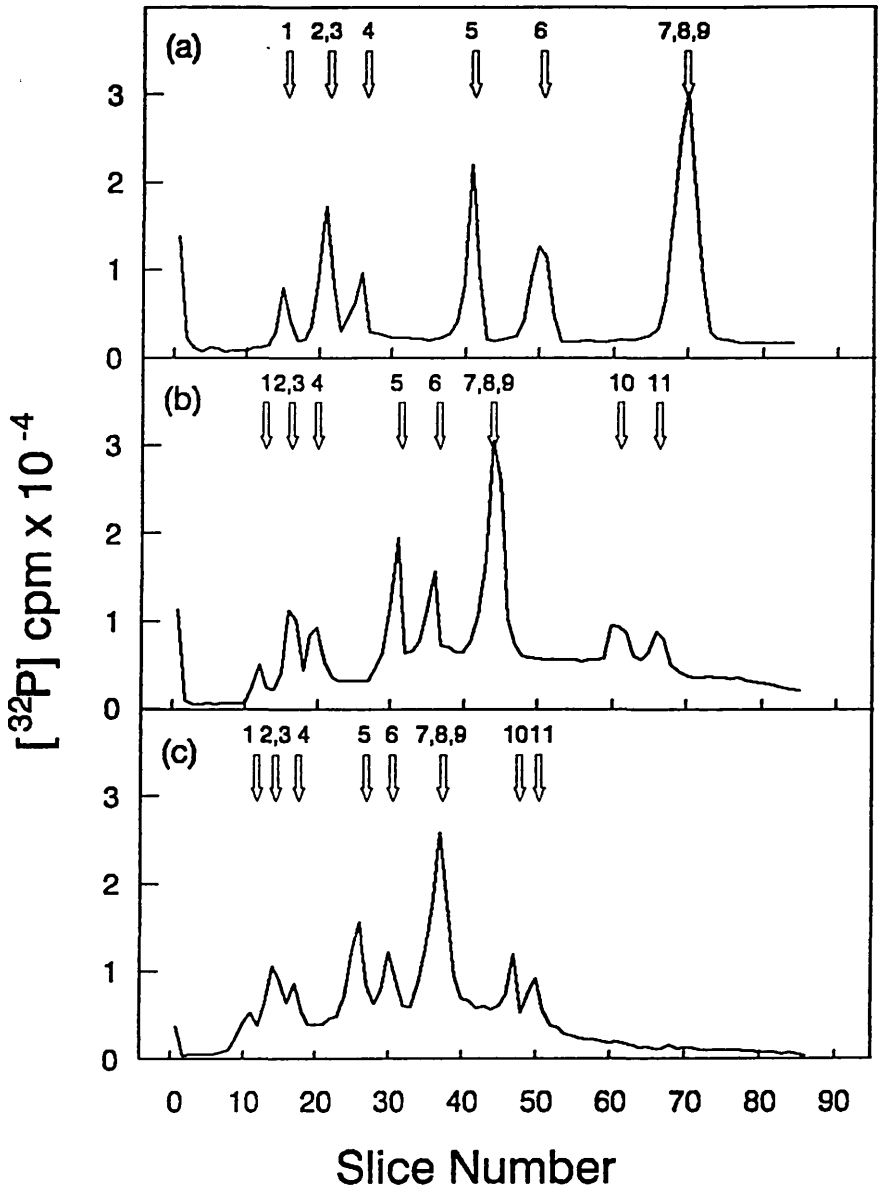


Fig 6.6: Separation of UK rotavirus ss-RNA transcripts, produced in vitro , by agarose gel electrophoresis in the presence of methlymercuric hydroxide. After electrophoresis for 16h at either (a) 2.0mA, (b) 1.5mA or (c) 1.25mA the gels were fractionated into 2mm slices and the amount of radioactivity contained in each fraction was determined by Cerenkov counting. The numbered arrows denote the positions of the transcripts. Electrophoresis was from left to right.

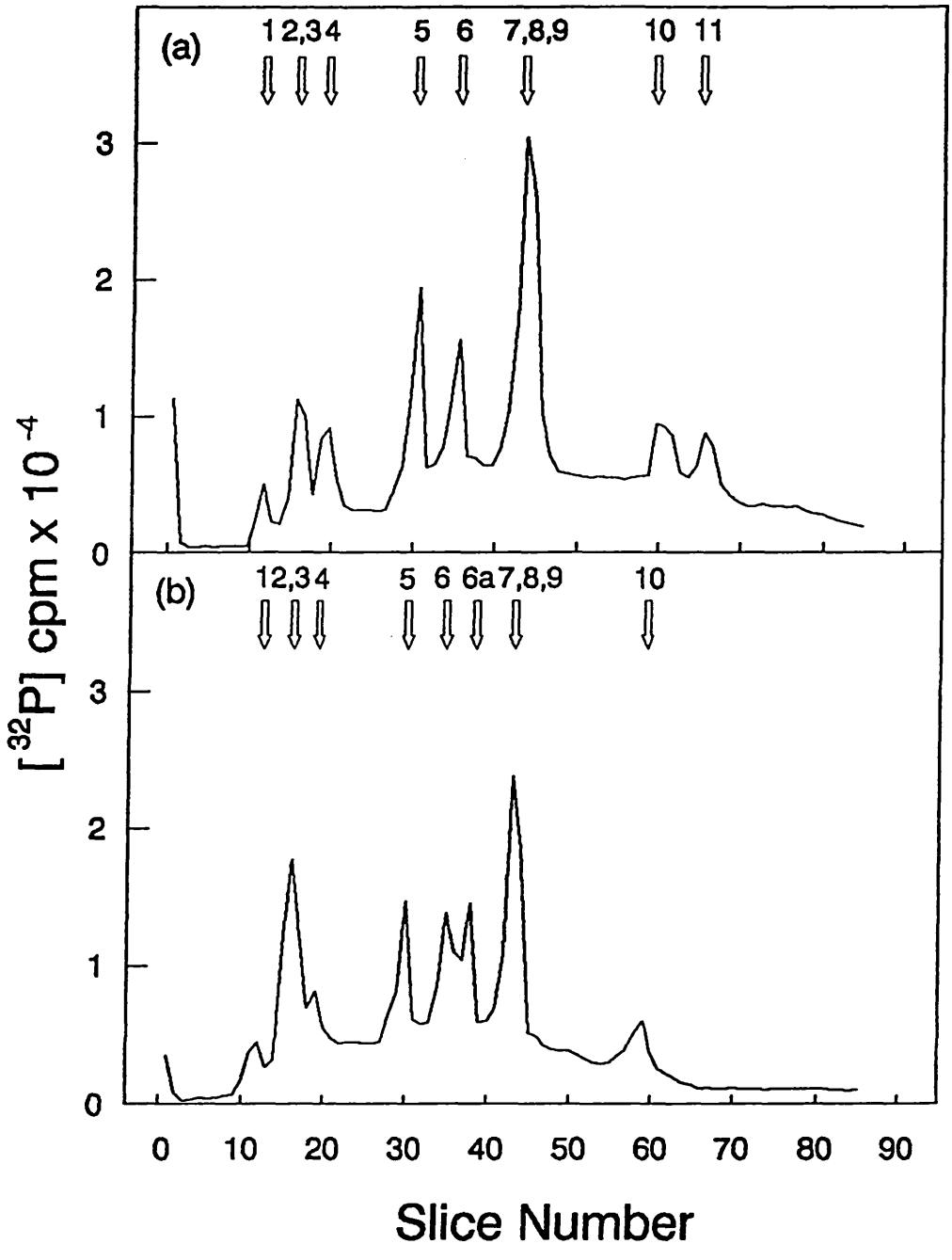


Fig 6.7: Separation of the ss-RNA transcripts, produced by UK (a) and C7-183 (b) by agarose gel electrophoresis in the presence of methylmercuric hydroxide. The distribution of radioactivity was determined by Cerenkov counting after fractionation of the gels into 2mm slices. The positions of the transcripts for both strains are denoted by the numbered arrows. Electrophoresis was from left to right.

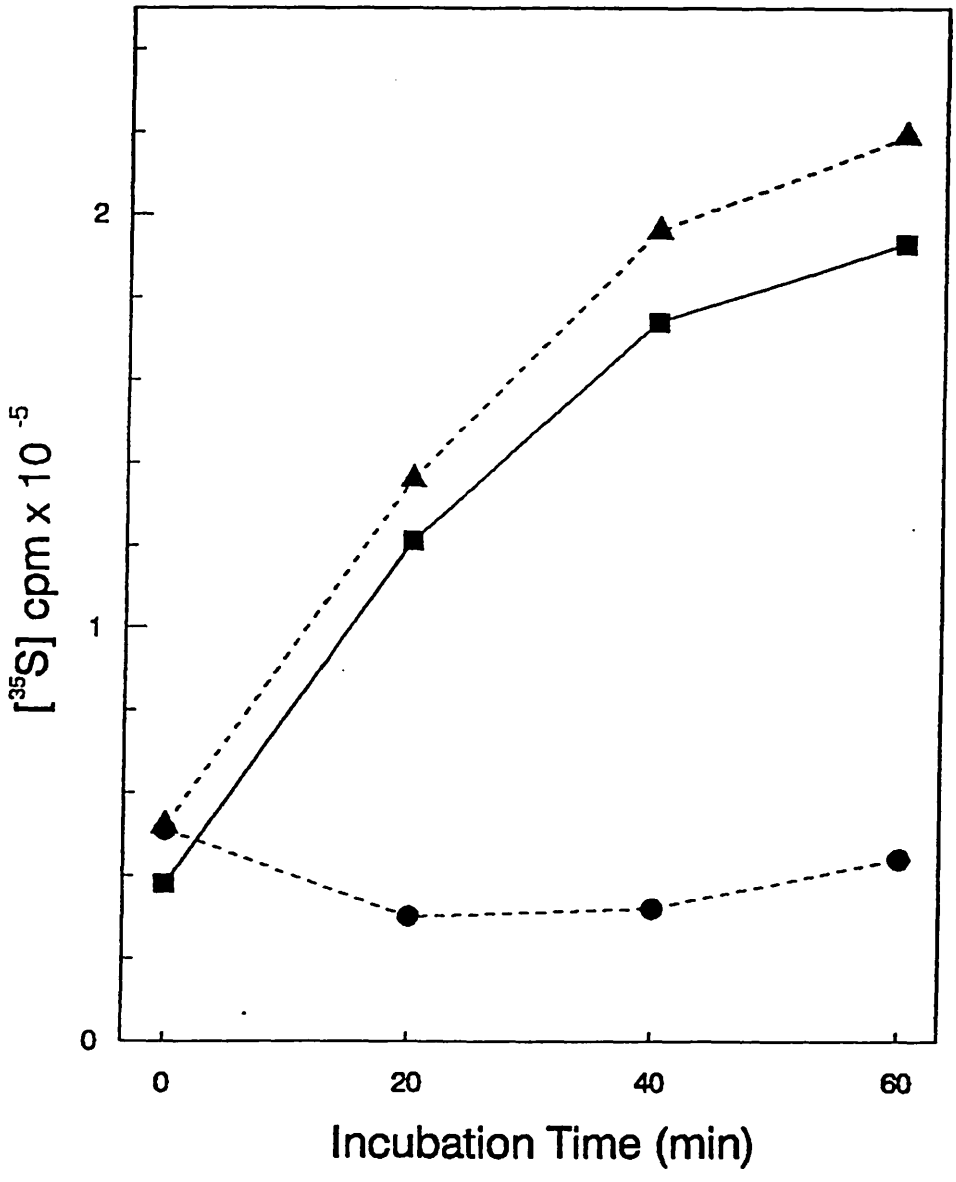


Fig 6.8: Rate of incorporation of [^{35}S]-met into acid precipitable material after priming rabbit reticulocyte lysate preparations with either UK ■—■ or C7-183 ▲···▲ denatured genomic ds-RNA compared with an unprimed ●···● lysate. Samples were removed at 0, 20, 40 and 60min after the addition of the viral RNA.

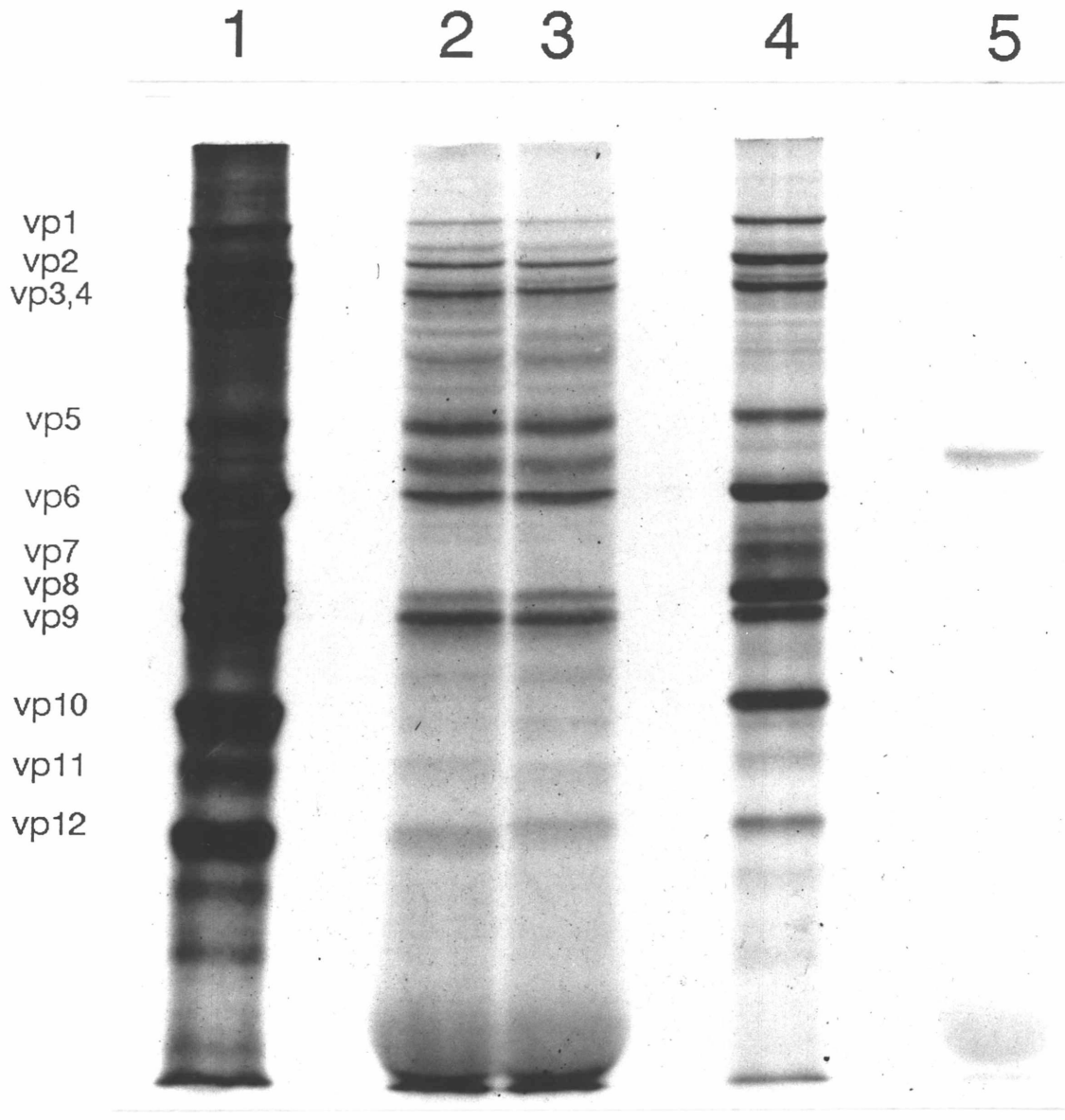


Fig 6.9: Analysis of the polypeptides produced during the *in vitro* translation of denatured rotavirus genomic RNA. Rabbit reticulocyte lysates were primed with either denatured C7-183 (track 2) or UK (track 3) RNA or unprimed (track 5) and incubated for 60 min. The polypeptides synthesised were compared with the intracellular polypeptides produced in MA104 cells by C7-183 (track 1) and UK (track 4) after separation by electrophoresis in a 10% polyacrylamide gel. The positions of the virus induced intracellular polypeptides are labelled vp1-vp12.

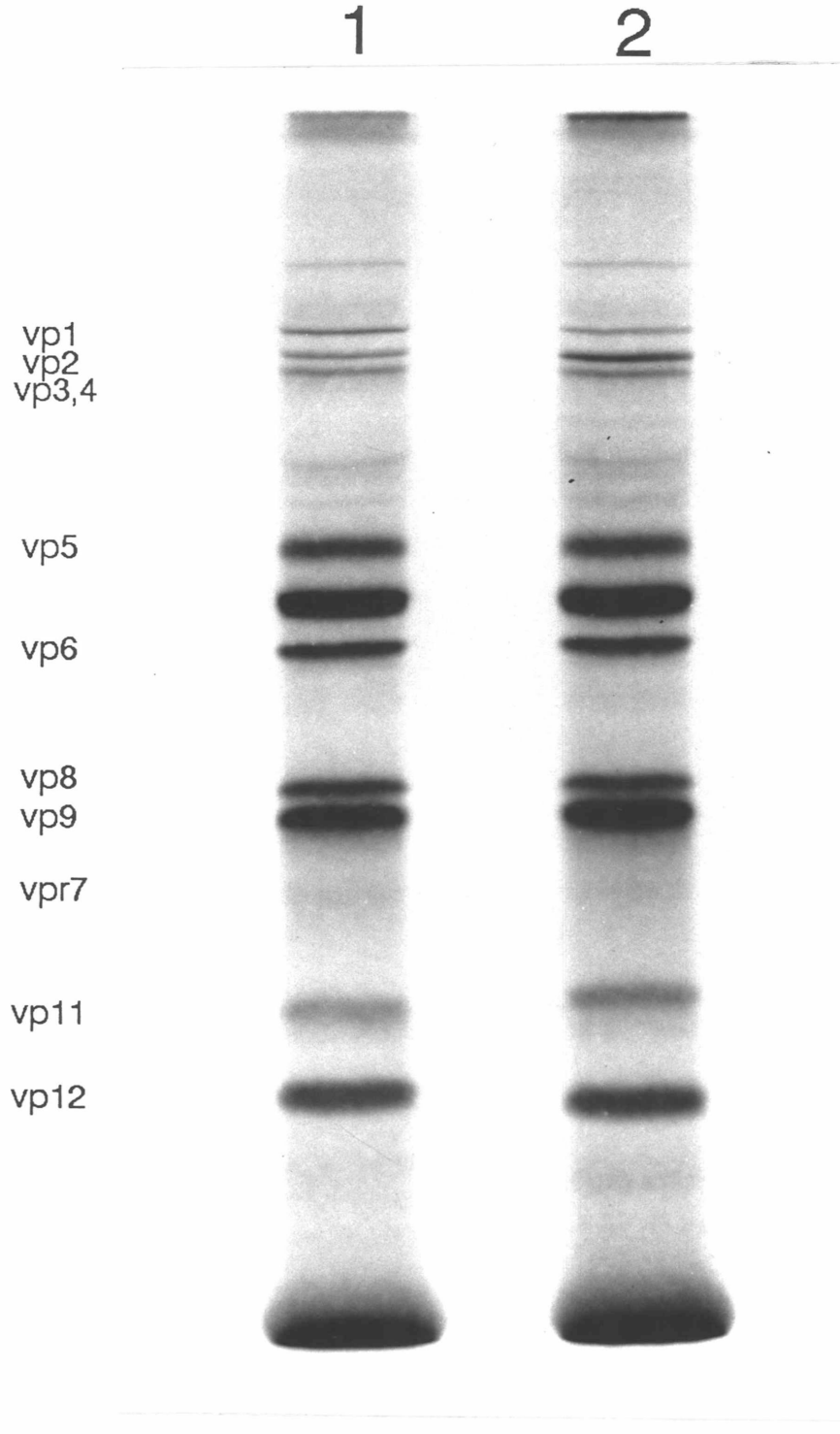


Fig 6.10: Analysis of the translation products of rabbit reticulocyte lysates primed with *in vitro* transcribed rotavirus ss-RNA. The polypeptides synthesised in lysates primed with ss-RNA transcripts from C7-183 (track 1) and UK (track 2) were separated by electrophoresis in a 10% polyacrylamide gel. The positions of the viral polypeptides are labelled vp1-vp12.

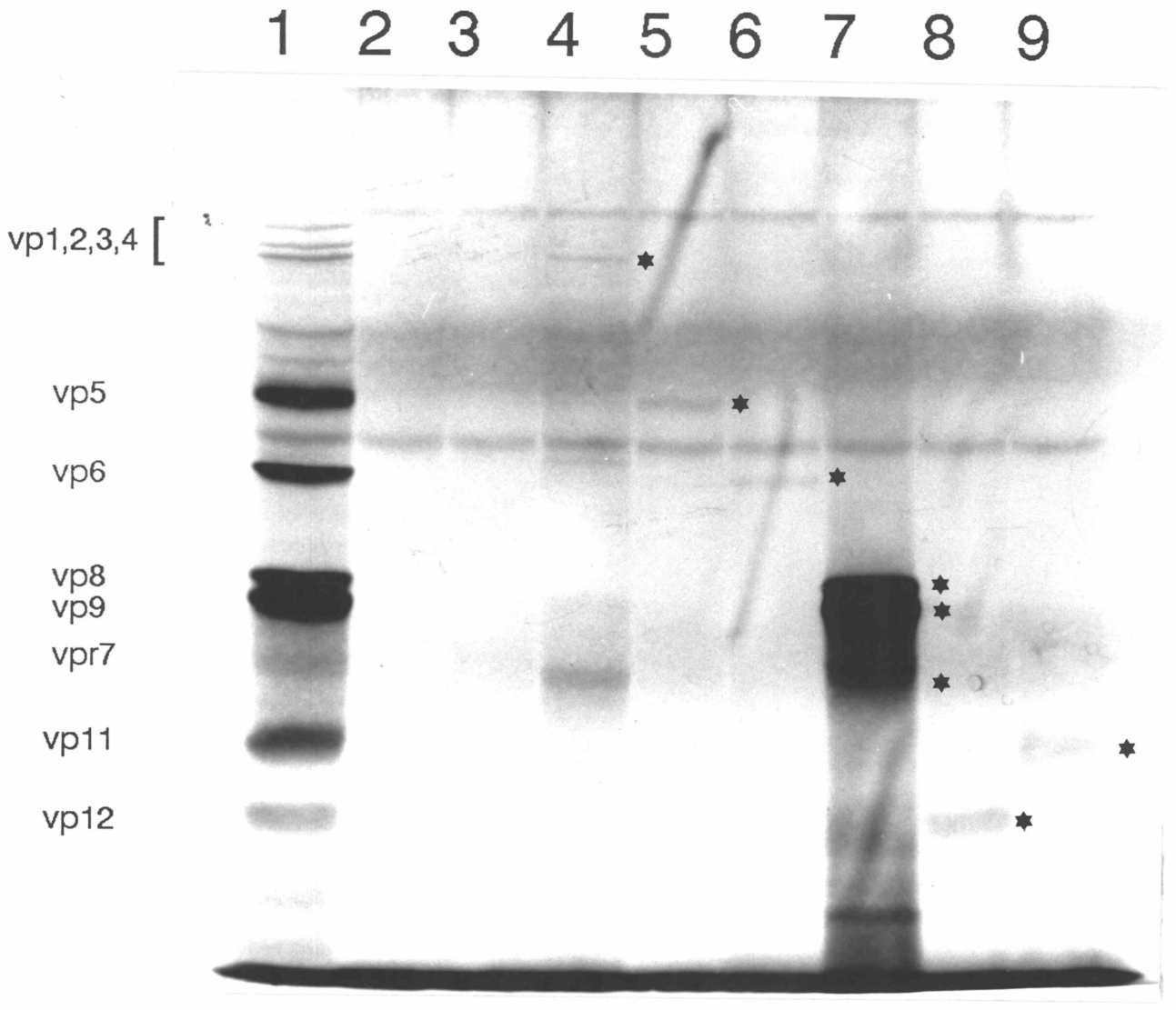


Fig 6.11: Polyacrylamide gel electrophoresis of the *in vitro* translation products of ss-RNA transcripts of UK rotavirus. The translation products from the total transcription mixture (track 1) and transcripts 1, 2+3, 4, 5, 6, 7+8+9, 10 and 11 (tracks 2-9 respectively) are shown. The positions of the primary gene products from the total transcription mixture are labelled vp1-vp12. The position of the products from the separated transcripts are labelled *.

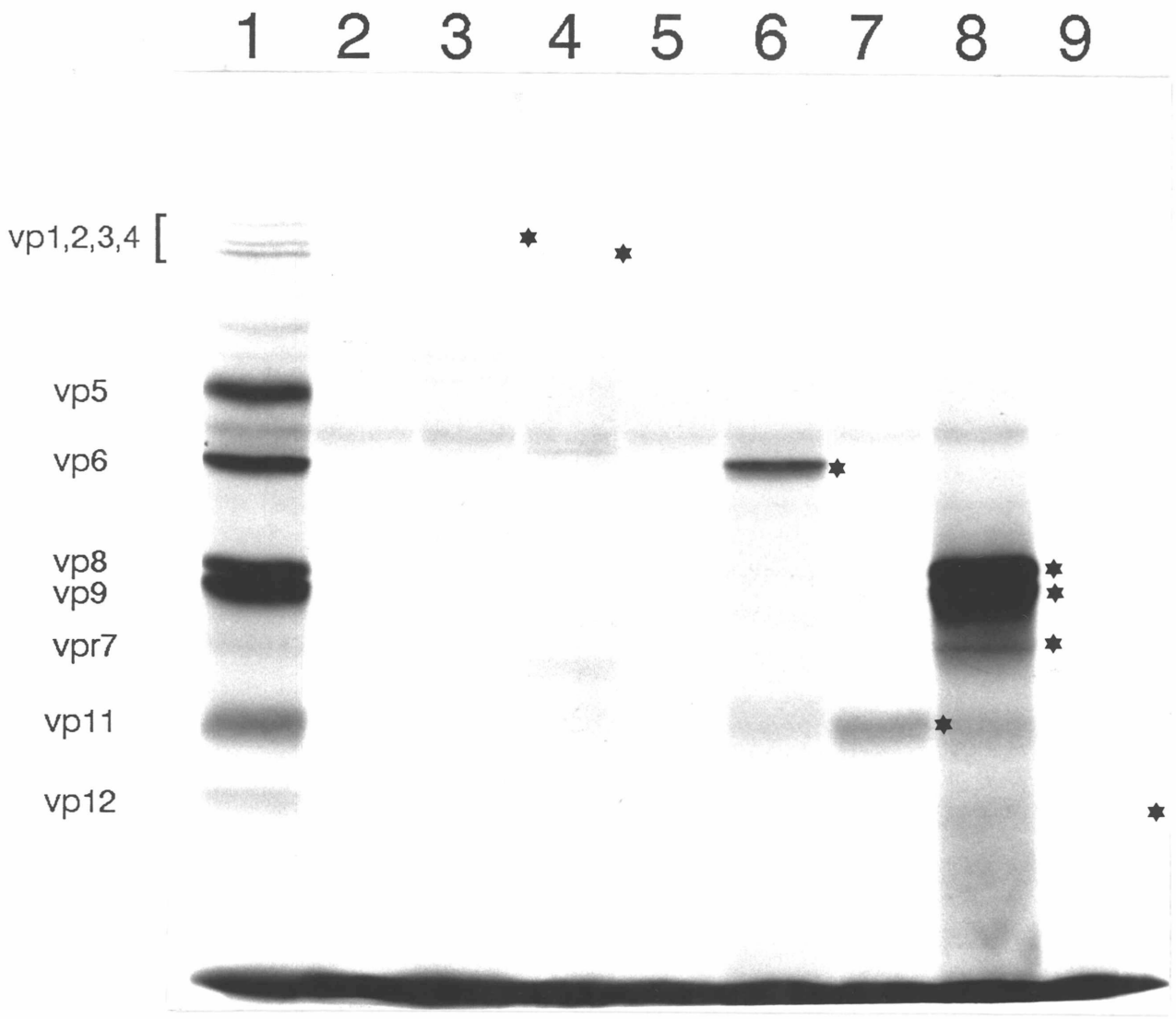


Fig 6.12: Polyacrylamide gel electrophoresis of the *in vitro* translation products of ss-RNA transcripts of C7-183 rotavirus. The translation products from the total transcription mixture (track 1) and transcripts 1, 2+3, 4, 5, 6, 6a, 7+8+9 and 10 (tracks 2-9 respectively) are shown. The positions of the primary gene products from the total transcription mixture are labelled vp1-vp12. The position of the products from the separated transcripts are labelled *.

7.0 ROTAVIRUS VIRULENCE

7.1 MATERIALS AND METHODS

7.1.1 Viruses

Two bovine rotavirus strains were used in these experiments, CP-1 which was virulent in 7 day old calves and C3-160 which was avirulent (see Section 5).

7.1.2 Comparison of the plaque sizes of C3-160 and CP-1

Plaque assays for C3-160 and CP-1 were set up as described in Section 3.1.5 with 6 petri dishes for each $\log_{10}$ dilution. Two petri dishes for each virus at each dilution were stained with neutral red at 8, 32 and 56h post inoculation. The plaque sizes for each virus were measured after 16h staining.

7.1.3 Effect of trypsin on the growth of C3-160 and CP-1

Monolayer cultures of MA104 cells were inoculated with either C3-160 or CP-1 and maintained with medium either trypsin-free or containing 0.5μg trypsin/ml. Cultures were harvested at 48h post inoculation and frozen at -70^{0}C. Each virus was passed twice in either trypsin-containing or trypsin-free medium. Each virus pass was assayed for infectivity by the fluorescent focus assay, described in Section 5.1.2, either with or without 0.5μg trypsin in the assay medium.

7.1.4 Chromium release from MA104 cells infected with C3-160, UK and CP-1 viruses

Confluent monolayers of MA104 cells in Roux flasks were incubated for 24h in serum-free medium. The cells were removed with trypsin/versene and washed x2 with serum-free medium containing 1% bovine serum albumin. The final cell suspension was adjusted to 1 x 10^{6}cells/ml. Aliquots of 1ml of cell suspension were centrifuged at 1000rpm for 2min, the medium discarded and the cells resuspended in 0.5ml medium containing either C3-160, UK or CP-1 virus at a multiplicity of 10.

Control cells were resuspended in virus-free medium. To each cell suspension was added 150μCi [^{51}Cr] as sodium chromate (sp. act. 250-500 mCi/mg Cr; Amersham International plc, Amersham, Bucks) and the suspensions rolled for 1h at 37^{0}C. The cells were then pelleted and washed x 3 with cold medium containing 5% foetal calf serum. The final cell pellet was resuspended in 4ml of serum containing medium prewarmed to 37^{0}C. Each cell suspension was inoculated into 96 well flat bottomed microtitre plates, 100μl containing 2.5 x 10^4 cells were added to each well, and incubated at 37^{0}C. One plate was used for each time period and contained control uninfected and virus infected cells. Plates were removed at 0hr and then at 3h intervals until 24h and the medium in the wells was mixed by gently tapping the edges of the plate prior to centrifuging the plates to pellet the non-adherent cells. To 3 wells of each of the virus infected or control cells 10μl 10% SDS was added; the SDS lysed the cells to give an estimate of the total number of [^{51}Cr] counts in the wells. Aliquots of 50μl were removed from 3 untreated and 3 SDS treated wells for each test and counted in a gamma counter. The % [^{51}Cr] release was calculated by dividing the mean cpm for the 3 untreated wells x 100 by the mean cpm for the 3 SDS treated wells.

7.1.5 Production of reassortant viruses containing C6-160 and CP-1 genome segments

Monolayers of MA104 cells were inoculated with a mixture of C3-160 and CP-1 viruses both at a multiplicity of 10. The cultures were incubated at 37^{0}C for 24h then frozen at -70^{0}C. The progeny virus was diluted and inoculated onto MA104 monolayers in petri dishes to produce virus plaques which were visualised 3 days later with neutral red staining. The agar and cells from well separated plaques were excised, mixed with 1ml medium and frozen at -70^{0}C. To produce enough virus for genotyping (see below) 100μl aliquots of each picked plaque were inoculated into tubes containing MA104 cell monolayers which were rolled at 37^{0}C. The virus was harvested when the monolayers showed extensive cpe.

7.1.6 Genotyping the progeny viruses

To determine the parental origin of all the eleven genome segments of the potential C3-160/CP-1 reassortants, viral RNA was extracted directly from the tissue culture fluid from the infected tube cultures. To 1.5ml of tissue culture fluid was added 300µl 10% SDS, 300µl 1M NaCl, 250µl 0.1M EDTA and 50µl 1M Tris/HCl pH7.2. After thorough mixing 2.5ml phenol/chloroform (1:1 $^{v}/_{v}$) was added and the two phases mixed. The two phases were separated by centrifugation at 3,500 rpm for 15min and the aqueous phase removed. One tenth volume of 2.5M sodium acetate was added to the aqueous phase and the RNA precipitated overnight with 2.5 volumes of ethanol at -20^{0}C. The RNA was pelleted at 10,000g for 30min and redissolved in 50µl distilled water. Aliquots (25µl) of each RNA preparation were taken for labelling with [^{32}P]-pCp and T_4 RNA ligase by the method described earlier. The viral genome segments were separated by electrophoresis through 10% polyacrylamide gels. On each gel, RNA segments from the two parental strains, C3-160 and CP-1, were also separated. Viruses shown to be reassortant strains were then plaque purified twice and genotyped for a second time.

7.1.7 Experimental infection of calves with C3-160/CP-1 reassortant strains

Gnotobiotic calves, 7 days of age, were inoculated orally with 3ml tissue culture fluid containing the cloned reassortant virus. Daily faecal samples were collected and assayed for virus excretion. Changes in faecal colour and anorexia were recorded but faecal outputs could not be assessed because all calves used in these experiments were female.

7.1.8 Growth of reassortant viruses at 37^{0}C and 39^{0}C

Monolayers of MA104 cells in 25cm^{2} plastic tissue culture flasks were infected with the viruses and submerged in water baths at either 37^{0}C or 39^{0}C. Cultures were harvested 48h after infection, frozen at -70^{0}C, thawed and the virus titres assayed by the fluorescent focus assay described earlier.

7.2 RESULTS

7.2.1 Plaque sizes of C3-160 and CP-1 rotaviruses

The increase in the size of C3-160 and CP-1 plaques with respect to the length of incubation is shown in Table 7.1. The C3-160 plaques were at least 50% larger than those of CP-1 after 1, 2 and 3 days incubation.

7.2.2 Effect of trypsin on the growth of C3-160 and CP-1 rotaviruses

The titres of virus produced during two passes of C3-160 and CP-1 in MA104 cells either in the presence or absence of trypsin are shown in Table 7.2. Titres of virus from the fluorescent focus assay performed by the published method, i.e. without trypsin in the assay medium, showed that both strains produced less virus when passed in the absense of trypsin but titres were maintained when trypsin was incorporated into the culture medium. The decrease in titre was more pronounced in the CP-1 cultures compared with C3-160, such that no infectious virus was detected after just one CP-1 pass in MA104 cells in the absence of trypsin. The effect of adding trypsin to the assay medium, however, was clearly seen. Although the titres of virus grown in the presence of trypsin remained within the error range for the assay, the titres of virus grown in the absence of trypsin increased 100-fold. This suggested that virus was still produced when trypsin was omitted from the medium but required protease treatment in order to be infectious. The fact still remained, however, that CP-1 was less able than C3-160 to grow in the absence of trypsin and was clearly seen after only one cell culture pass.

7.2.3 Release of [^{51}Cr] from MA104 cells infected with either C3-160, UK or CP-1 rotaviruses

The results of this experiment are shown in Fig 7.1. There was a linear release of [^{51}Cr] from uninfected cells to reach 25% after 24h. Cells infected with the three viruses showed increased release of [^{51}Cr] reaching a maximum of 80%.

Both C3-160 and UK strains gave similar release profiles with the increase from control values to maximum release occuring over a 9-12h period, although UK lagged 3h behind C3-160. CP-1 gave a slower rate of [^{51}Cr] release taking twice as long, 18h, to reach maximum values. It was interesting to note that cpe and [^{51}Cr] release were observed at the same time post infection (Table 7.3). The increase in the extent of the cpe with all three viruses mirrored their [^{51}Cr] release profiles.

7.2.4 Analysis of virus clones isolated from cell cultures infected with both C3-160 and CP-1 rotaviruses

Before attempting the production of reassortant viruses containing genome segments of either C3-160 or CP-1 it was necessary to show that the parental origin of each of the segments could be assigned. RNA was extracted from both isolates and the genome segments of each and a mixture of both RNAs were separated by electrophoresis. The results are shown in Fig 7.2. Nine of the eleven segments of both viruses migrated differently so that they could be distinguished. Only segments 2 and 8 could not be differentiated by this procedure. This method, however, produced enough information to be used as a first screen for the detection of reassortants.

To produce reassortant viruses, cell cultures were infected with both strains simultaneously. The progeny virus were titrated by plaque assay to a dilution at which well separated plaques could be isolated. The individual clones were grown in rolled tube cultures to produce enough virus for genome profile analysis. It was found that enough RNA for analysis could be directly extracted from 1.5ml of the culture fluid. A total of 32 clones were analysed in this way and their genome profiles are shown in Figs 7.3a, 7.3b, 7.3c and 7.3d. Of the 32 clones 29 were successfully analysed and yielded 9 reassortants (31%) and of the rest 17 clones were CP-1 (58.6%) and 2 were C3-160 (7%). The other clone A8 contained both C3-160 and CP-1 segments 3 and 4 which were visible as double bands suggesting that it contained a mixture of viruses. A summary of the parental origins of the genome segments for the reassortants is shown in Table 7.4. It was interesting to

note that for all the reassortants isolated during this experiment CP-1 segments predominated positions 1, 3 and 7 whereas C3-160 segment 4 was the most common. For the other segment positions neither parental strain predominated.

Although the detection of the genome segments after 3' terminal labelling is very sensitive, the random direction of emission of the high energy β particles released during the decay of [^{32}P] increases the width of each band on the autoradiograph. Electrophoresis of unlabelled RNA and detection of the segments by ethidium bromide staining requires larger amounts of viral RNA but the narrower band widths allow smaller differences in segment migration to be detected. This method was used to determine the parental origin of reassortant genome segments 2 and 8. However, as larger amounts of RNA were required for this method only those reassortants used to experimentally infect calves, A7, A9 and A10, were analysed. By using 1-2μg of RNA it was possible to visualise all the segments up to the 7,8 and 9 triplet, but more important the band width for each segment was narrow enough to differentiate C3-160 and CP-1 segment 8 on gels electrophoresed for 16h at 20mA and segment 2 on gels electrophoresed for 40h at 20mA. The results are shown in Figs 7.4a and 7.4b and the segment assignments are recorded in Table 7.4.

7.2.5 Experimental infection of calves with reassortant viruses

Due to the expense of producing and maintaining gnotobiotic calves only three viruses were used in these experiments, A7, A9 and A10. The results of these infections are shown in Fig 7.5. Both A7 and A10 viruses did not produce any clinical signs of infection whereas A9 produced symptoms similar to the virulent CP-1 infection. Although A7 and A10 appeared to be avirulent like C3-160 there was one feature that distinguished the two types of infection, namely that virus was excreted at high titre following C3-160 inoculation but with A7 and A10 no virus could be detected in the faeces of the infected animals. To determine whether infection had indeed occurred serum samples taken from the calves were tested for the presence of rotavirus antibody. Both animals seroconverted with serum antibody

levels similar to the control C3-160 infected calves. As a second check the calves were challenged three weeks after the first infection with the virulent CP-1. Both calves were found to be resistant to the second challenge.

As it was possible that the A7 and A10 infected calves were excreting defective non-infectious virus in their faeces, attempts were made to extract viral ds-RNA from daily faecal samples from both these calves and from the A9 infected calf. Viral RNA was detected in the samples from the A9 infected calf on days 3-8 (Fig 7.6), coincident with the detection of cell culture infectious virus. No viral RNA could be detected in the faeces of either the A7 or the A10 infected calves.

7.2.6 Growth of the parental and reassortant viruses at 37^0C and 39^0C

The temperature of the small intestine of calves is 39^0C so it was possible that the reassortants A7 and A10 contained a defect which restricted their growth at this temperature. The two parental viruses, C3-160 and CP-1, and the reassortants, A7, A9 and A10 were grown for one pass in MA104 cells at either 37^0C or 39^0C and the titres of virus produced compared (Table 7.5). All the viruses yielded less progeny at 39^0C with titres up to 1 $\log_{10}$ pfu/ml lower than the 37^0C levels. However, this small decrease did not suggest that temperature sensitivity was the reason for the lack of growth of the reassortants in infected calves.

7.3 DISCUSSION

This section describes experiments aimed at finding markers that could be used to identify rotavirus strains of different virulence and to examine the molecular basis of rotavirus virulence.

Three properties of rotaviruses were examined using two cloned strains, one virulent and one avirulent. The first property examined was the respective diameter of plaques produced by the two strains in MA104 cells. The plaques of the avirulent C3-160 strain were 50% larger than virulent CP-1 which would suggest that C3-160

grew, spread and killed the cells quicker than CP-1. Biggs and Milne (1972), working with Marek's disease virus of chickens and turkey herpes virus, found that naturally occurring avirulent strains of these viruses produced small plaques in chicken kidney cell monolayers, whereas the virulent strains gave large plaques. However, in chick embryo fibroblast cell cultures Cho (1976) found the converse to occur, that virulent strains of Marek's disease virus formed smaller plaques than avirulent strains. This apparent contradiction emphasises the need to characterise each virus/cell combination if plaque size is used diagnostically to differentiate virulent and avirulent viruses. Plaque size variation may be a method of assessing the virulence potential of bovine rotavirus strains, however, many more isolates of known virulence would need to be tested to validate the method.

The ability of these rotavirus strains to replicate in cell culture in the absence of trypsin was the second property to be examined. When the virus yields were assayed, both viruses apparently produced much less progeny virus when grown in the absence of trypsin when compared to the yields of virus grown with trypsin. However, the fluorescent focus assay used to estimate the virus yields does not include trypsin. When trypsin was included in the assay medium, comparison of the titres of progeny virus showed that although the titre of CP-1 was much less when the virus was grown without trypsin, removal of trypsin from the medium when growing C3-160 appeared to have little effect. It was evident, therefore, that although much of the progeny produced during the growth of C3-160 in the absence of trypsin required protease activation to be infectious the number of virions produced without trypsin was not significantly lower than that produced with trypsin. Even when assayed with trypsin, CP-1 yields were significantly lower when the virus was grown without trypsin. This suggested that, with this strain, trypsin, besides activating the progeny virus, may play another crucial role in the virus replication cycle. These results showed clearly the requirement of protease activation for the growth of rotavirus in cell culture. Both ortho- and paramyxoviruses require proteolytic cleavage of a surface proteins to become infectious. In paramyxoviruses the fusion protein must be cleaved into two subunits for infectivity (Homma and Ohuchi, 1973; Schneid and Choppin, 1974). The cleavage occurs with a trypsin-like

enzyme which is contained in some cell types and not others (Appleyard and Davis, 1983) and for this reason Sendai virus grown in chicken amnion cells is infectious for other cells whereas virus grown in MDBK or L-929 cells is not. Mutants of Sendai virus in which the fusion protein was cleaved by different proteases were found to have a different host range (Silver *et al.*, 1978; Schneid ad Choppin, 1976). Comparative studies of several isolates of Newcastle disease virus, another member of the paramyxovirus group, showed that the proteolytic activation of virulent strains was achieved in a wide variety of cell types, whereas for avirulent strains this activity was restricted to relatively few cell types (Nagai *et al.*, 1976; Nagai and Klenk, 1977). The current results with rotavirus would suggest that MA104 cells do not contain a protease capable of activating either virulent or avirulent strains of the virus. It may be that other cell types grown in culture could be used which would show differences between rotaviruses of varying virulence. With orthomyxoviruses, the haemagglutinin must be cleaved to render the viruses infectious (Bosch *et al.*, 1979). These workers also showed a correlation between the pathogenicity of strains of avian influenza virus and their ability to grow in cell culture without the addition of trypsin to the culture medium. Virulent strains grew without added trypsin whereas avirulent strains only grew in chick fibroblasts when trypsin was added. As trypsin was required for the growth of both virulent and avirulent strains of rotavirus it would appear that this characteristic cannot be used to differentiate rotavirus strains of different pathogenicity.

The ability of CP-1 and C3-160 strains to alter the permeability of MA104 cell membranes was the third feature tested. It has been suggested that lytic viruses, i.e. those that produce alterations in cell morphology leading to characteristic cytopathic effect, produce an increase in the permeability of infected cell membranes which ultimately leads to cell death (Carrasco and Smith, 1976; Contreras and Carrasco, 1979; Carrasco and Esterban, 1982). The release of absorbed chromium was used as a measure of membrane permeability in rotavirus infected cells. The results presented showed that avirulent C3-160 increased MA104 cell membrane permeability and induced changes in cell morphology at an earlier time post infection than did virulent CP-1. These results were contrary to the situation *in*

vivo as rotaviruses cause enteritis by infecting and destroying enterocytes (Mebus *et al.*, 1977; Wyatt *et al.*, 1976; Theil *et al.*, 1978) leading to malabsorption (Graham *et al.*, 1982). This shows the need to select the correct cell/virus combination if this type of test is to be diagnostic. Organ cultures, although not widely used, offer an alternative to cell monolayer cultures when the natural host cell of the virus is a highly differentiated cell type. In bovine pharyngeal organ cultures Burrows (1972) showed that virulent strains of foot and mouth disease virus grew faster and to higher titres in the pharyngeal epithelial cells than attenuated strains. It may be possible to use organ cultures of intestinal tissue to differentiate virulent and avirulent strains of rotavirus or perhaps a different cell culture system.

Other tests have been developed to differentiate virulent and avirulent strains of viruses. Taguchi and Siddell (1985) showed that mouse hepatitis viruses, members of the coronavirus family, with high and low virulence for mice had different sensitivities to interferon, the low virulence strains being the most sensitive. Interferon is induced following rotavirus infection of calves (Vanden Broecke *et al.*, 1984) and therefore differences in the sensitivity of growth of different strains may correlate with virulence. The use of monoclonal antibodies to differentiate viruses of differing virulence should gain widespread popularity due to the simplicity of the resulting diagnostic test, e.g. ELISA and latex agglutination tests. Monoclonal antibody based diagnostic tests have been developed to detect differences in virulence of strains of feline infectious peritonitis virus (Fiscus *et al.*, 1987) and Newcastle disease virus (Erdei *et al.*, 1987).

Following the findings of this project a second avirulent bovine strain has been identified (Bridger & Pocock, 1986). There is, therefore, a need to identify virulence markers for rotavirus so that an accurate diagnosis of rotavirus as the causal agent in an outbreak of enteritis can be made. The two potential markers identified here, increased plaque size and release of intracellular [^{51}Cr] by the avirulent strain, will need to be tested on more strains, both virulent and avirulent, to determine their applicability. However, this type of test would not be suitable for

testing field isolates as they both require prior isolation of the virus strains in cell culture. It is possible that an ELISA test could be developed if the gene(s) and hence the viral protein(s) responsible for virulence determination is evaluated.

Viruses that contain segmented genomes can reassort genetic information when individual cells become infected with two different virus strains. If the two virus strains are selected on the basis of a unique viral characteristic then the gene responsible for that character will be associated with the reassortant viruses which contain it. Work with reoviruses has revealed the gene segments responsible for many of the viral characteristics including those coding for the reovirus haemagglutinin (Weiner *et al.*, 1978) and the major neutralising antigen (Weiner and Fields, 1977) and the genes that specify the site of intestinal disease (Rubin *et al.*, 1986) and the spread of the virus from the intestine (Kauffman *et al.*, 1983). The molecular basis of reovirus neurotropism (Tyler *et al.*, 1985) and virulence (Fields *et al.*, 1980; Fields, 1982) have also been elucidated using virus genome reassortment. Genes involved in virus virulence have also been identified for the arenavirus, lymphocytic choriomeningitis virus (Riviere and Oldstone, 1986) and bunyaviruses (Beaty *et al.*, 1982). Although the initial experiments suggested that the virulence of influenza virus was determined solely by the haemagglutinin, analysis of genetic reassortants of strains of differing virulence revealed that influenza virus virulence is polygenically controlled (Mayer *et al.*, 1973; Rott *et al.*, 1976) and depends on the parental origin of the viral RNA synthesis genes (Scholtissek, 1979). Reassortant rotavirus strains have also been used to identify the genes that code for the viral haemagglutinin (Kalica *et al.*, 1983), neutralisation and subgroup antigens and virus growth restriction in cell culture (Kalica *et al.*, 1981; Greenberg *et al.*, 1983a). In an attempt to gain an understanding of the molecular basis of rotavirus virulence, reassortant strains, produced from dual infections of cell cultures with CP-1 and C3-160, were characterised both genotypically and for the virulence phenotype. The frequency of reassortant isolation, 31%, seen in this study was similar to that found by Gombold and Ramig (1986) who described a frequency of 38% after *in vivo* inoculation of mice with two rotavirus strains. The non-random reassortment of certain genome segments has

been described previously for rotavirus (Gombold and Ramig, 1986; Graham *et al.*, 1987), reovirus (Wenske *et al.*, 1985) and influenza virus (Lubeck *et al.*, 1979). The extensive studies of Graham *et al.* (1987) on the reassortment of two rotaviruses in two different cell cultures revealed that host cell factors contributed to the resulting gene reassortment frequencies. However, the factors involved and the basis of the selection of certain genes remain unknown.

To evaluate the virulence of the reassortants, calves were inoculated and observed for clinical signs of rotavirus infection. Two reassortants, A7 and A10, gave no clinical signs whereas the third, A9, gave a clinical picture similar to that of a virulent infection. Reference to the table 8.4, showing the parental origins of each of the genome segments, revealed that both A7 and A10 inherited only 2 genome segments each from the avirulent C3-160 virus, segments 4 and 8 in A7 and 4 and 9 in A10. Therefore, by a process of elimination it seemed that gene 4 was implicated in virulence determination and this was reinforced by the finding that A9 inherited this gene from CP-1. A9 also possessed segments 2, 5, 6 and 11 from C3-160, which would suggest that these genes are not involved in rotavirus virulence. However, the calves infected with the two "avirulent" reassortants did not excrete any progeny virus and could therefore not be classified as avirulent, as defined for the parental C3-160 strain. The reasons for the apparent abortive infection *in vivo* with these reassortants are unclear. An analogous situation has been described for influenza. Although the haemagglutinin has been shown to be a major virulence determinant, mixing the parental genes whose products are involved in the viral RNA polymerase complex resulted in the generation of non-pathogenic strains (Rott *et al.*, 1979). Patton and Gallegos (1988) found that rotavirus replicase particles, isolated from infected cells, contained three structural polypeptides, vp1, vp2 and vp6 and two nonstructural proteins vp8 and vp9. Although gene segment 1, which encodes vp1, was derived from CP-1 in the three reassortants tested in calves, the genes encoding the other 4 components had mixed parentage. The possibility exists therefore that viruses containing only certain gene constellations will produce a viable viral RNA replicating system and that this may also be cell type dependant.

This could explain why the reassortants gave a productive infection in MA104 cells and an abortive infection in calf enterocytes and would suggest that rotavirus virulence is a polygenic characteristic.

The growth of the non-pathogenic influenza reassortants was found to be temperature sensitive above 37^0C and as the body temperature of chickens is 41^0C this restricted their growth *in vivo* (Rott *et al.*, 1982). The reassortant rotaviruses were tested for growth temperature sensitivity at 39^0C, the temperature of the calf small intestine, but no difference was found between any of the strains and the wild types. Ogawa and Ueda (1981) still considered the haemagglutinin to be the major virulence determinant of influenza and vp4 may be its counterpart in rotaviruses. It is interesting that both the influenza haemagglutinin and rotavirus vp4 are both surface virion proteins that require proteolytic cleavage to activate the viral infectivity.

Work on the comparison of rotavirus strains isolated from symptomatic and asymptomatic infections of children by RNA/RNA hybridisation has shown that gene 4 was conserved within the two groups but differences occured between the groups (Flores *et al.*, 1986). Subsequent sequence data of gene 4 from isolates of the two groups confirmed this (Gorziglia *et al.*, 1986). Serotypic analysis of strains from asymptomatic infections from several countries showed that these viruses were not confined to a single serotype but spread over all the known serotypes (Hoshino *et al.*, 1985b). This would suggest that vp7, the major neutralising antigen, is not involved in virulence determination. Using reassortant rotavirus strains Offit *et al.* (1986a) also provided evidence for the involvement of gene 4 in virulence determination. However, their definition of virulence was different in that the two viruses chosen for reassortment both caused disease and although a lower titre of one strain produced diarrhoea when compared with the other, it was this dose effect that was attributed to gene 4. Also, as a heterologous system was used, i.e. bovine and simian rotaviruses were used to infect mice, this introduced the possibility that host factors may have caused the dose effect. Analysis of the reassortants produced between a virulent and an avirulent bovine strain, reported here, also incriminated

gene 4. However, the lack of production of progeny virus following infection with some of the reassortants would suggest that other factors are involved. It may be that analysis of more reassortants would show that virulence determination in rotaviruses is similar to influenza viruses and requires the correct gene constellation to provide fully infectious virulent and avirulent reassortant strains. The fact that these reassortants grow readily in cell culture but not in the calf highlights the care required when assumptions are made, concerning virulence, based on *in vitro* studies without confirmation *in vivo*.

Elucidation of the molecular basis of rotavirus virulence requires further experimentation. However, the data produced will not only be relevant to rotaviruses and their interaction with the intestinal epithelium but will also help in the understanding of the pathogenic processes involved in viral enteric disease.

It has been proposed that reassortant influenza viruses (Snyder *et al.*, 1986) and rotaviruses (Midthun *et al.*, 1985; Clark *et al.*, 1986a) could be used as live vaccines. To test this possibility, the calves infected with the three reassortant rotaviruses were challenged with a virulent strain. All three calves were protected against challenge. This was somewhat surprising since two of the reassortants produced no progeny virus after infection suggesting that either abortive replication occurred in the cells originally infected by the inoculum or that infection spread from cell to adjacent cell without the release of virus particles. More work is needed to determine the site and extent of this replication in order to define the requirements for protection more clearly. The advantage of this type of live virus vaccine would be that only the recipient would be infected because no virus would be excreted. This may be important as it has already been shown that immune compromised children can have enteritis of extended duration after rotavirus infection (Pedley *et al.*, 1984).

Table 7.1

Effect of length of incubation on the plaque size of C3-160 and CP-1 rotavirus strains.

	Plaque diam.	
Hours post inoculation	C3-160	CP-1
24	1.0 [1]	0.5
48	3.5	2.0
72	4.5	3.0

[1] Mean diameter of 10 individual well spaced plaques measured to the nearest 0.5mm

Table 7.2

Effect of trypsin on the growth of C3-160 and CP-1 rotavirus strains in MA104 cells over two cell culture passes.

	Virus titre (log_{10} ff ml^{-1})			
	C3-160		CP-1	
Cell culture pass	+trypsin	-trypsin	+trypsin	-trypsin
a) Assayed without trypsin				
1	6.50	3.50	6.17	< 1.50
2	6.17	2.50	5.50	< 1.50
b) Assayed with trypsin				
1	6.17	5.50	5.83	2.83
2	5.50	4.83	5.50	1.83

Table 7.3

Appearance of virus induced c.p.e. in MA104 cell cultures infected with C3-160, UK or CP-1 rotaviruses.

	Cells infected with		
Hours post infection	C3-160	UK	CP-1
3	- [1]	-	-
6	-	-	-
9	+	-	-
12	++	+	+
15	+++	+++	++
18	++++	++++	++
21	++++	++++	+++
24	++++	++++	++++

[1] - no visible c.p.e.

\+ some cells beginning to contract and become rounded

++ c.p.e. affecting up to 40% of the cells

+++ c.p.e. affecting between 40 and 80% of the cells

++++ c.p.e. affecting >80% of the cells

Table 7.4

Summary of the parental origin of each of the genome segments of reassortant viruses isolated from cell cultures infected with a mixture of C3-160 and CP-1 strains of rotavirus.

Reassortant Virus	Genome Segment Number										
	1	2	3	4	5	6	7	8	9	10	11
A3	▲[1]	–[2]	■[3]	▲	■	▲	▲	–	■	▲	▲
A7	■	■	■	▲	■	■	■	▲	■	■	■
A9	■	▲	■	■	▲	▲	■	■	■	■	▲
A10	■	■	■	▲	■	■	■	■	▲	■	■
A15	■	–	■	▲	▲	■	■	–	▲	▲	■
A19	▲	–	▲	▲	▲	▲	■	–	■	▲	▲
A24	■	–	■	▲	■	■	■	–	▲	▲	■
A29	■	–	■	▲	▲	■	■	–	■	■	■
A30	■	–	■	■	■	▲	■	–	■	■	■

[1] ▲ C3-160 [2] – Not determined [3] ■ CP-1

Table 7.5

Growth of reassortants and parental C3-160 and CP-1 rotavirus strains at 37 °C and 39°C.

	Incubation temperature		
Virus strain	37°C	39°C	Ratio 39°/37°
C3-160	3.57 [1]	0.34	0.09
CP-1	2.97	1.47	0.49
A7 [2]	4.52	0.51	0.11
A9 [2]	2.53	0.30	0.12
A10 [2]	7.95	0.34	0.04

[1] Virus titre (p.f.u. x 10^{-7} ml^{-1})

[2] Reassortant strains

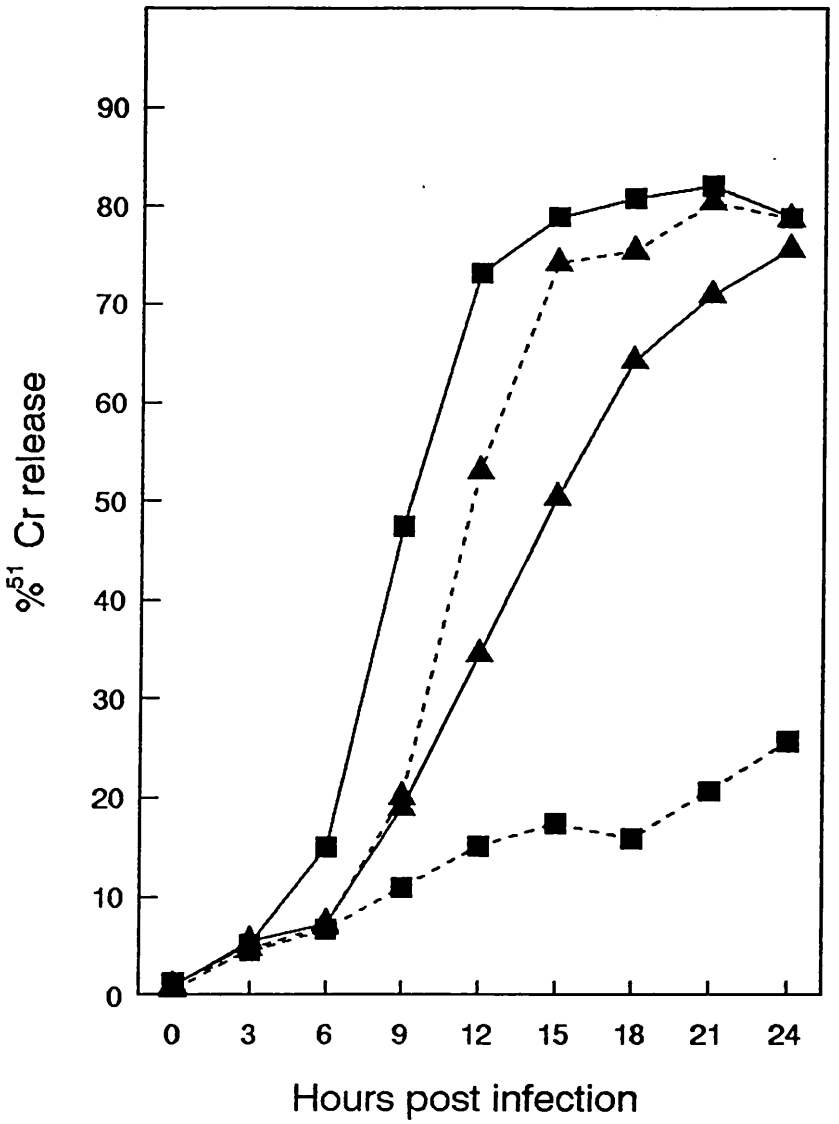


Fig 7.1: Release of [^{51}Cr] from MA104 cells during infection with rotavirus. MA104 cells, labelled with [^{51}Cr], were either infected with CP-1 (▲—▲), UK (▲···▲) and C3-160 (■—■) or left uninfected as controls (■···■). Cultures were harvested at 3h intervals during the 24h period post infection to determine the amount of [^{51}Cr] released from the cells into the medium.

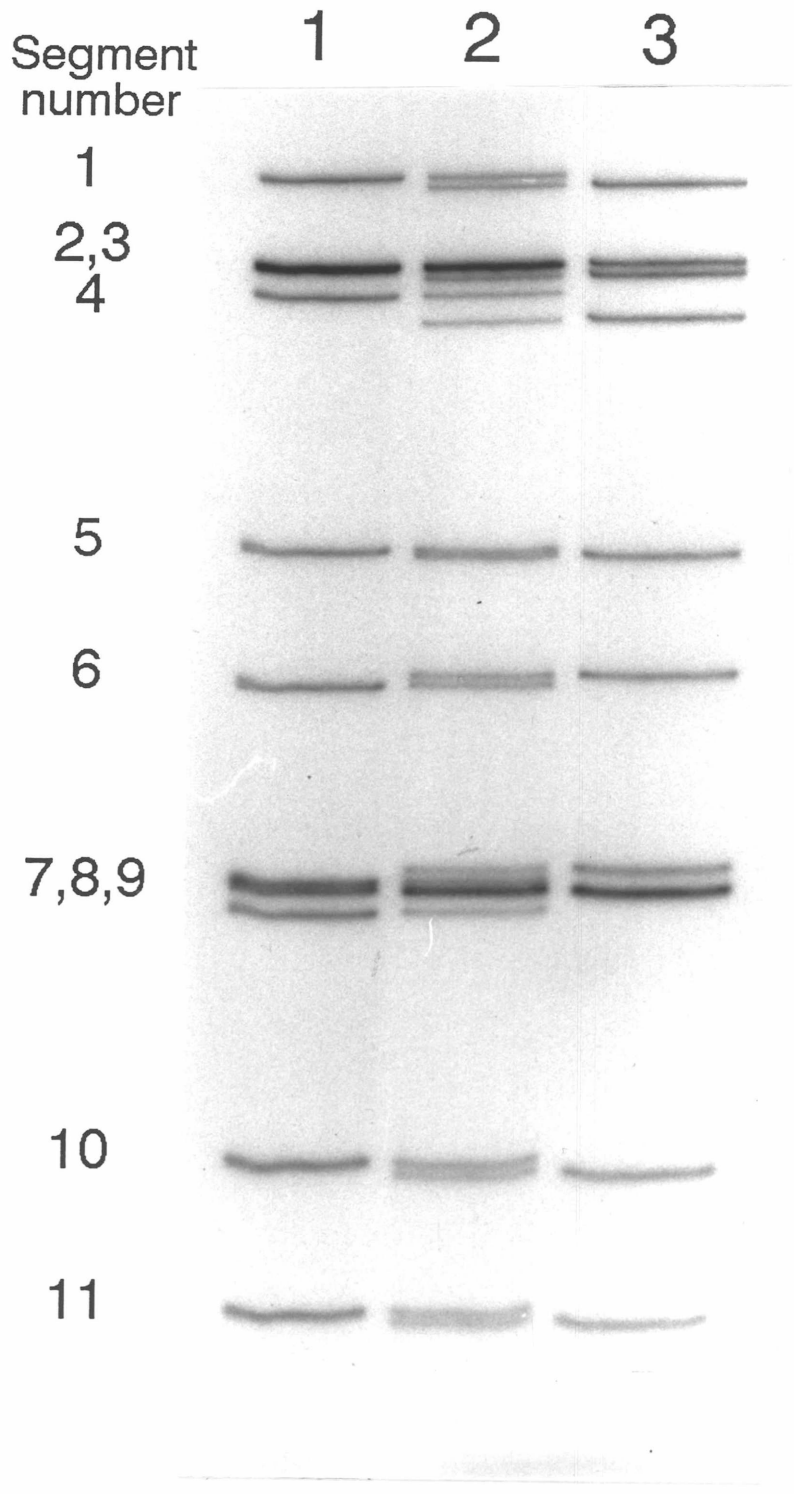


Fig 7.2: Comparison of the genome profiles of CP-1 and C3-160. The genomic RNA was 3'-terminal labelled with [^{32}P] and the segments separated by electrophoresis. Track 1 contained CP-1 RNA, track 3 C3-160 RNA and track 2 a mixture of CP-1 and C3-160 RNAs. The genome segments are labelled on the left of the figure.

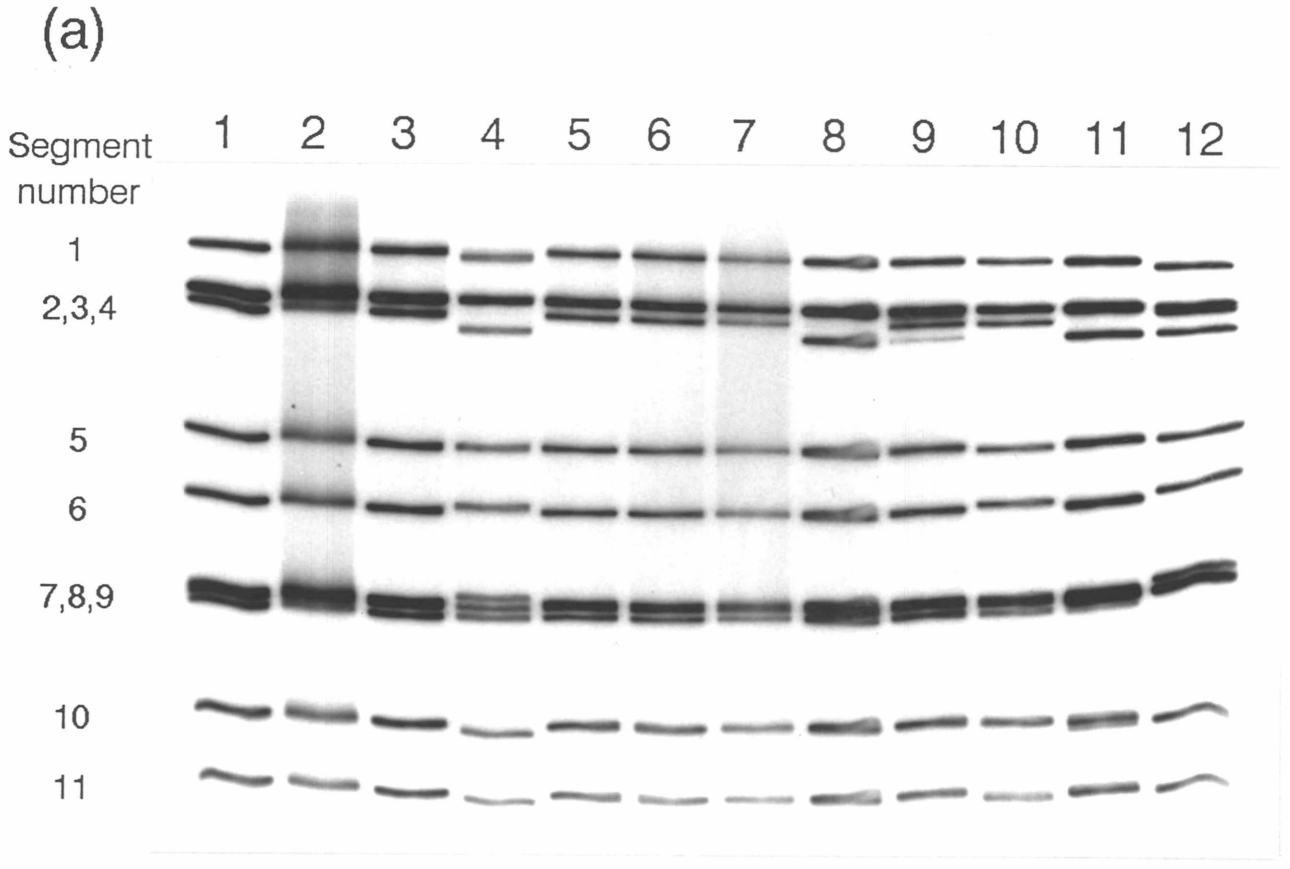


Fig 7.3: Genome profiles of cloned virus from a CP-1/C3-160 mixed infection of MA104 cells.

Fig 7.3(a): Track 1 contained CP-1 RNA, track 12 C3-160 RNA and tracks 2-11 RNA from clones A1-A10.

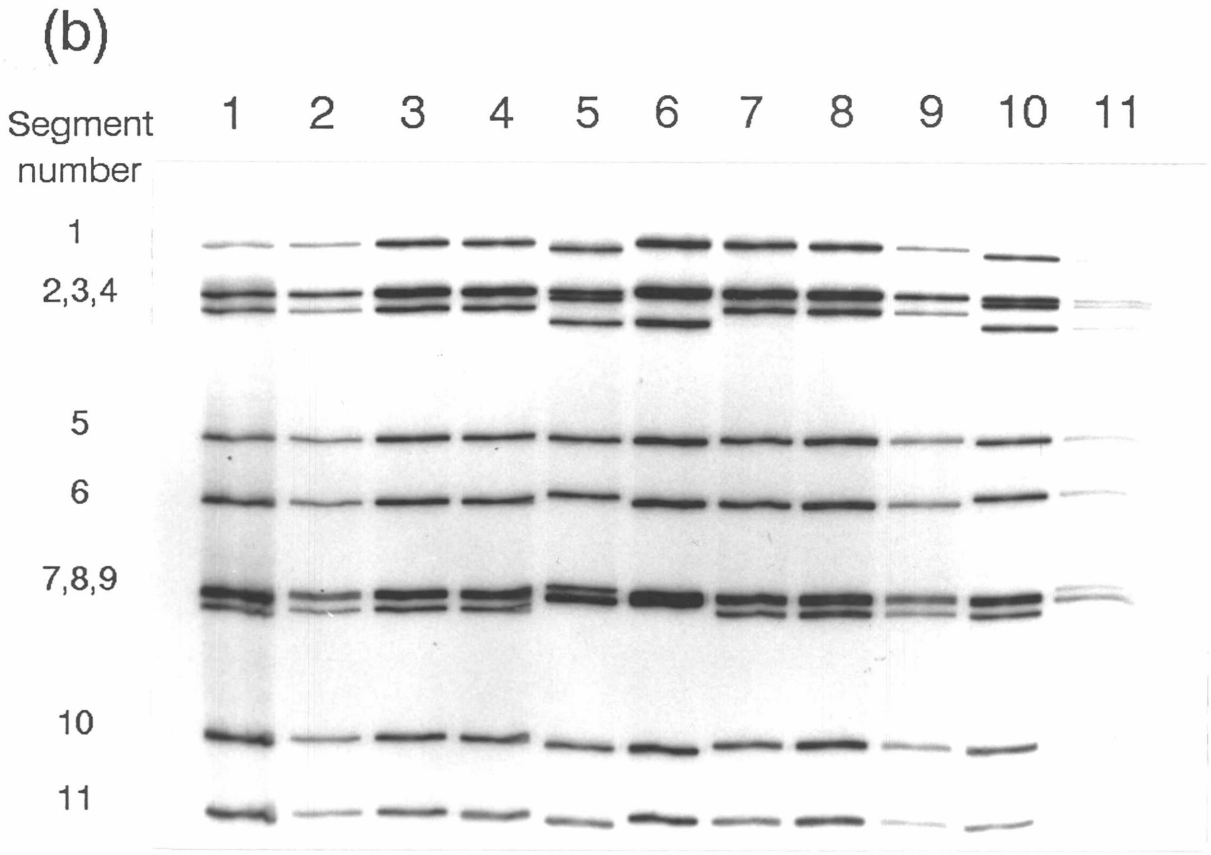


Fig 7.3(b): Track 1 contained CP-1 RNA, track 11 C3-160 RNA and tracks 2-10 RNA from clones A11-A19.

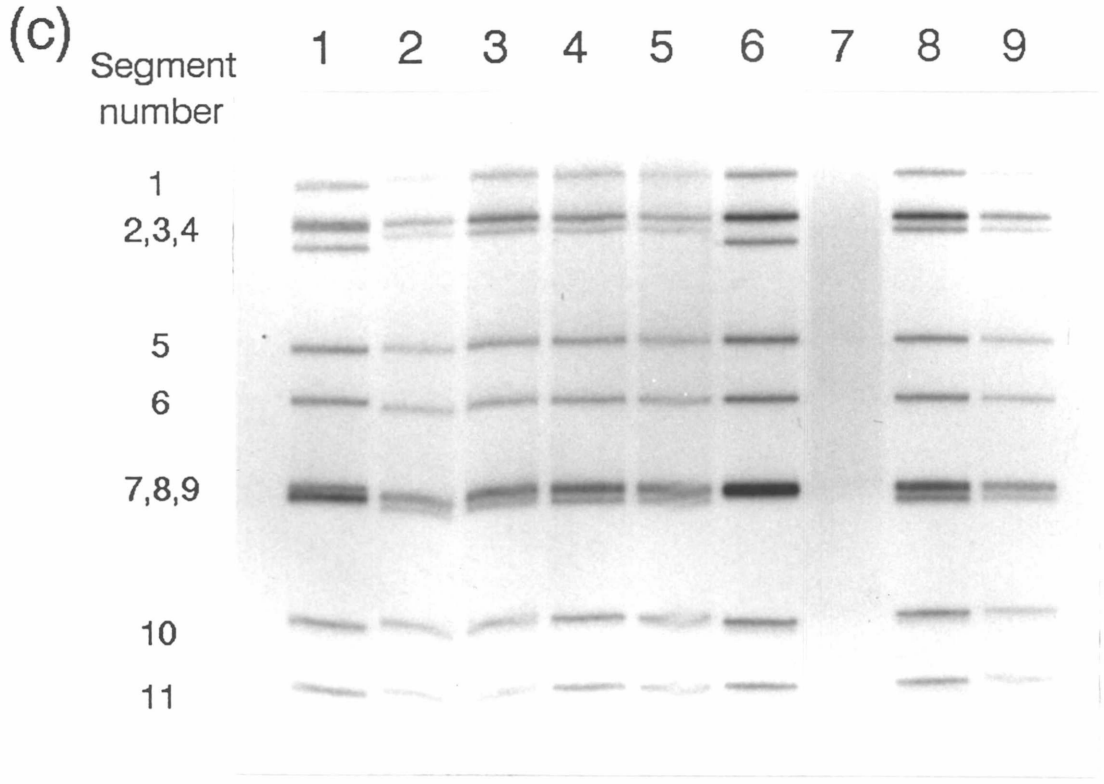


Fig 7.3(c): Track 1 contained C3-160 RNA, track 9 CP-1 RNA and tracks 2-8 RNA from clones A20-A26.

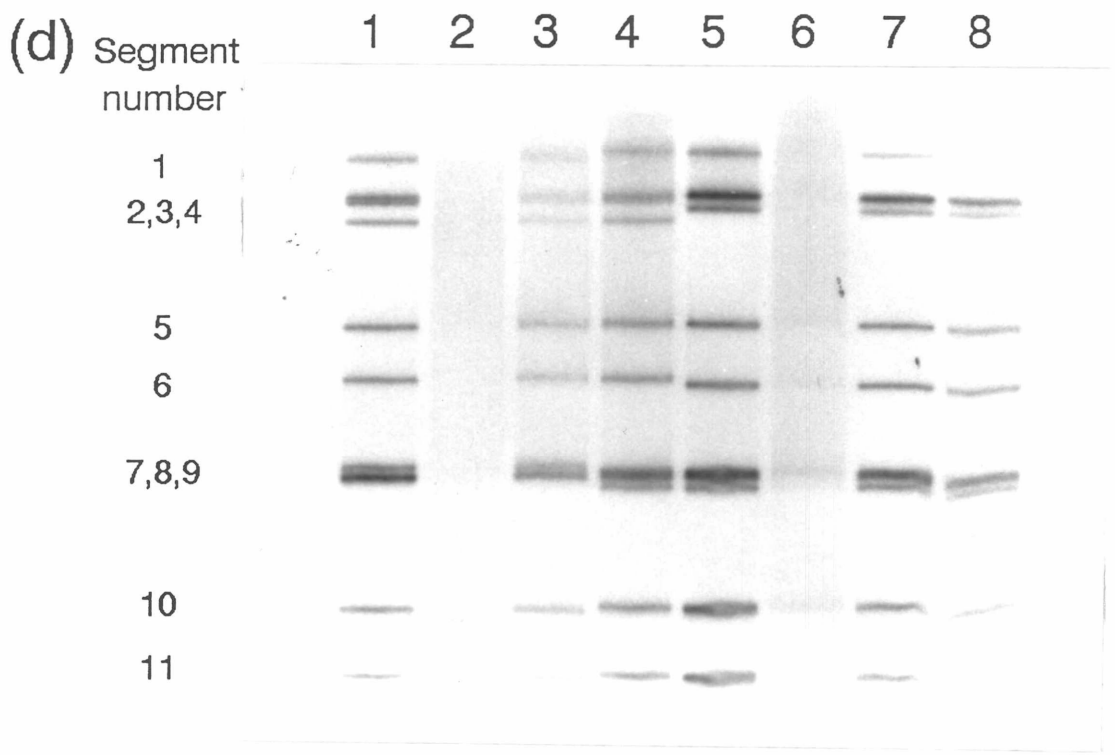


Fig 7.3(d): Track 1 contained C3-160 RNA, track 8 CP-1 RNA and tracks 2-7 RNA from clones A27-A32.

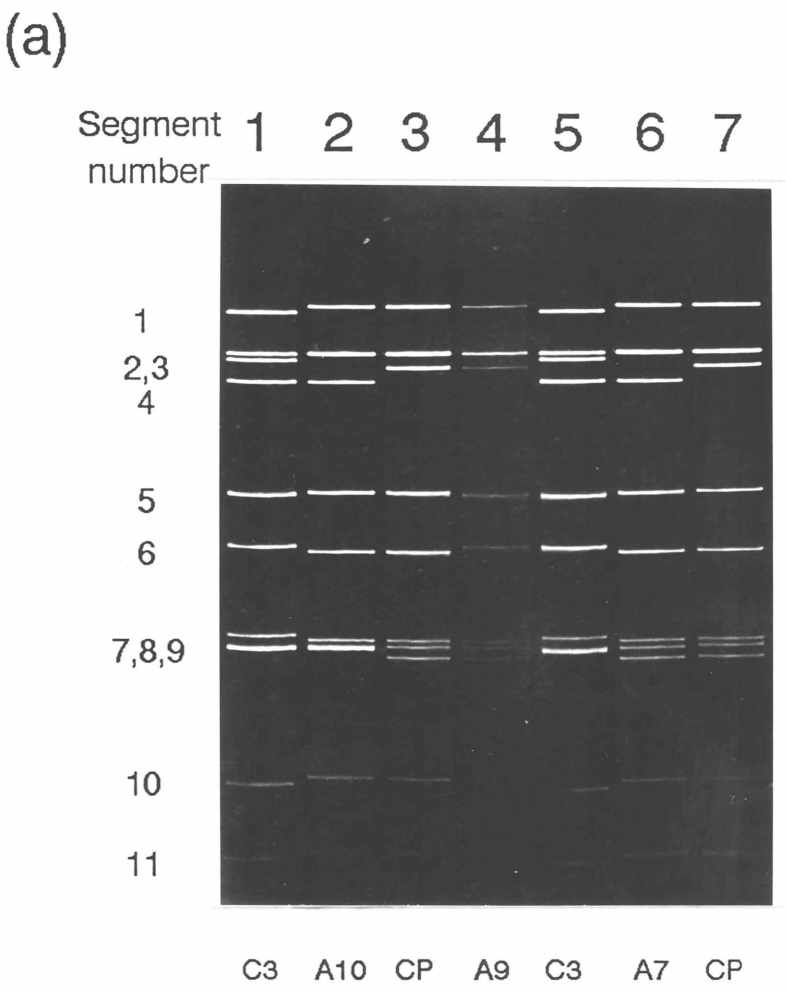


Fig 7.4a: Determination of the parental origin of genome segments 2 and 8 of reassortant strains A7, A9 and A10. Unlabelled virion RNA was electrophoresed at 20mA in 10% polyacrylamide gels for (a) 16h, for segment 8 evaluation or (b) 40h for segment 2. The genome segments were visualised by UV light after ethidium bromide staining. The track order for both gels was 1 and 5, C3-160; 3 and 7, CP-1; 2, A10; 4, A9; 6, A7 RNAs. The positions of the genome segments are shown on the left of the figure.

(b)

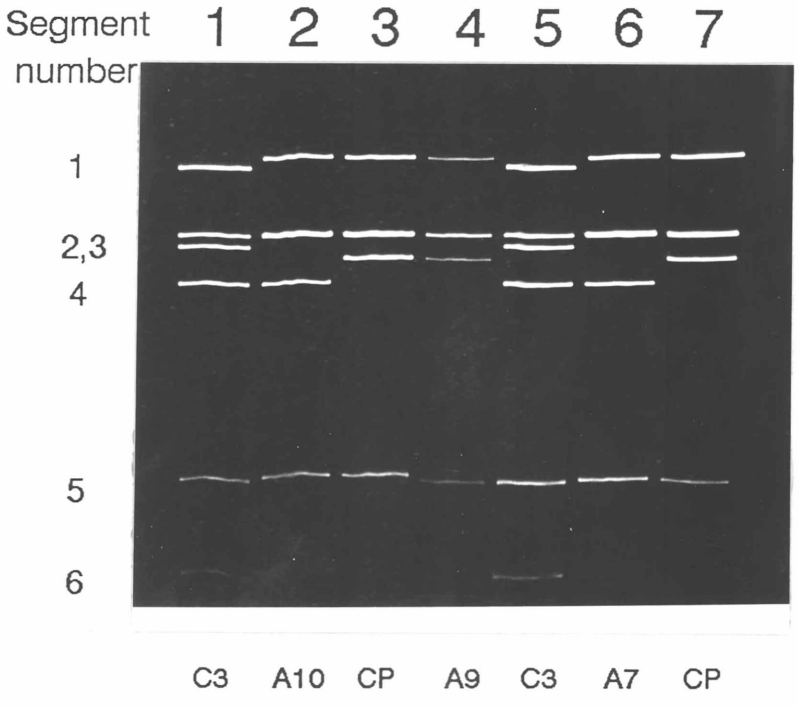


Fig 7.4b: For legend see Fig 7.4a on previous page.

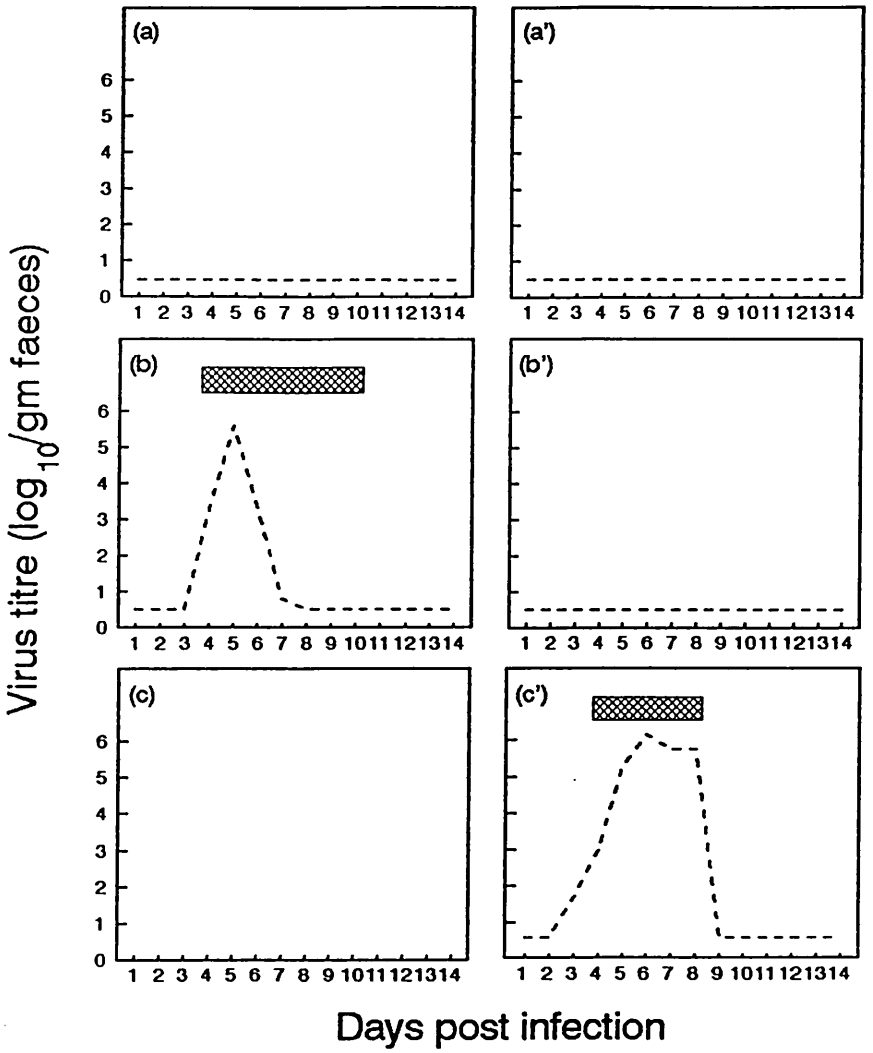


Fig 7.5: Results from the experimental infection of 7 day old calves with reassortant rotavirus strains. Calves were inoculated orally with (a) A7 or A10, (b) A9 and (c) uninfected. At 3 weeks post inoculation all calves were challenged with CP-1 and the challenge data are shown in panels (a'), (b') and (c'). Daily recorded parameters were virus excretion ----- and faecal colour change ▒▒▒

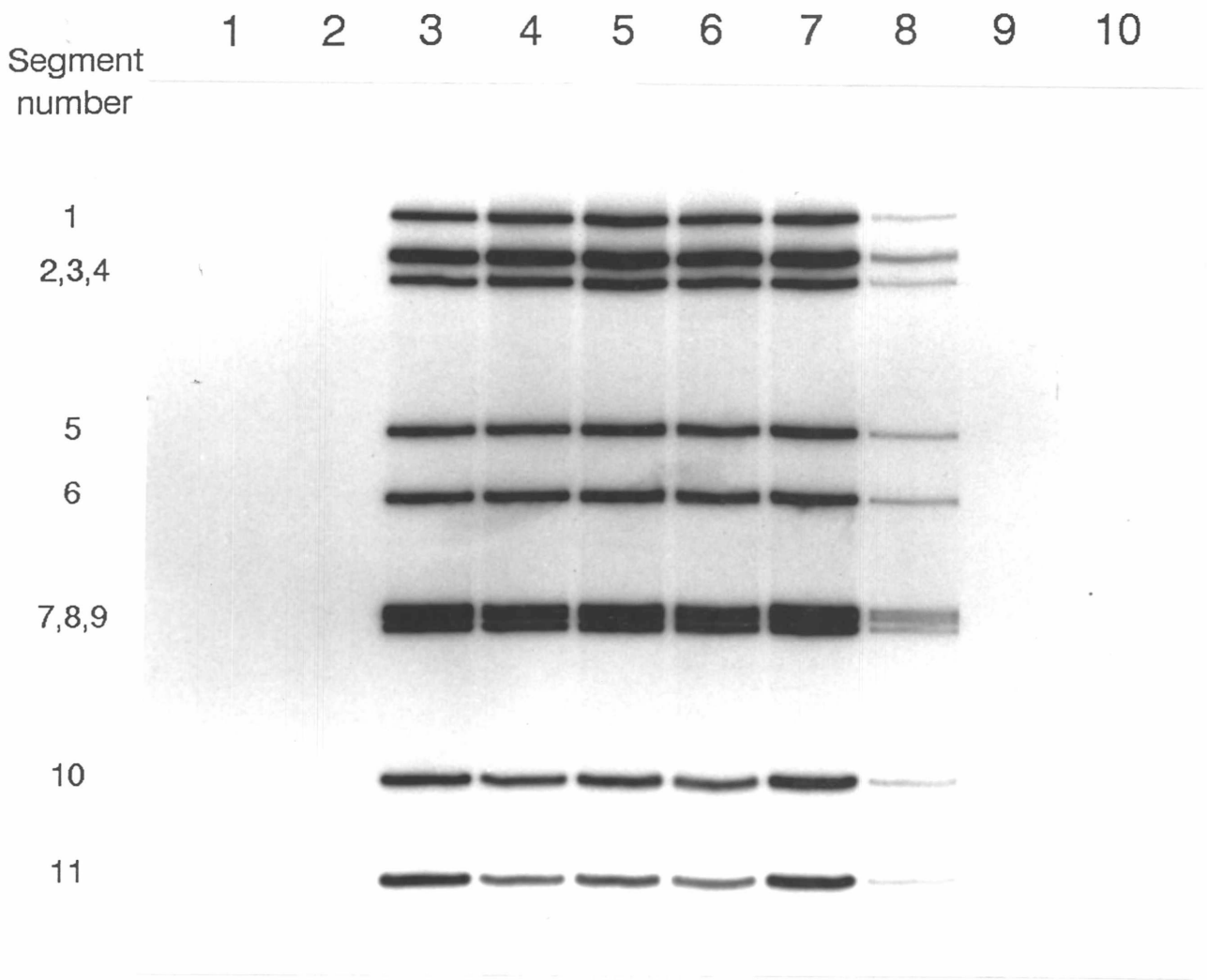


Fig 7.6: Genome profiles of virus excreted in daily faecal samples by a calf infected with rotavirus reassortant A9. Tracks 1-10 contained RNA extracted from faecal samples obtained on days 1-10 post inoculation.

SECTION 8.0 CONCLUSIONS

8.1 Rotavirus genome profile analysis.

The methods used in this project for the generation of rotavirus genome profiles were found to be both sensitive and reproducible. Although the 3'-terminal labelling of the genome segments prior to electrophoresis was the method used here, there have been many reports of the successful application of silver staining for the visualisation of genome segments thereby allowing wider application of this technique. Genome profile analysis is currently being used in hospital and veterinary laboratories as a diagnostic test for the detection of virus in faeces. The profiles generated will also provide important epidemiological data, however, caution is required when interpreting the results. As already discussed co-migration of genome segments does not indicate identity. This has been shown by hybridisation, RNA/RNA (Flores *et al*, 1982b) or RNA/DNA (Street *et al*, 1982; Pedley & McCrae, 1984), and single dimension (Clarke & McCrae, 1982) or two dimension oligonucleotide mapping of genome segments (Desselberger *et al*, 1986). Recent sequence data would suggest that, although large variations in electrophoretic mobility are a result of variation in the molecular size of the genome segment, some of the minor mobility differences observed are a function of changes in the base composition of the segment. For example the nucleotide sequence of segment 6 from 6 different rotavirus isolates have been described, simian SA11 (Estes *et al*, 1984), human Wa (Both *et al*, 1984), bovine RF (Cohen *et al*, 1984), human 1076, porcine Gottfried and equine H-2 (Gorziglia *et al*, 1988) and, with the exception of the H-2 strain which has 6 extra bases contained within the open reading frame (ORF), all the genome segments contained 1356 bases with a single ORF 1181 bases long. Also, Kantharidis *et al* (1988) analysed the nucleotide sequences of segment 4 from bovine UK, human RV-5 and simian SA11 strains and reported %A+T values of 57.7%, 68.0% and 63.4% respectively. More work is required to provide direct evidence to link the variation in electrophoretic mobility to the base composition of the segment.

The finding that current methods for analysing genome profiles are not very sensitive in detecting infections involving more than one rotavirus genotype was important. The current literature would suggest that mixed infections are rare, but this may be a false assumption. If this is so then the possibility of producing recombinant virus strains by reassortment, during mixed infection, would be increased.

Analysis of the genome rearrangement of segment 11 in the C7 type viruses revealed that these viruses produced a 'normal' segment 11 gene product. However, the finding that genome rearrangement can result in the production of an 'abnormal' product (Hundley *et al*, 1985) suggests that this process, along with genomic drift and shift, could contribute to the evolution and antigenic change of rotaviruses. Elucidation of the mechanism of genome rearrangement will increase the understanding of rotavirus replication.

8.2 Antigenic Variation Between Rotaviruses.

Although there have been attempts to serotype human rotaviruses there is no classification scheme for viruses from other species. One of the reasons for this, is the finding of strains which cross serologically in one direction only, i.e. antibody to strain A will neutralise both strain A and B but antibody to strain B does not neutralise strain A. Initially it was assumed that only vp7, the surface glycoprotein, was involved in virus neutralisation but it has since become clear that the other surface protein vp4 is also involved (Greenberg *et al*, 1983b; Hoshino *et al*, 1985a; Taniguchi *et al*, 1985; Offit & Blavat, 1986). As the two proteins are capable of independant segregation in different virus strains (Hoshino *et al*, 1985a), it has been suggested that rotaviruses should be classified in a similar way to influenza viruses which would take into account the neutralisation moieties of both proteins. One of the main uses of the classification of viruses by the analysis of antigenic variation is if this variation correlates with protection against infection and/or disease. Recently Offit *et al* (1986b) showed that monoclonal antibody against either vp4 or vp7 was capable

of protecting passively against rotavirus disease and therefore it would seem that a serological classification of rotaviruses based on both vp4 and vp7 would be useful. More work is needed to determine the number of vp4 and vp7 variants that are in circulation so that suitable reagents can be obtained to facilitate this type of classification.

8.3 Immunity of Rotavirus Infection.

In the main rotavirus causes disease in the neonate and therefore passive protection against disease, by the transfer of antibody contained in colostrum and milk from dam to neonate, is very important. This was thought to be all that was necessary for calves because rotaviruses caused disease only in the first 2 weeks of life. However, the finding of Bridger & Pocock (1986) of rotavirus strains capable of causing disease in older animals means that protection is required for a longer period. Immunisation of the dam to prolong lactogenic immunity has proved successful in cattle (Snodgrass *et al*, 1980b; Saif *et al*, 1983) but until widespread vaccination is achieved it will not be known how effective the protection will be against the strains which infect the older animal. Also passive protection relies on the effective neutralisation of the virus in the gut lumen and antigenic variation could be a problem.

Active immunity against rotavirus is less well understood. Initially it was thought that, as with passive protection, active immunity correlated with serotype i.e. strains of the same serotype cross-protected whereas strains from different serotypes did not (Gaul *et al*, 1982; Woode *et al*, 1983). However doubt was cast on this when protection was found between viruses of different serotypes (Wyatt *et al*, 1983; Bishop *et al*, 1986; Torres *et al*, 1986). It therefore seems that viral antigens other than the surface proteins may play a role in active immunity. Recently Offit & Dudzik (1988) showed that rotavirus specific cytotoxic T lymphocytes were capable of lysing syngeneic cells infected with either homologous virus or viruses from different serotypes. The finding in this study that an infection with the avirulent strain C3-160 protected against

challenge with an antigenically distinct but virulent strain CP-1 was significant. There have been several attempts to use live rotavirus vaccines to elicit active immunity. The vaccine strains used have been either strains from a heterologous species e.g. a bovine virus for use in humans (Delem *et al*, 1984; Clark *et al*, 1986b) or reassortant viruses, usually between homologous and heterologous parental strains (Midthun *et al*, 1985; Clark *et al*, 1986b). These vaccines have met with mixed success with the lack of results probably due to the level of attenuation of the vaccine strains in the heterologous host leading to poor immune responses. The possibility of using an avirulent strain in the homologous species which is capable of providing wide protection would seem advantageous. More work on this aspect is required to investigate the feasibility of this approach. Also the results from infections with the reassortant viruses A7 and A10, produced from virulent and avirulent parents, which did not produce disease or progeny virus after infection but protected the animal against challenge with virulent virus are interesting and these viruses should be investigated further and their use as vaccine candidates tested. This type of investigation would also determine the level of infection required to elicit a protective immune response and if the infection was found to be abortive could provide extra data concerning the replication of rotaviruses.

8.4 Rotavirus Virulence.

The discovery of a naturally occuring avirulent strain of rotavirus is important as it allows investigations into the pathogenesis of rotavirus disease and the molecular basis of rotavirus virulence. Following the findings of this project a second avirulent bovine strain has been identified (Bridger & Pocock, 1986). There is, therefore, a need to identify virulence markers for rotavirus so that an accurate diagnosis of rotavirus as the causal agent in an outbreak of enteritis can be made. The two potential markers, increased plaque size and release of intracellular [^{51}Cr] by the avirulent strain, will need to be tested on more strains, both virulent and avirulent, to determine their applicability. However, this type of test is not suitable for testing field isolates as they both

require prior isolation of the virus strains in cell culture. It is possible that an ELISA test could be developed if the gene(s) and hence the viral protein(s) responsible for virulence determination is evaluated. Work on the comparison of rotavirus strains isolated from symptomatic and asymptomatic infections of children by RNA/RNA hybridisation has shown that gene 4 was conserved within the two groups but differences occured between the groups (Flores *et al*, 1986). Subsequent sequence data of gene 4 from isolates of the two groups confirmed this (Gorziglia *et al*, 1986). Serotypic analysis of strains from asymptomatic infections from several countries showed that these viruses were not confined to a single serotype but spread over all the known serotypes (Hoshino *et al*, 1985b). This would suggest that vp7, the major neutralising antigen, is not involved in virulence determination. Using reassortant rotavirus strains Offit *et al* (1986a) also provided evidence for the involvement of gene 4 in virulence determination although their definition of virulence was different and a heterologous system was used, introducing the possibility that host factors may have caused the dose effect. Analysis of the reassortants produced between a virulent and an avirulent bovine strain, reported here, also incriminated gene 4. However, the lack of production of progeny virus following infection with some of the reassortants would suggest that other factors are involved. It may be that analysis of more reassortants would show that virulence determination in rotaviruses is similar to influenza viruses and requires the correct gene constellation to provide fully infectious virulent and avirulent reassortant strains. The fact that these reassortants grow readily in cell culture but not in the calf highlights the care required when assumptions are made, concerning virulence, based on *in vitro* studies without confirmation *in vivo*.

More work is required to elucidate the molecular basis of rotavirus virulence, however, the data produced be relevant to the interaction of rotaviruses with the intestinal epithelium and assist in the understanding of the pathogenic processes involved in viral enteric disease.

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SECTION 10: APPENDICES

(Published papers)

10.1

ANTIGENIC VARIATION IN PORCINE AND BOVINE ROTAVIRUSES

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ABSTRACT

Two levels of antigenic variation in rotaviruses are described - variation in the group antigens of porcine rotaviruses and variation in the neutralization (type-) specific antigens of bovine rotaviruses.

By cross-immunofluorescence between classical porcine rotaviruses and two atypical porcine rotaviruses, the existence of three separate rotavirus groups - A, B and C - is proposed. Examination of porcine and bovine field sera indicated that rotaviruses belonging to all three groups are prevalent in pigs and exist in cattle in the U.K.

Analysis by neutralization of four bovine rotaviruses (all belonging to the proposed group A) indicated that, based on 20-fold differences in activity of convalescent and hyperimmune sera, two or three serotypes exist but that intermediate strains may also occur.

The significance of these findings is discussed.

INTRODUCTION

Antigenic variation between rotaviruses was suggested soon after their recognition (6,17) although, at this time, all known rotaviruses had been found to share a common group antigen. In 1977, Thouless et al. (13) demonstrated that these classical rotaviruses from different animal species could be distinguished by neutralization but, subsequently, neutralization tests have also detected differences in rotaviruses from single animal species i.e. man and birds (9,13,15,18,19).

Although it had become accepted that all viruses with rotavirus morphology shared a common group antigen, some recent isolates have failed to cross-react. To date, these atypical rotaviruses have been detected in pigs, birds and man (2,8,10,11,12) but cross-relationships, if any, of these atypical rotaviruses are unknown. In pigs, two atypical rotaviruses have been described, one from the U.K. (5) and one from America (1).

The present paper reports two aspects of antigenic variation in rotaviruses - firstly, a comparison of the two atypical porcine rotaviruses by immunofluorescence for the presence of common antigens and, secondly, examination of variation in neutralization antigens between classical rotaviruses isolated from one animal species i.e. cattle.

MATERIALS AND METHODS

Viruses - The porcine rotaviruses used were: the classical viruses A/SW20/21 (17) and the A/OSU strain obtained from E.H. Bohl after 50 passages mainly in MA104 cells; atypical rotaviruses - B/NIRD-1 identified in the faeces of a diarrhoeic pig in the United Kingdom (2) and C/Cowden, identified in the United States (12).

Four classical bovine rotaviruses, related by their group antigen, were studied - TC, the virus reported in cell culture in 1975 (4), PP and CP (Bridger et al., manuscript in preparation), and 160/222. Excluding TC, viruses were adapted to grow in MA104 cells with the aid of trypsin; PP and CP were cloned by terminal dilution whereas TC and 160/222 were plaque-purified.

Antisera - Convalescent antisera to the porcine rotaviruses were raised in gnotobiotic piglets by oral administration of bacteria-free faecal filtrates (S. Pedley, J.C. Bridger, J.F. Brown and M.A. McCrae, in press J. Gen. Virol.).

For the bovine rotaviruses, TC, CP and PP, two convalescent ('A' and 'B' sera) and one hyperimmune ('C' serum) sera were raised in one gnotobiotic calf for each virus (J.C. Bridger et al., manuscript in preparation). 'A' sera were obtained 18-21 days after primary oral inoculation while 'B' sera were obtained 14-28 days after a second oral inoculation. 'C' sera were prepared by intramuscular inoculation of the same calf with partially-purified antigen combined with Freund's incomplete adjuvant.

Indirect Immunofluorescence Test - The A/OSU rotavirus antigen was MA104 cells infected eighteen hours previously and fixed in 80% acetone. B/NIRD-1 and C/Cowden antigens were sections of small intestinal tissue taken from piglets orally infected eighteen hours previously, snap frozen in liquid nitrogen, sectioned and fixed in acetone. Dilutions

of the sera to be tested were allowed to react with antigen for one hour at room temperature before being replaced by the appropriate anti-species immunoglobulin conjugated with fluorescein isothiocyanate (Nordic Immunological Laboratories Ltd.). The titre of the serum was taken as the highest dilution which produced fluorescing cells. To assess the prevalence of antibody, sera were tested at 1:20 dilution.

Virus Neutralization Test - A fluorescent focus-reduction test (3) was used with MA104 cells to determine 50% end-points. To minimize dilution error, sera to be tested were diluted so that a maximum of 12 serial doubling dilutions was not exceeded. Titres were based on results obtained on 2 to 5 separate occasions when the variation between tests was 1 to 6-fold (mean 2-fold).

RESULTS

Group Antigen Variation

a) Comparison of three porcine rotaviruses. - Reciprocal cross-immunofluorescence tests showed no heterologous reactions between classical porcine rotavirus and two atypical rotaviruses, whereas, in homologous tests, fluorescence was obtained with all three sera at high dilution (Table 1). Thus, we suggest the existence of three groups, A, B and C.

Table 1. Antigenic relationship of 3 porcine rotaviruses by indirect immunofluorescence. *: Highest serum dilution giving immunofluorescence.

Antiserum to	Virus		
	A/OSU	B/NIRD-1	C/Cowden
A/SW20/21	2.5×10^3*	<20	<40
B/NIRD-1	<10	2×10^3	<20
C/Cowden	<10	<10	2×10^3

b) Prevalence of antibody. - By indirect immunofluorescence, a high proportion of sera collected from pigs aged 5 weeks to several years at the Institute's farm, possessed antibodies to groups A, B and C rotaviruses (Table 2). Overall, the percentage of sera from younger animals positive to groups B and C rotaviruses was lower than that to group A, whereas all sera were positive for all viruses at 6 months of age. Analysis of 16 sera from colostrum-fed calves at the Institute showed that under half were positive to groups B and C rotaviruses, although all had antibodies to group A rotavirus (Table 3).

Type-Specific Variation - Reciprocal cross-neutralization of three cloned bovine rotaviruses (TC, CP and PP) with convalescent and hyperimmune sera indicated that they were antigenically heterogeneous (Table 4). Comparison of TC and PP viruses with their reciprocal sera showed differences in the ratio of homologous:heterologous titres to range from 57 to >2290 (Table 5). Thus, it is suggested that these two bovine rotaviruses are separate serotypes on the basis that a minimum of

Table 2. Prevalence of rotavirus antibody in the pig herd at IRAD, Compton, 1981, by indirect immunofluorescence. *: Number of sera positive/number tested.

Age (weeks)	Rotavirus Group		
	A	B	C
5	10/10*	1/10	4/10
12	10/10	9/10	8/10
26	10/10	10/10	10/10
Sows	7/7	6/7	6/7

Table 3. Prevalence of rotavirus antibody in calves at IRAD, Compton, 1982, by indirect immunofluorescence. *: Number of sera positive/number tested.

Age	Rotavirus Group		
	A	B	C
1-9 days	16/16*	3/16	7/16

Table 4. Cross-neutralization between four bovine rotaviruses. *: Reciprocal of dilution giving 50% reduction in fluorescing foci. A and B: Convalescent sera; C: Hyperimmune serum.

Serum		Rotavirus			
		Standard			Field
		TC	CP	PP	160/222
TC	A	804*	220	14	<29
	B	6,327	674	18	94
	C	405,065	9,150	636	5,050
CP	A	28	6,762	4,725	<20
	B	242	7,547	15,682	40
	C	436	164,038	350,132	640
PP	A	<10	3,110	5,005	<46
	B	<10	32,032	22,897	<43
	C	762	286,916	298,437	3,700
Serotype		1	2?	2	3?

Table 5. Differences in neutralizing activity between homologous and heterologous sera for four bovine rotaviruses. *: Homologous titre of that serum ÷ heterologous titre. A and B: convalescent sera; C: hyperimmune serum.

Serum		Rotavirus			
		Standard			Field
		TC	CP	PP	160/222
TC	A	1	4*	57	>28
	B	1	9	351	67
	C	1	44	637	80
CP	A	242	1	1	>338
	B	31	1	0.5	189
	C	376	1	0.5	256
PP	A	>500	2	1	>109
	B	>2,290	1	1	>533
	C	392	1	1	81

a 20-fold difference in neutralization is required to define a serotype (7), i.e. the TC strain as serotype 1 and PP as serotype 2.

Comparison of the viruses CP and PP with their reciprocal sera failed to separate them from one another, suggesting that CP is a member of the proposed serotype 2. Extension of this comparison to include each of the three TC sera indicated that CP and PP were not identical, however - CP showed a stronger relationship to TC than PP (Table 5). With the two convalescent TC sera (A and B), the ratio homologous: heterologous titre for CP virus was less than 20-fold indicating a strong relationship to the proposed serotype 1 virus. The relationship of CP to TC was one-way (based on 20-fold differences) as none of the CP sera cross-reacted within 20-fold. Thus the CP virus seemed to be intermediate between TC and PP, the proposed serotypes 1 and 2.

With TC, CP and PP sera, a fourth bovine rotavirus, 160/222, was found to be distinct (Tables 4 and 5). Whether this virus represents a third distinct serotype or has a one-way cross with any of the standard viruses will be established when results from the reciprocal cross are available.

DISCUSSION

Two levels of antigenic variation between rotaviruses were studied - group and neutralization antigen variation. Two atypical porcine rotaviruses, which did not possess the original group antigen of classical rotaviruses, were unrelated by a group antigen test. Antibody to both atypical viruses was found to occur commonly in pigs in the United Kingdom, and to be present in British cattle. Secondly, four bovine rotaviruses, which shared the original rotavirus group antigen, showed differences in neutralization tests which suggests division into serotypes based on 20-fold differences in serum titres.

Thus we propose that, at least for porcine rotaviruses, three distinct groups exist based on group antigens - group A: rotaviruses with the originally-described group antigen which comprise the majority of rotaviruses studied to date (e.g. OSU strain and the four bovine viruses studied here); group B: the group antigen of the British NIRD-1 strain and group C: the group antigen of the American Cowden strain. Examination of the nucleic acid of these viruses by both genome profile and terminal fingerprint analyses has also shown that the viruses are distinguishable (S. Pedley, J.C. Bridger, J.F. Brown and M.A. McCrae, in press).

Although there have been no publications on group C viruses in pigs in the United Kingdom or group B and C viruses in cattle, the demonstration of antibody in these species suggests that they may be widespread here. Whether the atypical rotaviruses from man and birds belong to groups B and C or additional groups remains to be established but 15 sera collected from children in the U.K. and an experimental serum to an avian atypical rotavirus failed to cross-react with B/NIRD or C/Cowden (J.C. Bridger and J.F. Brown, personal observations). The importance of atypical rotaviruses in porcine diarrhoea remains to be assessed but both cause diarrhoea in experimental piglets (1, J.C. Bridger, personal observation). Because of the absence of serological cross-reaction, the serologically-specific tests which have been used to date to assess the prevalence of rotaviruses in porcine diarrhoea will have failed to detect the atypical viruses.

In contrast to the porcine rotaviruses, differences between the bovine rotaviruses (group A) based on neutralization were not always so clear-cut - differences ranged from nil to greater than 2000-fold. The two viruses, CP and PP, which showed differences only by neutralization with TC sera, are also different in virulence - CP is virulent for calves but avirulent for pigs and PP vice versa (J.C. Bridger, manuscript in preparation). It was possible to differentiate serotypes based on 20-fold differences but CP virus was intermediate between types 1 and 2. Analysis of many more cloned rotaviruses will be needed before it can be established if a continuous antigenic spectrum exists but preliminary results indicate that this is possible.

The significance of neutralization differences between rotaviruses in vivo remains to be assessed but we have observed that, in cross-protection experiments in calves, heterologous bovine rotaviruses may fail to cross-protect whereas all of 5 calves challenged with homologous virus did not develop clinical disease or excrete virus. In vitro, in a screening neutralization test with 100 infectious foci of TC, CP or PP viruses and sera at 20 times the homologous titre, complete neutralization was only obtained in homologous tests and between PP and CP viruses (which showed no differences on reciprocal neutralization). All other combinations consistently showed incomplete neutralization to varying degrees. In contrast we have noticed that many field bovine rotaviruses have some cross-relationship with the TC-virus but whether this affinity is close enough for TC antibody to provide adequate protection under natural conditions remains to be assessed.

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Variation in virulence of bovine rotaviruses

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SUMMARY

Forty-six gnotobiotic calves aged less than 16 days or 42–116 days were infected with three strains of bovine rotavirus designated C3-160, CP-1 and PP-1. Each virus was passaged and cloned in cell culture (cloned viruses) but CP-1 and PP-1 were also used before culture (faecal viruses).

Infection of calves aged less than 16 days with faecal or cloned CP-1 caused disease whereas cloned C3-160 and faecal or cloned PP-1 caused subclinical infections. The clinical signs of disease were change in faecal colour to pale yellow or cream, increase of 2- to 7-fold in the volume of faecal output and, usually, anorexia. With the virulent CP-1 virus and the avirulent C3-160, similar amounts of virus were excreted in the faeces for 4–6 days.

Infection of calves aged 56–116 days with faecal CP-1 produced disease of similar severity to that seen in calves aged 7–10 days infected with the same virus. No differences in clinical signs, virus excretion or levels of convalescent antibody were seen between the two groups. With cloned CP-1, 5 of 8 older calves developed disease but 3 showed only mild signs of infection.

It was concluded that two strains of rotavirus caused sub-clinical infections in young calves while a third was virulent in calves up to at least 116 days of age.

INTRODUCTION

Rotaviruses are accepted as enteric pathogens in many animal species. In the UK and North America they have been associated with disease in calves aged under 4 weeks, frequently in the second week of life (Woode & Bridger, 1975; Acres & Babiuk, 1978; D. J. Reynolds and J. H. Morgan, personal communication) but the occurrence of antibody in nearly 100% of cattle of all ages indicates that sub-clinical infections are common (de Leeuw *et al.* 1980; Schusser, Hinaidy & Bürki, 1982; McNulty & Logan, 1983). There are at least three possible explanations for these observations. Firstly, sub-clinical infections, which resulted in the development of serum antibody, were produced by feeding rotavirus antibody at the time of challenge with virulent virus (Snodgrass & Wells 1976; Bridger & Brown, 1981); this probably occurs frequently under natural conditions. Secondly, differences in virulence between isolates of rotavirus have been suggested in studies using intestinal loops (Carpio, Bellamyu & Babiuk, 1981) and infection with avirulent strains could account for sub-clinical infections. A third explanation could be an

age-related resistance to disease; virus may multiply and produce an immune response in calves of all ages but produce disease only in the young. Evidence to support this hypothesis was reported by Tzipori *et al.* (1981) with four rotaviruses isolated in Australia which caused disease only in calves aged less than 7 days but these results contrast with those obtained with a rotavirus studied in the United Kingdom which was said to cause disease in gnotobiotic and colostrum-deprived calves aged up to 56 days (Bridger & Woode, 1975).

The present paper examines the second and third explanations for sub-clinical infections. The virulence of three strains of bovine rotavirus in gnotobiotic calves infected with either uncultured (faecal) or cloned viruses is described. Firstly, infection of calves aged 16 days and under was studied with cloned rotaviruses CP-1, C3-160 and PP-1 plus faecal CP-1 and PP-1. Secondly, infection of calves aged 42–116 days with the faecal and cloned CP-1 strain is described.

MATERIALS AND METHODS

Viruses.

Bovine rotaviruses CP-1 and PP-1 were obtained from an outbreak of diarrhoea in colostrum-deprived calves at this Institute in 1973 (Woode *et al.* 1974). The faecal CP-1 inocula were prepared from faeces of a gnotobiotic calf inoculated with virus from the outbreak which had been passaged serially seven times in gnotobiotic calves; the faeces were filtered through 0·22 or 0·45 μm membranes (Millipore). The faecal PP-1 inocula were prepared as filtrates of diarrhoeic faeces from a gnotobiotic pig inoculated with the same outbreak material which had been passaged serially five times in gnotobiotic pigs. By electron microscopy, viruses other than rotaviruses were not observed in the faeces of these animals. Both faecal CP-1 and PP-1 were passaged and cloned in cell cultures as described previously (Bridger & Brown, 1984). Cloned CP-1 had been passaged serially a total of nine times in cell cultures and cloned PP-1 a total of eight times.

Rotavirus C3-160 was obtained from the Institute's herd in 1980 from a normal calf in the second week of life when there was no diarrhoea in the herd. It was adapted for growth in MA104 cells (continuous Rhesus monkey kidney cells, Microbiological Associates, USA) and cloned by three rounds of plaque purification (D. H. Pocock, personal communication). It had been passaged serially a total of 10 times in cell culture.

Calves and inoculation.

Forty-six gnobiotic calves were produced and reared on a milk based diet (Dennis, Davies & Hoare, 1976; Hoare, Davies & Dennis, 1976) They were infected as follows: 3, aged 7–10 days, and 18, aged 56–116 days, with faecal CP-1; 5, aged 8–12 days and 8, aged 42–85 days, with cloned CP-1; 6, aged 1–4 days, with faecal PP-1; 3, aged 4–16 days, with cloned PP-1; 3, aged 6–15 days with cloned C3-160. With the cloned viruses, these infections included three to five serial calf passes after culture. Calves were either bacteriologically sterile during the period of observation or were accidently contaminated with species of *Staphylococcus*, *Bacillus*, *Edwardsiella*, *Micrococcus*, *Citrobacter*, *Streptococcus* or *Enterobacter*. Accidental contamination of calves with these species did not result in changes in

faecal colour, faecal output or in anorexia. Serum samples were obtained at the time of inoculation and 3 weeks later. Before inoculation, the serum of each calf was free of rotavirus antibody. Calves were inoculated orally with 2 ml of either a faecal filtrate or cell culture fluid.

The daily faecal output of male calves and dry matter content of the faeces was determined as described previously (Bridger, Hall & Brown, 1984). The mean daily faecal output before infection was 320 g (S.D. $\pm$ 80 g). Samples of faeces, passed naturally, were collected from female calves and the colour and visible consistency of all faecal samples noted. Anorexia was assessed by a comparison of feeding habits before and after inoculation and was recorded as present when, for one or more feeds, calves did not consume the milk offered within 1 h; normally calves drank within 5–10 min.

Virus detection

Infectivity assays. Assays of faecal samples and cell culture fluids were conducted in duplicate in trypsin-free medium as described previously (Bridger & Brown, 1981) except that MA104 cells were used. Titres were expressed as $\log_{10}$ TCID50 per g of faeces or ml of culture fluid.

Electron microscopy. Three grams of faeces were diluted 1:4 with phosphate-buffered saline, centrifuged twice at 8000 *g* for 30 min and the supernatant fluid layered over 5 ml of 40% (w/w) sucrose solution followed by centrifugation at 83000 *g*. The resultant pellet was resuspended in phosphate buffered saline and examined by electron microscopy after staining with 2% potassium phosphotungstate (pH 6·0).

Antibody detection

Immunofluorescent activity was determined indirectly as described previously (Bridger, 1978) except that MA104 cells and rabbit anti-bovine immunoglobulin conjugated with fluorescein isothiocyanate (Nordic Laboratories Ltd) were used. Neutralizing activity was determined by a fluorescent focus reduction test with homologous virus (Bridger & Brown, 1984).

RESULTS

Comparison of the virulence of three rotavirus strains in calves aged 1–16 days

Oral inoculation of five calves aged 8–12 days and three calves aged 6–15 days with cloned viruses CP-1 and C3-160 respectively produced different clinical effects (Table 1). Similar levels of infectious virus and serum neutralizing antibody were found with both viruses but infection with C3-160 was sub-clinical in all three serial calf passes. In contrast, clinical signs developed in all three calf passes with CP-1. The presence of rotavirus in the faeces was confirmed for both viruses by electron microscopy, beginning 2–4 days after infection. The clinical effects of CP-1 before passage in cell culture (faecal CP-1) were similar to those after culture and cloning (compare Tables 1 and 2). Samples of C3-160 were not available before culture.

Sub-clinical infections also occurred in nine calves aged 1–16 days inoculated with $10^{2\cdot0}$–$10^{7\cdot5}$ TCID50 of PP-1, a virus from bovine faeces passaged in piglets. During three (cloned virus) or five (faecal virus) serial passages rotavirus was

Table 1. *Inoculation of young gnotobiotic calves with cloned bovine rotaviruses CP-1 and C3-160*

Virus	Age of calves (days)	Dose (log_{10} TCID50)	No. calves tested	No. calves positive (days duration)				Mean (range) of		
					Faecal changes					
				Anorexia	Colour	Daily output†	Virus excretion	Peak faecal output /day (g)	Peak virus excretion log_{10} TCID50 /g faeces)	Antibody titre in convalescent serum§
CP-1*	8–12	2·0–7·0	5	3 (1–3)	5 (6–7)	3‡ (3–6)	5 (4–6)	1570‡ (750–2250)	5·6 (4·5–6·6)	6800
C3-160*	6–15	4·0–6·0	3	0	0	0	3 (4–5)	374 (331–440)	6·4 (6·0–6·8)	1560 (1100–2180)

* CP-1 was passaged nine times in cell culture followed by three serial passes in calves. C3-160 was passaged 10 times in cell culture followed by three serial passes in calves.

† Above 500 g: in uninoculated controls daily faecal output averaged 320 g (standard deviation ±80 g).

‡ Three calves tested.

§ By neutralization of homologous virus. A single calf was tested for CP-1.

Table 2. *Inoculation of gnotobiotic calves of different ages with faecal virus, CP-1*

Age of calves (days)	Dose of virus (log_{10} TCID50)	No. calves tested	No. calves positive (days duration)				Mean (range) of		
				Faecal changes					
			Anorexia	Colour	Daily output*	Virus excretion	Peak faecal output /day (g)	Peak virus excretion log_{10} TCID50 /g faeces)	Antibody titre in convalescent serum§
7–10	5·0–5·3	3	3 (1–4)	3 (6–9)	3 (3–7)	3 (6)	1370 (900–2000)	6·2 5·7–6·7)	320 (160–640)
56–116	2·7–5·6	18	17 (1–7)	18 (5–9)	9† (5–7)	9† (5–6)	2000† (1250–2800)	5·9‡ (4·8–6·3)	640 (640)

* Above 500 g: in uninoculated controls, daily faecal output averaged 320 g (standard deviation ±80 g).

† Nine calves tested.

‡ Five calves tested.

§ Measured by indirect immunofluorescence; three calves tested per group.

detected in faeces of each calf for 1–4 days with peak titres per calf ranging from $10^{1.7}$ to $10^{5.7}$ TCID50 /g followed by seroconversion. There was no disease although two calves, at the second and third passages of the faecal virus, showed a slight colour change of the faeces which coincided with virus excretion.

With pathogenic infections produced by faecal and cloned CP-1 virus in young calves, clinical signs commenced within 2–3 days after inoculation. The first signs were anorexia and/or a colour change of the faeces from dark green or brown to pale yellow or cream, occasionally light green or grey. The amount of faeces produced was a good indication of the severity of disease; increased volumes above 500 g/day were produced for 3–7 days with maximum daily outputs of 2- to 7-fold above normal. In the majority of calves, the faeces were less fluid after infection than before, although some calves showed no visible change. Occasional individual samples were watery but these occurred sporadically. The dry matter content of the faeces was a poor indication of disease, ranging from 11·4–28·1 % (mean 17·2 %) before infection to 7·3–20·6 % (mean 13·3 %) after infection (data from 12 calves). Anorexia occurred in 6 of 8 calves and 2 inoculated with faecal CP-1 became severely ill; they were unable to rise without assistance and bellowed repeatedly. Milk was withheld and the calves recovered.

Influence of age on susceptibility to rotavirus CP-1

Faecal CP-1. Faecal CP-1 produced clinical disease of equal severity in calves aged 56–116 days as in calves aged 7–10 days (Table 2). In older calves the faeces became pale yellow in colour, the volume increased about 4- to 9-fold and all but one calf developed anorexia. Virus was excreted concurrently in the faeces at similar levels to younger calves and, by indirect immunofluorescence, the levels of rotavirus antibody in convalescent sera were similar to those of younger calves. Four of the 18 older calves were more severely affected. They were unable to rise without assistance and bellowed repeatedly. As with younger calves, they recovered after milk was withheld.

Cloned CP-1. Eight calves aged 42–85 days, were inoculated with the first to third calf passage after culture. Five, aged 42–85 days and inoculated with $10^{2.7}$ TCID50 (1 calf) or 10^{6}–$10^{7.6}$ TCID50 (4 calves) of the first and second calf passages developed disease indistinguishable from that after infection with faecal and cloned CP-1 in young calves. They excreted virus for 3–6 days with maximum levels of $10^{4.3}$–$10^{7.5}$ TCID50/g of faeces. One calf, aged 46 days and inoculated with $10^{2.7}$ TCID50 was killed *in extremis* 13 days after inoculation when it had been lethargic for 6 days and anorexic from day 2 after inoculation. *Streptoccus* sp. and *Staphylococcus* sp. were isolated from the faeces. In the remaining three calves, aged 45–70 days and inoculated with low doses of virus ($10^{2.0}$–$10^{4.0}$ TCID50) at the second and third calf passages after culture, only mild clinical signs developed. The colour of the faeces became slightly lighter and the maximum daily faecal outputs ranged from 583 to 790 g; anorexia was not observed. Rotavirus was detected for 2–4 more days with maximum levels of $10^{3.7}$–$10^{4.5}$ TCID50/g. *Staphylococcus* sp., *Bacillus* sp. and *Edwardsiella* sp. were isolated from the faeces. The mean titre of neutralizing antibody in the convalescent sera (8150) was similar to that in the clinically affected animals (8640).

Cloned CP-1 followed by faecal CP-1. The possiblity that faecal CP-1 contained a filterable agent other than rotavirus was examined by challenge of two gnotobiotic calves infected at 45 and 51 days of age with cloned CP-1. When challenged at 66 and 72 days of age with $10^{5\cdot0}$ TCID50 of faecal CP-1 no clinical signs or excretion of rotavirus occurred whereas two control calves aged 71 and 73 days developed diarrhoea. These results confirmed that rotavirus in the faecal CP-1 inocula was responsible for the disease in the older calves.

DISCUSSION

The results presented here show that two strains of rotavirus (C3-160 and PP-1) multiplied avirulently while another (CP-1) was virulent in calves of all ages. These observations support the hypothesis that infection with avirulent rotaviruses could account for natural sub-clinical infections but do not support the hypothesis that rotaviruses are pathogenic only in the young calf. In natural infections, rotaviruses have been associated with disease in calves aged up to 49 days (Sibalin, Szekely & Bürki, 1980; Van Opdenbosch & Wellemans, 1982) and in man, rotavirus-associated outbreaks of diarrhoea have been observed in adults (Linhares *et al.* 1981; Echeverria *et al.* 1983). In contrast, age-dependent resistance to disease have been reported in cattle and mice (Tzipori *et al.* 1981; Wolf *et al.* 1981; Riepenhoff-Talty *et al.* 1982; Sheridan *et al.* 1983). These data, together with the observations presented here, lead us to conclude that some strains cause disease in all ages, some infect all ages but cause disease only in the young, while other strains infect the young without disease.

The use of viruses which had been cloned in cell culture reduced the chance of the presence of adventitious viruses and mixed rotavirus populations. However, after cloning, CP-1 was pathogenic less consistently in older calves, 3 of 8 infected calves developed only mild clinical signs. This may have been due to attenuation by passage in cell culture or due to selection of a less virulent virus. The latter explanation is supported by the finding that, in neutralization tests with standard sera, the cloned virus was neutralized whereas the faecal virus was not (J. C. Bridger, personal observation). Whether the avirulent virus C3-160 was pathogenic before culture could not be tested but as CP-1 was not rendered avirulent in young calves by a similar number of passages in cell cultures, nor were two other rotaviruses (Bridger & Brown, 1984), this seems unlikely. In addition, Carpio, Bellamyu & Babiuk (1981) reported that rotaviruses were not readily attenuated by passage in culture.

The reason for the failure of cloned CP-1 to cause disease in 3 of 8 older calves while causing disease in 5 is unclear but these calves were inoculated with low doses, $10^{2\cdot0}$–$10^{4\cdot0}$ TCID50 of virus. Dose alone does not appear to determine the clinical outcome, however, as one older calf inoculated with $10^{2\cdot7}$ TCID50 and contaminated with *Streptococcus* sp. and *Staphylococcus* sp succumbed to infection. Other factors such as animal variation and nature of the bacterial flora (Gouet *et al.* 1978; Runnels *et al.* 1980) may contribute to the clinical outcome of infection, but on their own, these bacteria did not cause the clinical signs observed following infection with virulent rotavirus.

The avirulent strain, PP-1 was identified and isolated by passage in gnotobiotic

piglets. It originated from an outbreak of diarrhoea in cattle, was pathogenic for pigs at the first passage (Hall *et al.* 1976) and was found to be related antigenically to both bovine and porcine rotaviruses (Bridger & Brown, 1984). On subsequent serial passage of the faecal PP-1 from gnotobiotic pigs in calves, it was avirulent but its pathogenicity for calves may have been modified by passage in pigs. If so, then passage in a heterologous host may be a mechanism by which rotaviruses became avirulent naturally.

Thus, taking all the available evidence together, it is likely that the outcome of rotavirus infection depends on the immune status at the time of infection, the virulence of the infecting virus strain and the age of the infected animal. The reasons for differences in virulence between strains are unclear from the present study but may be elucidated by studies of the molecular basis of virulence and the pathology of virulent and avirulent virus infections.

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CHARACTERISATION OF ROTAVIRUS ISOLATES FROM SUB-CLINICALLY INFECTED CALVES BY GENOME PROFILE ANALYSIS

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ABSTRACT

Pocock, D.H., 1987. Characterisation of rotavirus isolates from sub-clinically infected calves by genome profile analysis. *Vet. Microbiol.*, 13: 27—34.

Rotaviruses isolated from 43 sub-clinically infected calves from a single farm were analysed by genome profile analysis. The isolates showed genomic variation and eight different profiles were observed, including one which was atypical for Group A rotaviruses.

The 3' terminal labelling method for the analysis of genome profiles used in this study required only 1 ng of viral RNA, an increase of 1000-fold in sensitivity over ethidium bromide staining for detecting all rotavirus genome segments. However, dual infections involving two rotaviruses with distinct profiles could not be detected if the concentrations of the viruses differed by > 10-fold.

INTRODUCTION

Although rotaviruses are frequently associated with acute diarrhoea in man and animals, sub-clinical infections are common (Chrystie et al., 1978; de Leeuw et al., 1980). In a recent longitudinal survey McNulty and Logan (1983) reported the detection of rotavirus in 79% of calves during the first 3 weeks of life and of these 80% showed no clinical signs. A similar survey carried out on children found that rotavirus shedding was detected to the same degree in children with or without diarrhoea (Barron-Romero et al., 1985). The reasons for sub-clinical reactions to infection have not been fully elucidated, but there is experimental evidence that oral administration of antibody during virulent rotavirus infection eliminates any clinical signs of the infection (Snodgrass and Wells, 1976; Bridger and Brown, 1981). Differences in virulence among bovine rotaviruses have also been reported (Carpio et al., 1981) but naturally occurring avirulent strains have not yet been isolated and characterised.

Since genomic diversity among rotaviruses was found to be extensive

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(Kalica et al., 1978; Rodger and Holmes, 1979), genome profile analysis has been used to characterise virus isolates. To improve the sensitivity of this technique Clarke and McCrae (1981) described a method in which the viral RNA was 3′ terminal labelled prior to electrophoresis, so that the RNA could then be detected in the polyacrylamide gels by autoradiography instead of ethidium bromide staining. This report investigates the use of this technique for the characterisation of rotaviruses isolated from sub-clinically infected calves, as the first stage in a study of asymptomatic rotavirus infections in calves.

MATERIALS AND METHODS

Viruses

The UK strain of bovine rotavirus (Bridger and Woode, 1975) was grown in MA104 cells with serum-free Eagles MEM containing 0.5 μg ml^{-1} trypsin (Sigma Chemical Co. Ltd., Type 3).

Isolates of bovine rotavirus were obtained from 43 sub-clinically infected calves on a farm where such infections were common (Reynolds, 1983). Rotavirus was detected in the faecal samples of these animals by enzyme-linked immunosorbent assay (ELISA) (Reynolds et al., 1984).

Extraction of rotavirus RNA

Ten ml of a 10% faecal suspension were extracted twice with 5 ml fluorocarbon (Arklone P, ICI Ltd.) and the virus pelleted from the aqueous phase by centrifugation at 75 000 × *g* for 1 h. Cell culture grown virus was similarly pelleted from supernatant fluids after clarification at 5000 × *g* for 20 min. Virus pellets were resuspended in buffer (20 mM NaCl; 2 mM $CaCl_2$; 0.5% SDS; 25 mM Tris/HCl pH 7.4) and Proteinase K (Sigma Chemical Co. Ltd., Protease Type XI) was added to give a final concentration of 500 μg ml^{-1}. The mixture was incubated at 37°C for 30 min and extracted twice with 2 vol phenol:chloroform (1:1 v/v). Sodium acetate (pH 5.5) was added to the aqueous phase to give a final concentration of 0.3 M and the RNA was precipitated with 67% ethanol. After centrifugation at 10 000 × *g* for 30 min, the RNA pellet was dissolved in 0.1 ml distilled water and stored at −20°C. RNA concentrations were determined by spectrophotometry: $10D_{260\ nm}$ was assumed equivalent to 50 μg ml^{-1} (Gaillard and Joklik, 1982).

3′ terminal labelling of rotavirus RNA

The method of RNA labelling was essentially that described by Clarke and McCrae (1981), except that 2 μg RNase-free bovine serum albumin (Miles Laboratories Ltd.) was added to the reaction mixture to prevent adsorption

of the T_4 RNA ligase to the reaction vessel. Unincorporated radioactivity was removed by chromatography on Whatman CF11 cellulose columns.

Electrophoresis of rotavirus RNA

Electrophoresis was carried out on 10% polyacrylamide gels with a 3% stacking gel using the discontinuous buffer system described by Laemmli (1970), at either 40 mA constant current for 5—6 h or overnight at 20 mA. The gels were either stained with a 1 μg ml^{-1} aqueous solution of ethidium bromide (EB) for 1 h to detect unlabelled RNA or dried onto filter paper and autoradiographed when ^{32}P-labelled RNA was used.

Analysis of mixtures of two rotaviruses with distinct profiles

RNA extracted from the UK strain and from an isolate with a C7 profile was adjusted to give equal concentrations as estimated by spectrophotometry. Mixtures containing a constant amount of C7 RNA and varying amounts of UK strain RNA were prepared and 3′ terminally labelled as above. Samples of the labelled ds-RNA mixtures containing 10 000 c.p.m. were electrophoresed on a 10% gel.

RESULTS

Comparison of 3′ terminal labelling and ethidium bromide staining methods for detecting rotavirus RNA

By 3′ terminal labelling of 5 ng of viral RNA, all 11 rotavirus segments were detected after autoradiographic exposure for 16 h (Fig. 1a). The amount of RNA could be reduced to 1 ng if the exposure time was extended to 3—4 days (data not shown). A comparable gel stained with ethidium bromide (Fig. 1b) showed that the smallest amount of RNA which gave a complete pattern was 1 μg, i.e. there was a 1000-fold difference in sensitivity between the two methods.

Genome profile analysis of field isolates

Genome profiles were obtained from the rotavirus-positive faecal samples of all 43 calves, and the patterns fell into eight types, C1—C8 (Fig. 2). There was clear evidence of genomic variation and this variation was not confined to any segment or group of segments. The profile type C7 differed from the normal pattern produced from Group A rotaviruses as no band was observed in the normal Segment 11 region of the gel but a band was evident between Segments 6 and 7. Viruses with these profiles were detected in the faecal samples of eight calves by an ELISA specific for Group A rotaviruses indicating that they must belong to this group. Profiles

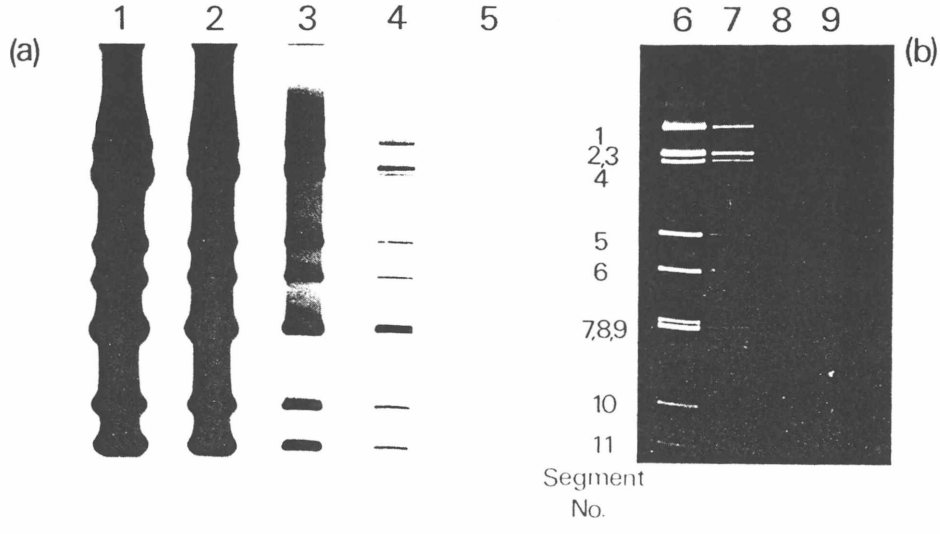


Fig. 1. Detection of different amounts of rotavirus genomic ds-RNA after separation of individual segments by electrophoresis through 10% polyacrylamide gels. Samples containing known amounts of ds-RNA were: (a) terminally labelled with [^{32}P] p-C-p and after PAGE the bands were detected by autoradiography for 16 h, or (b) electrophoresed and the bands visualised by UV illumination of the gel after staining with 1 μg ml^{-1} aqueous ethidium bromide. Lanes 1—5 contained 3.125 μg, 625 ng, 125 ng, 25 ng and 5 ng, and Lanes 6—9 contained 5 μg, 1 μg, 200 ng and 40 μg ds-RNA, respectively.

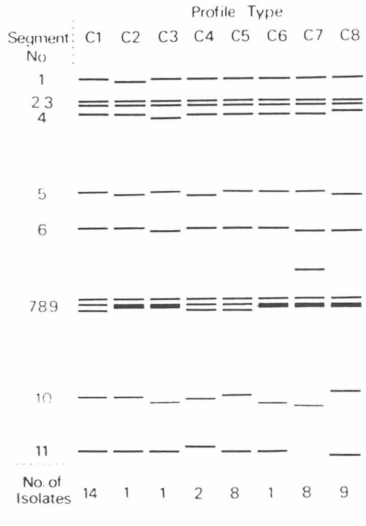


Fig. 2. Diagrammatic representation of the genome profiles of isolates identified during the study.

containing the expected 11 segments of ds-RNA were found from 42 samples. One profile, however, had an extra four bands suggesting that this calf had been infected with two viruses having C4 and C5 type profiles.

Dectection of mixed infections with bovine rotavirus

In this study only one sample showed evidence of mixed infection with two rotaviruses. It was therefore important to determine whether mixed infections were rare or whether the technique simply could not detect them. To investigate the latter, mixtures containing known amounts of ds-RNA from two rotavirus isolates with disparate genome profiles (UK strain and a C7 type) were used to simulate the type of samples that might be recovered from calves concurrently infected with these two viruses. After 3′ terminal labelling the RNA segments were analysed by electrophoresis (Fig. 3). Only samples b to f showed evidence of being mixed

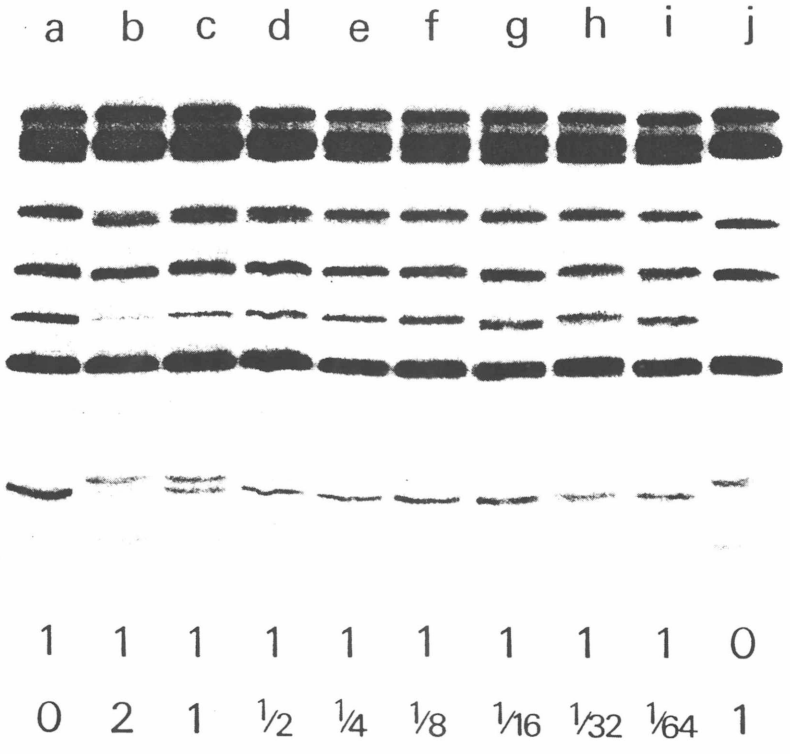


Fig. 3. Evaluation of genome profile analysis for the detection of heterogeneous mixtures of rotavirus RNA. Lanes a—j contained mixtures of C7 type and UK bovine rotavirus ds-RNA mixed in the proportions shown. The mixtures were terminally labelled and samples containing equal numbers of counts were electrophoresed.

profiles, and meant that in a faecal sample containing two rotaviruses if one virus was present at a concentration 10-fold less than the other, i.e. 1 $\log_{10}$ dilution, it would not be detected.

DISCUSSION

Rotaviruses isolated from 43 sub-clinically infected calves were characterised by genome profile analysis. The 3' terminal labelling of rotavirus ds-RNA used in this study was found to require only 1 ng of viral RNA to give a genome profile after electrophoresis. Comparison of this technique with ethidium bromide staining for the detection of rotavirus ds-RNA segments showed it to be 1000-fold more sensitive, allowing the successful analysis of all the ELISA positive samples used. Herring et al. (1982) used silver staining to visualise the genome segments and reported an increase of 20-to 40-fold over ethidium bromide. However this method suffers the same problem as ethidium bromide staining in that low molecular weight segments are difficult to detect, because they both depend on the total weight of RNA present in each band whereas the 3' terminal labelling method relies on the number of molecules of each segment.

Genomic variation was observed between isolates, and this was not confined to any particular genome segment or group of segments, a finding similar to that reported by Rodger and Holmes (1979) for rotaviruses from calves with enteric disease. Eight different profiles were observed and all were typical for Group A rotaviruses (Pedley et al., 1983), except for the C7 type which was typified by the lack of a band in the Segment 11 region and the appearance of a band between Segments 6 and 7. Variation in the position of Segment 11 has been reported for Subgroups 1 and 2 of human Group A rotaviruses (Kalica et al., 1981), although not to the extent seen with the C7 isolates. Rotaviruses with unusual genome profiles have been detected in man and animals (Pedley et al., 1983; Snodgrass et al., 1984), but these isolates have distinct group antigens and their genome profiles lack a triplet of Segments 7, 8 and 9, having similar electrophoretic mobilities, characteristic of Group A viruses. Therefore, the detection of C7 profile viruses by a Group A specific ELISA and the presence of the 7, 8 and 9 segment triplet in their genome profiles both confirm their assignment to Group A. The detection of these viruses highlights the caution needed if genome profile analysis alone is used to assign isolates to a particular rotavirus group.

The detection of calves dually infected with rotaviruses by the appearance of extra bands in genome profiles was found to depend on the relative rates of excretion of the two virus strains. It is therefore possible that other rotavirus variants were present in the samples but remained undetected, and underlines the requirement of cloning these isolates before further experimental work, e.g. animal inoculation, is undertaken.

Work is now in progress to adapt these genetic variants of rotavirus

for growth in cell culture so that further antigenic and virulence characterisation can be carried out. One isolate, with a C3 profile, has been isolated and cloned in cell culture and shown by inoculation into gnotobiotic calves to be avirulent (Bridger and Pocock, 1986).

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Key words: *rotavirus/bovine/genome profile*

Isolation and Characterization of Two Group A Rotaviruses with Unusual Genome Profiles

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SUMMARY

Analysis of the genomic dsRNA of rotaviruses isolated from calves with subclinical infections has revealed eight calves excreting group A viruses with unusual genome profiles. Two of these virus strains, C7-176 and C7-183, were grown and cloned by plaque purification in cell culture. Examination of their genome profiles after cloning showed that the unusual pattern had been retained. They were without RNA segment 11 but had an extra band (6a) migrating between segments 6 and 7. However, they contained the triplet of segments 7, 8 and 9, of similar size, which is characteristic of group A rotaviruses. The number and mol. wt. of the intracellular polypeptides induced by these viruses were similar to those of the bovine group A rotavirus UK strain. Analysis of the RNA transcripts produced by the transcription *in vitro* of purified C7-183 virus showed that segment 6a produced a large RNA transcript of corresponding size. After isolation, this transcript was translated in a rabbit reticulocyte lysate preparation and yielded a single polypeptide, vp11, equivalent to the product of segment 11 of rotaviruses with typical genome profiles.

INTRODUCTION

The rotavirus genome comprises 11 segments of dsRNA. All rotaviruses were thought to contain a common group-specific antigen, until several viruses were described which did not possess this antigen and were called atypical. Pedley *et al.* (1983) defined three rotavirus groups, group A containing the original viruses and groups B and C the atypical viruses. Since then two further groups of atypical rotaviruses have been described (Pedley *et al.*, 1986). Besides serological differences between the group A viruses and viruses of the other groups, Pedley *et al.* (1986) found that the genome profiles were distinct. The genome of the group A viruses was defined as comprising four high mol. wt. dsRNA segments (1 to 4), five middle-sized segments (5 to 9) including a distinctive triplet of segments (7 to 9) and two smaller segments (10 and 11). Although variation in genome profile has been observed with group A viruses, in most cases this basic pattern has been maintained. In 1980, however, Espejo *et al.* described rotavirus isolates in which the electrophoretic migration of segments 10 and 11 was less than that seen in other isolates and called them short profile viruses. This was followed by a description of the super short profile (Hasegawa *et al.*, 1984) in which segment 10 migrated just below the segments 7, 8 and 9 triplet. In the same year, Pedley *et al.* (1984) described the isolation of group A rotaviruses, from chronically infected immunodeficient children, whose genome profiles contained additional RNA segments migrating between segments 1 and 7. These additional segments contained sequences homologous to normal rotavirus segments. More recently there have been reports (Besselaar *et al.*, 1986; Pocock, 1986; Thouless *et al.*, 1986) of group A rotaviruses from man, calves and rabbits, in which segment 11 was absent but an extra middle-sized segment between segments 6 and 7 was observed.

This paper describes the isolation in cell culture of two bovine rotaviruses with unusual genome profiles, analysis of the virus-induced intracellular polypeptides, characterization of the viral RNA transcription products and the coding assignment of the aberrant dsRNA segment.

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METHODS

Viruses and cell cultures. The three bovine rotaviruses used in this study were the UK strain and two isolates (C7-176 and C7-183) which had unusual RNA profiles (Pocock, 1986). The latter two isolates were detected by a group A-specific enzyme-linked immunosorbent assay (Reynolds *et al.*, 1984).

The continuous rhesus monkey kidney cell line MA104 (Microbiological Associates, Los Angeles, Ca., U.S.A.) was grown in Eagle's MEM containing 5% foetal calf serum. Prior to inoculation with rotavirus, confluent monolayers of cells were incubated for 16 h with serum-free medium.

Virus isolation and cloning. The methods for the primary isolation of rotavirus described by Fukusho *et al.* (1981) and Murakami *et al.* (1983) were used, including the treatment of faecal virus with trypsin prior to inoculation onto rolled cultures of MA104 cells.

Faecal suspension (10%) containing either C7-176 or C7-183 were extracted with an equal volume of fluorocarbon (Arklone-P, ICI). The virus was pelleted from the aqueous phase by centrifugation at 75000 *g* for 90 min, resuspended in 0·02 M-Tris–HCl buffer (TB) pH 7·4 and stored at −70 °C. This material was diluted tenfold with serum-free medium containing 5 μg/ml trypsin (type XI, Sigma) and incubated at 37 °C for 30 min, prior to inoculation onto tube cultures of MA104 cells. After adsorption the inoculum was removed, the cell sheets were washed and incubated in serum-free medium containing 0·5 μg/ml trypsin. The tubes were rolled at 37 °C and observed daily for c.p.e. When the c.p.e. affected > 50% of the cell sheet the tubes were frozen at −70 °C, thawed, the cell debris was removed by low speed centrifugation and the supernatant stored at −70 °C for further passage.

After three serial passages the viruses were cloned three times by plaque purification. Plaques were produced in Petri dishes of MA104 cells under serum-free medium containing 0·5 μg/ml trypsin and 0·6% agarose (Miles). One passage in tube cultures was performed between each plaque passage.

Viral RNA analysis. Cell culture-grown virus was concentrated by centrifugation at 75000 *g*, the viral RNA was extracted and 3′ terminally labelled with [^{32}P]pCp by the method of Clarke & McCrae (1981) as described by Pocock (1986). The RNA was electrophoresed in 10% polyacrylamide gels at 20 mA for 16 h using the discontinuous buffer system of Laemmli (1970). The RNA bands were located in the dried gels by autoradiography on Fuji RX X-ray film.

Intracellular viral polypeptide analysis. Confluent monolayers of MA104 cells were inoculated with the virus isolates at a m.o.i. between 1 and 10 and incubated at 37 °C for 16 h. After incubation for 1 h in methionine-free medium the cells were pulse-labelled for 3 h in methionine-free medium containing 25 μCi/ml L-[^{35}S]methionine ([^{35}S]Met; >800 Ci/mmol, Amersham). At the end of the labelling period the medium was removed, the cell sheets were washed with cold isotonic (6%) glucose and the cells lysed in Laemmli's sample buffer [62·5 mM-Tris-HCl pH 6·8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol containing bromophenol blue (BPB) as marker]. The samples were boiled for 2 min and the DNA was sheared by passage through a 25-gauge needle. Electrophoresis was carried out in 10% polyacrylamide gels (Laemmli, 1970) at 100 V constant voltage until the BPB dye band was 1 cm from the bottom of the gel. The gels were soaked in En^{3}Hance (New England Nuclear) before drying, and fluorographed at −70 °C with preflashed X-ray film.

Apparent mol. wt. for the viral polypeptides were determined by comparison with standard protein mol.wt. markers electrophoresed on the same gel. The protein standards used were a high mol. wt. standard mixture (Sigma) containing myosin (mol. wt. 205000), β-galactosidase (116000), phosphorylase B (97400), bovine serum albumin (66000), ovalbumin (45000) and carbonic anhydrase (29000) with the addition of lysozyme (14300).

Transcription of rotavirus RNA in vitro. The transcription *in vitro* of rotavirus genomic dsRNA segments by the virion-associated RNA-dependent RNA polymerase was carried out as described by Cohen (1977) and Mason *et al.* (1980).

UK and C7-183 viruses grown in cell culture were pelleted by centrifugation at 75000 *g* and resuspended in 0·02 M-TB pH 7·4. The concentrated virus was layered onto preformed CsCl gradients (density 1·1 to 1·45 g/ml) and centrifuged at 100000 *g* for 4 h. After fractionation the virus-containing band was detected by A_{260} measurement and the relevant fractions were dialysed against sterile distilled water and stored at −70 °C. The concentration of virus was calculated on the basis that 5·4 A_{260} was equivalent to 1 mg virus/ml as determined for reovirus (Lai & Joklik, 1973).

The method of Flores *et al.* (1982) was used for the transcription *in vitro* of rotavirus RNA. Briefly, 100 μl containing 10 to 20 μg of purified virus was added to 150 μl of reaction mixture containing 100 mM-TB pH 8·0, 2·5 mM-CTP and -GTP, 10 mM-ATP, 0·625 mM-UTP, 10 mM-$MgCl_2$, 0·5 mM-*S*-adenosyl methionine, 0·1% Macaloid (NL Industries, New Jersey, U.S.A), 50 μCi [α-^{32}P]UTP (3000 Ci/mmol; Amersham) and incubated at 43 °C for 5 h. The virus was removed by centrifugation at 75000 *g* for 30 min and the RNA recovered from the supernatant by phenol/chloroform extraction and ethanol precipitation.

Separation and isolation of individual rotavirus RNA transcripts. Rotavirus RNA transcripts, from transcription *in vitro*, were treated with 5 mM-methylmercuric hydroxide (Ventron, F.R.G) for 5 to 10 min at room temperature and electrophoresed in 2% low melting point agarose gels (Gibco/Bethesda Research Laboratories) with borate buffer pH 8·2 (50 mM-boric acid, 5 mM-sodium borate, 10 mM-sodium sulphate). Gels were cast in 0·6 cm diam.

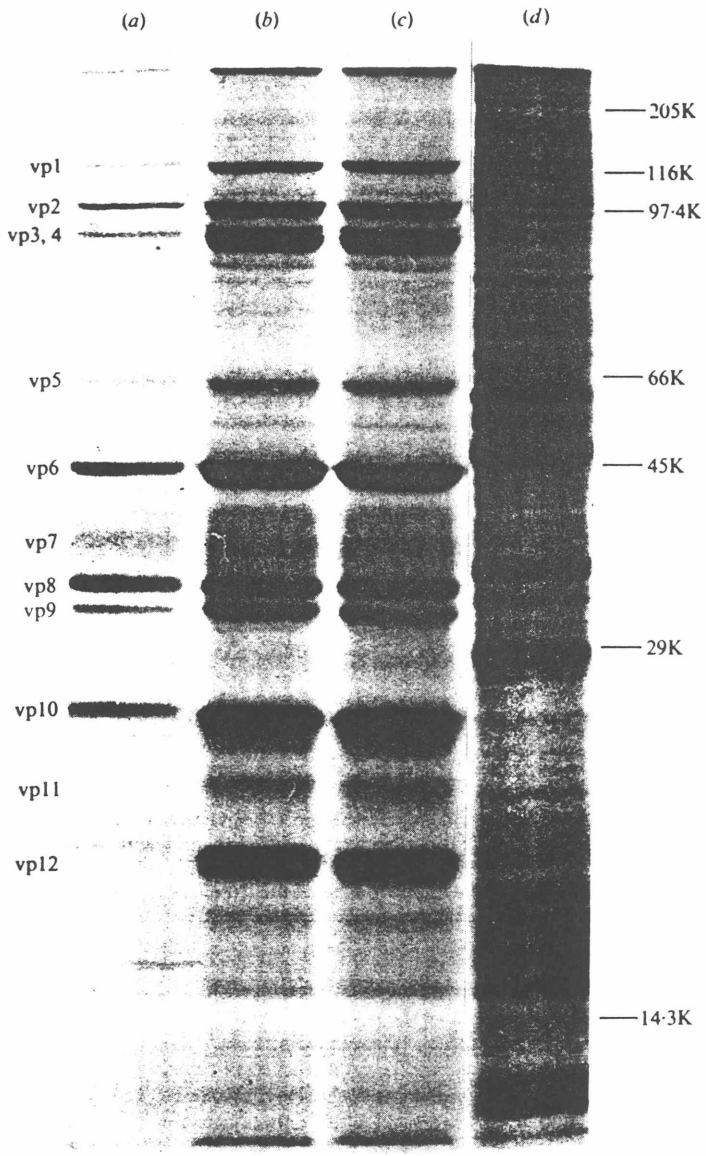


Fig. 2. [^{35}S]Methionine-labelled intracellular polypeptides induced by UK (*a*), C7-183 (*b*) and C7-176 (*c*) rotaviruses and uninfected control cells (*d*) were separated by electrophoresis and detected by fluorography. Polypeptides are labelled on the left of the figure with the notation used by McCrae & Faulkner-Valle (1981) and the positions of the mol. wt. standards are shown on the right.

Virus-induced intracellular polypeptides

Twelve viral polypeptides, labelled vp1 to vp12, were identified in cells infected with the UK strain (Fig. 2), in agreement with the published results for this strain (McCrae & Faulkner-Valle, 1981) and simian rotavirus SA11 (Ericson *et al.*, 1982). Both vp3 and vp11 were seen as faint bands, with vp3 migrating just above the more prominent vp4 band. This pattern of polypeptides was also found for the C7-176 and C7-183 strains, although differences in the migration of vp4, vp10 and vp11 differentiated them from the UK strain. The polypeptide vp11,

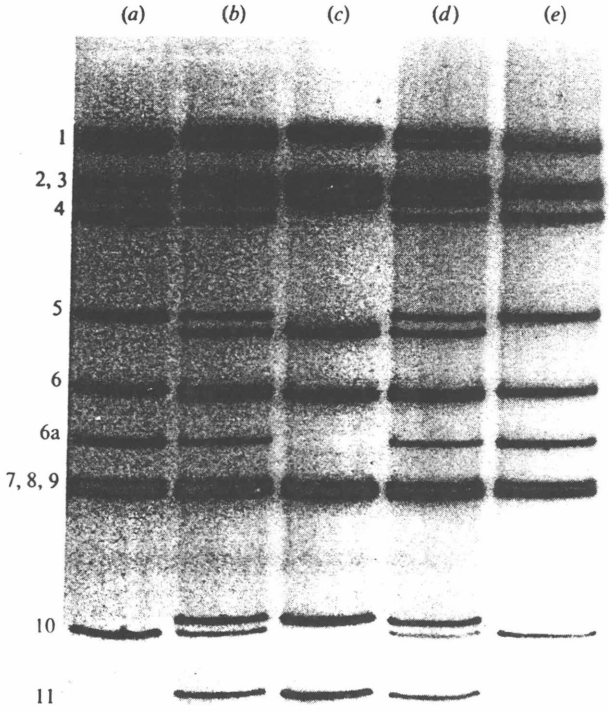


Fig. 1. Genome profiles of cloned C7-176, C7-183 and UK rotaviruses. ^{32}P-labelled genome segments from C7-183 (*a*), UK (*c*) and C7-176 (*e*) with mixtures of C7-183 and UK RNAs (*b*) and C7-176 and UK RNAs (*d*) were separated by electrophoresis in 10% polyacrylamide gels. The positions of the genome segments of the UK rotavirus strain are numbered on the left of the figure. 6a refers to the position of the C7-176 and C7-183 aberrant segment.

glass tubes 18 cm long and electrophoresed at 1·5 mA/tube for 16 h. The gels were removed and 2 mm slices analysed by Cerenkov counting to determine the position of the RNA bands. RNA was recovered from the gel pieces by electroelution and concentrated by ethanol precipitation.

Translation of isolated rotavirus RNA transcripts. Individual rotavirus RNA transcripts were translated with a rabbit reticulocyte lysate preparation (Anglian Biotechnology, Colchester, U.K.) following the manufacturer's protocol. Prior to translation the RNA was treated with 5 mM-methylmercuric hydroxide for 5 to 10 min at room temperature and translation reactions were performed at 30 °C for 1 h in the presence of 1 μCi/μl [^{35}S]Met. Translation products were analysed by diluting 5 μl translation mixture with 50 μl of Laemmli's sample buffer and electrophoresis at 100 V in 10% polyacrylamide gels.

RESULTS

Genome profiles of the cloned viruses

Rotaviruses C7-176 and C7-183 grew readily in cell cultures and produced extensive c.p.e. at the first passage. After cloning, the genomic RNA profiles showed that both viruses had retained their unusual profiles (Fig. 1), with the absence of a band in the segment 11 region of the gel and an extra band between segments 6 and 7, labelled 6a. The triplet of segments 7, 8 and 9, characteristic of group A rotaviruses, was retained by these strains. When compared to the UK strain these profiles also showed the type of genomic variation previously reported (Rodger & Holmes, 1979) with differences in segments 1, 3, 4, 5, 6 and 10. When compared to each other the only variation observed was a small difference in mobility of segment 3 (see lanes *a* and *e* in Fig. 1).

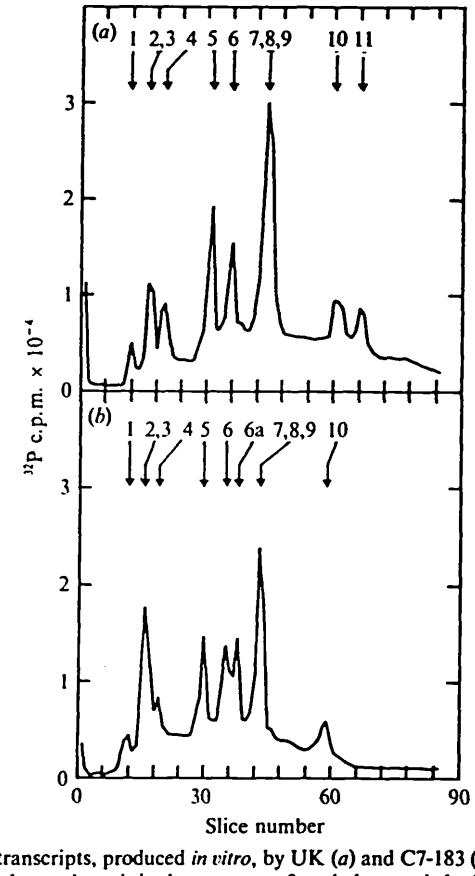


Fig. 3. Rotavirus RNA transcripts, produced *in vitro*, by UK (*a*) and C7-183 (*b*). The transcripts were separated by agarose gel electrophoresis in the presence of methylmercuric hydroxide. The distribution of radioactivity was determined by Cerenkov counting after fractionation of the gels into 2 mm slices. The positions of the transcripts for both strains are denoted by the numbered arrows. Electrophoresis was from left to right.

shown to be the translation product of rotavirus segment 11 (Dyall-Smith & Holmes, 1981; McCrae & McCorquodale, 1982*a*) was detected and therefore encoded by an alternative segment. Other protein bands seen in the profiles of these strains corresponded to bands found in the cell controls.

Viral RNA transcripts produced by UK and C7-183 viruses

Electrophoretic separation of UK and C7-183 viral RNA transcripts (Fig. 3) showed that the patterns produced reflected the size range of the genomic dsRNA profiles. The C7-183 transcript that migrated between the UK transcripts 6 and 7, labelled 6a, was assumed to be the product of the aberrant genomic segment 6a.

Coding assignment of UK and C7-183 RNA transcripts 6 to 11

UK and C7-183 RNA transcripts 6 to 11 were isolated from agarose gel slices by electroelution, with recoveries of 80 to 90%. No attempt was made to separate RNA transcripts 7, 8 and 9, from either virus, as under the conditions used they migrated as a single band. Translation of the total transcript mixtures from both viruses gave similar patterns (Fig. 4). Besides the polypeptides labelled vp1 to vp12, other minor bands were seen that did not appear in the unprimed translation mixture. These bands were not consistently observed and their

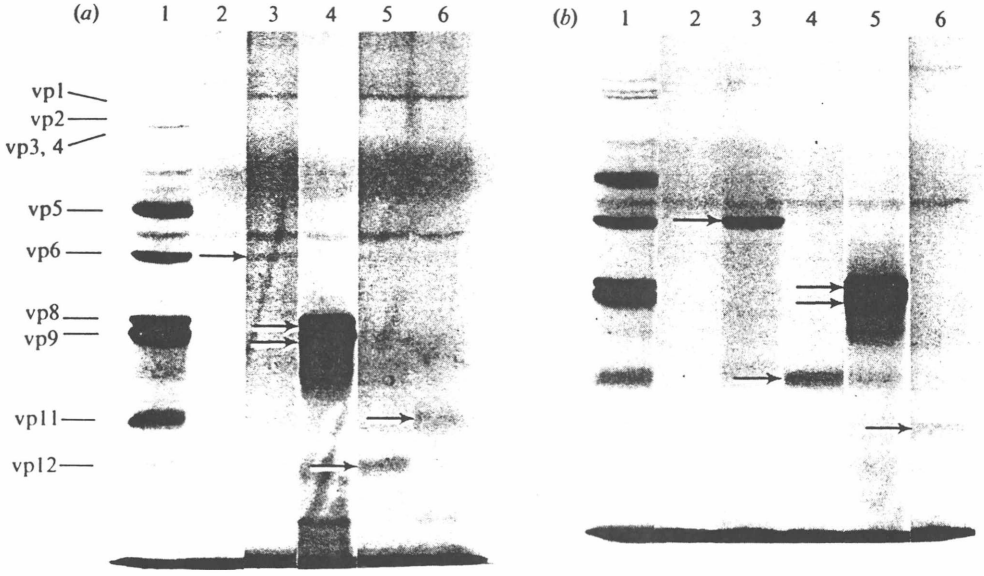


Fig. 4. Polyacrylamide gel electrophoresis of the translation products of RNA transcripts from (*a*) UK and (*b*) C7-183 rotaviruses. The lanes in (*a*) contained the translation products (arrowed) from (1) total transcription mixture, (2) no added RNA, (3) transcript 6, (4) mixture of transcripts 7, 8 and 9, (5) transcript 10 and (6) transcript 11. The lanes in (*b*) contained the products (arrowed) from (1) total transcription mixture, (2) no added RNA, (3) transcript 6, (4) transcript 6a, (5) mixture of transcripts 7, 8 and 9, and (6) transcript 10. vp1 to vp12 indicate the expected positions of the primary gene products for UK virus (McCrae & McCorquodale, 1982*a*).

origin remains obscure. Translation of UK RNA transcripts 6, 10 and 11 produced polypeptides corresponding to vp6, vp12 and vp11 respectively, in agreement with published data (McCrae & McCorquodale, 1982*a*). The mixture of transcripts 7, 8 and 9 produced apparently only two polypeptide bands vp8 and vp9; however vpr7, the precursor of vp7 and the other product of this triplet, has been shown to have a mol. wt. similar to vp9 (McCrae & Faulkner-Valle, 1981). C7-183 transcripts 6 and 10 were translated to vp6 and vp12 and the mixture 7, 8 and 9 to vp8, vp9 and presumably vpr7 in agreement with those of the UK virus. Transcript 6a was shown to have the same coding assignment as UK transcript 11 and produced vp11, suggesting that in C7-183 the genomic segment 11 had been replaced by segment 6a.

DISCUSSION

This paper describes the characterization of two bovine group A rotaviruses, C7-176 and C7-183, having unusual genome profiles with no segment 11 but an extra segment between segments 6 and 7. The number and sizes of the intracellular polypeptides induced by these strains were similar to those produced by the UK virus, suggesting that the aberrant genome segment coded for a normal sized viral protein. Analysis of the transcription products of C7-183 *in vitro* revealed an abnormally large RNA transcript which when translated produced a single viral polypeptide, vp11. This polypeptide has been shown to be the product of UK segment 11 (McCrae & McCorquodale, 1982*a*) and therefore the unusual genome profiles of C7-176 and C7-183 resulted from an increase in size of the normal rotavirus segment 11.

Comparison of the genome profiles of group A rotaviruses with rotaviruses from other serogroups (Pedley *et al.*, 1986) showed that the triplet of segments 7, 8 and 9 appeared to be diagnostic for group A viruses. Even though C7-176 and C7-183 showed unusual genome profiles for group A viruses, the characteristic triplet was still evident. So far few viruses from other serogroups have been analysed and therefore a new rotavirus isolate should not be assigned to a particular serogroup on its genome profile alone.

Variation in the size of segment 11 has been seen before in group A rotaviruses but not to such a large extent. In investigations of human rotaviruses, Espejo *et al.* (1980) and Rodger *et al.* (1981) showed that the viruses could be divided into two groups, termed short and long electropherotypes, according to their genome profiles. By gene coding assignment, this division was later shown to be due to an increase in the size of segment 11 in one group resulting in the short electropherotype (Dyall-Smith & Holmes, 1981). This could also explain the super short RNA patterns described recently for other human rotavirus strains (Hasegawa *et al.*, 1984; Albert, 1985). However, the increase in size of segment 11 in both the short and super short viruses was not as great as seen with C7-176 and C7-183.

Other group A rotaviruses with abnormal genome profiles were isolated from chronically infected immunodeficient children (Pedley *et al.*, 1984). The RNA segments of increased size were derived from the segments of normal size by concatemer formation. When concatemeric segments were introduced into the UK virus by gene reassortment, Allen & Desselberger (1985) found that they performed the normal function of the replaced bovine rotavirus gene. Viruses with similar concatemeric segments were also produced by cell culture passage of UK rotavirus at high m.o.i. (Hundley *et al.*, 1985). This type of genomic rearrangement could explain the occurrence of the unusual genome segments seen in C7-176 and C7-183.

With methods now available for the molecular cloning and sequencing of rotavirus genomic segments (McCrae & McCorquodale, 1982*b*; Both *et al.*, 1982) and sequence data for segment 11 of both UK bovine and Wa human strains (Imai *et al.*, 1983; Ward *et al.*, 1985) it should be possible to determine the nature of the extra RNA joined to these unusual segments and these experiments are under way.

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