

THE MOLECULAR DIAGNOSIS OF *ECHINOCOCCUS MULTILOCULARIS* IN MAN

By

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ABSTRACT

The metacestode stage of the tapeworm *Echinococcus multilocularis* is the causative agent of the disease 'alveolar echinococcosis' (AE). The symptoms of the disease are often confused with those of hepatic carcinoma and correct diagnosis is usually only possible when the condition is well advanced, making this one of the most lethal parasitic infections known to man.

The antigens currently used in AE immunodiagnostic tests can contain cross-reactive components and are not readily available. Recombinant DNA techniques can provide a potentially limitless source of pure, well-characterised parasite antigens and, consequently, a metacestode stage cDNA library was constructed using the expression vector λ gt11 to provide requisite antigens. Differential screening with pools of human anti-*E. multilocularis* and anti-*E. granulosus* infection sera was performed and the immunodiagnostic potential of two putatively *E. multilocularis*-specific clones, designated EM2 and EM4, was assessed.

The fusion-peptides were purified under denaturing conditions and their testing with pools of human infection sera showed both to be putatively species-specific. However, when panels of individual human anti-*E. granulosus* and anti-*Taenia solium* infection sera were used to test the EM2 antigen, specificity was substantially reduced. Furthermore, testing with individual sera from proven *E. multilocularis* human cases showed that a number of these sera failed to recognise this antigen. Denatured and non-denatured EM4 β -gal fusion-peptide also demonstrated poor diagnostic sensitivity on testing with individual human anti-*E. multilocularis* infection sera. In an effort to improve this, the vector pGEX-1 was used to express the EM4 peptide as a glutathione-S-transferase fusion-peptide which could be readily purified by affinity chromatography using non-denaturing conditions. Subsequent testing of this fusion with panels of individual human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* infection sera showed that it demonstrated 100% diagnostic specificity but that sensitivity was not significantly improved.

The EM4 β -gal fusion-peptide was shown by immunoblotting to be recognised by mouse anti-*E. multilocularis* infection sera 42-70 days postinfection, indicating its potential for early diagnosis. The EM2 fusion

was not recognised 168 days postinfection.

Immunoblotting with anti-EM2 and anti-EM4 β -gal fusion-peptide antibody identified a 70 kDa native EM2 antigen and 62, 49 and 44 kDa excreted/secreted native antigens sharing EM4 epitopes. Further testing revealed that native antigens related to those of EM4 are present in taeniids other than *E. multilocularis*, despite the lack of anti-EM4 antibody in human anti-*E. granulosus* and anti-*T. solium* infection sera. In *E. granulosus*, a possible reason for this has been shown to be the lack of excretion/secretion of these antigens.

The genomic organisation of the EM2 and EM4 genes and its conservation has been assessed by Southern blot hybridisation and mRNA transcripts have been putatively identified by Northern blot analysis. Immunoprecipitation of *in vitro* translation products indicates that post-translational processing of a precursor molecule may give rise to the three EM4 native antigens. Furthermore, the full nucleotide sequence of the EM4 cDNA molecule has been obtained and the derived amino acid sequence has shown no significant homology with existing protein sequences.

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CHAPTER 1

1 INTRODUCTION

1.1 GENERAL

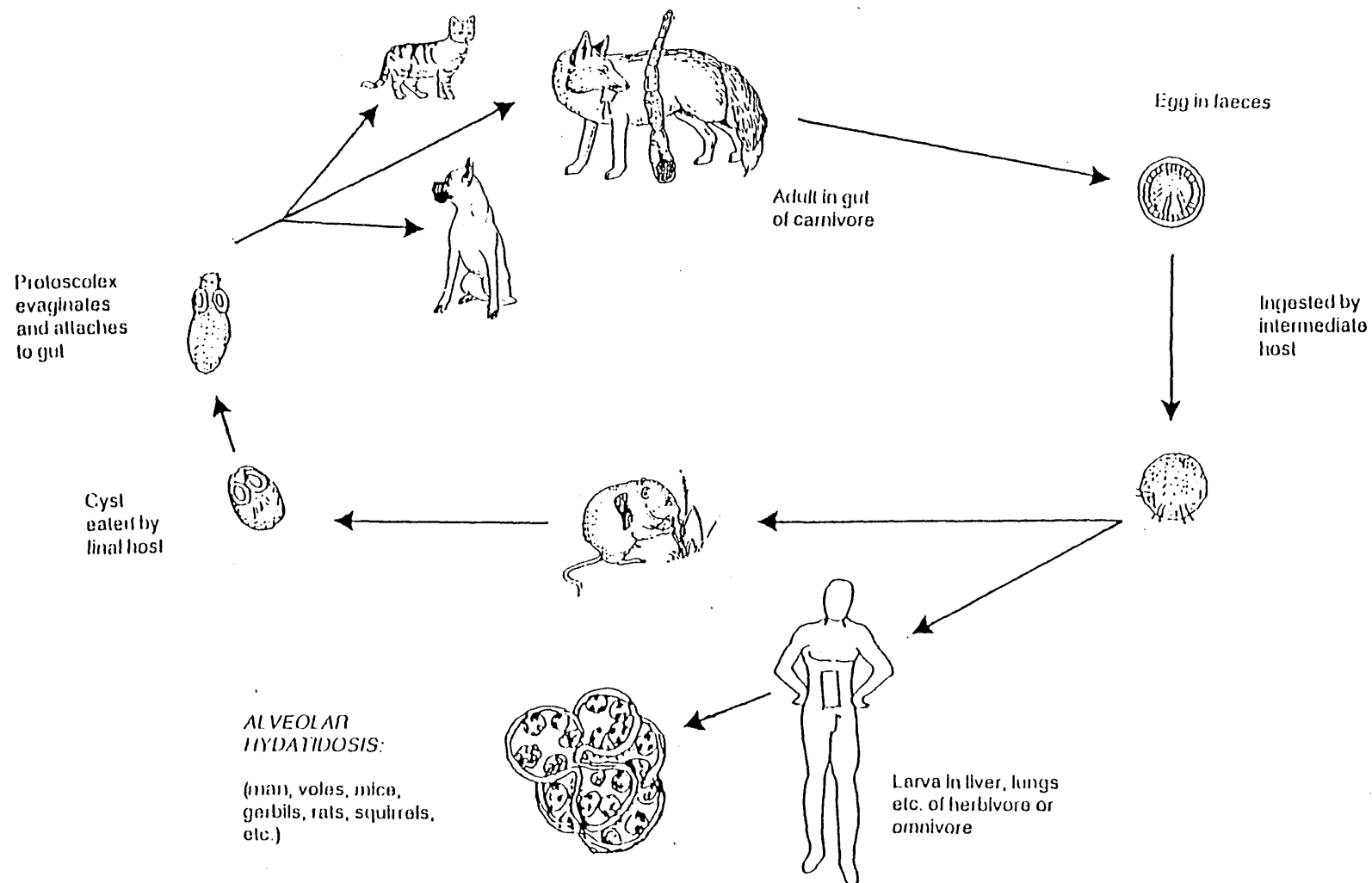
1.1.1 HISTORY, CLASSIFICATION AND DISEASE

Echinococcosis (hydatid disease), caused by the larval stages of *Echinococcus granulosus* and *E. multilocularis* has been recognised and treated since ancient times. It was described in the Talmud (200 AD), was known to Hippocrates and was treated by Artaeus, a Roman physician, by aspiration (Morris, 1986). *E. multilocularis*, *E. granulosus*, *E. oligarthrus* and *E. vogeli* are tapeworms belonging to the Class *Cestoda* and the Family *Taeniidae*. The species *E. multilocularis* and *E. granulosus* are the most extensively studied as the larval or metacestode stage of these organisms can develop within man to produce the disease conditions known, respectively, as 'alveolar' (AE) and 'cystic' (CE) echinococcosis.

1.1.2 LIFE CYCLE

The life cycle of *Echinococcus* spp. requires two hosts, a carnivorous definitive host and a herbivorous or omnivorous intermediate host. The definitive host of *E. multilocularis* can be a fox or domestic dog or cat (Fig. 1.1). The adult worm is only a few millimetres in length and is comprised of a specialised attachment organ or 'scolex', possessing suckers and hooks for fixing to the gut wall, and usually no more than five proglottids. Sexually mature proglottids self fertilise to produce eggs, each of which contains a single embryo. These eggs are passed out with the faeces and are infective to the intermediate host, which can be a small rodent or man. The egg hatches in the stomach releasing the oncosphere which is then activated within the small intestine. Penetration of the gut mucosa is facilitated by the action of special oncospherical hooks and by lytic secretions. On entering the circulation, transportation to the site of predilection occurs. This is primarily the liver where the metacestode begins development to form an alveolar cyst. In the natural cycle i.e. when

Figure 1.1 Life cycle of *E. multilocularis* (After McManus and Smyth, 1986).



man is not the intermediate host, protoscoleces are produced within the cyst, by means of asexual reproduction. These larvae originate from an internal germinal membrane that forms the innermost layer of the cyst wall. Protoscoleces are not usually present in human infections (see below, section 1.1.4). To complete the cycle, protoscoleces are ingested upon killing and eating of the intermediate host. They are at first invaginated and inactive but are then stimulated to evaginate by a number of largely unknown factors. Bile salts are, however, thought to be the major trigger (Smyth, 1967). The protoscoleces then become embedded within the crypts of Lieberkühn of the small intestine, where the adult worm develops (Thompson, 1986).

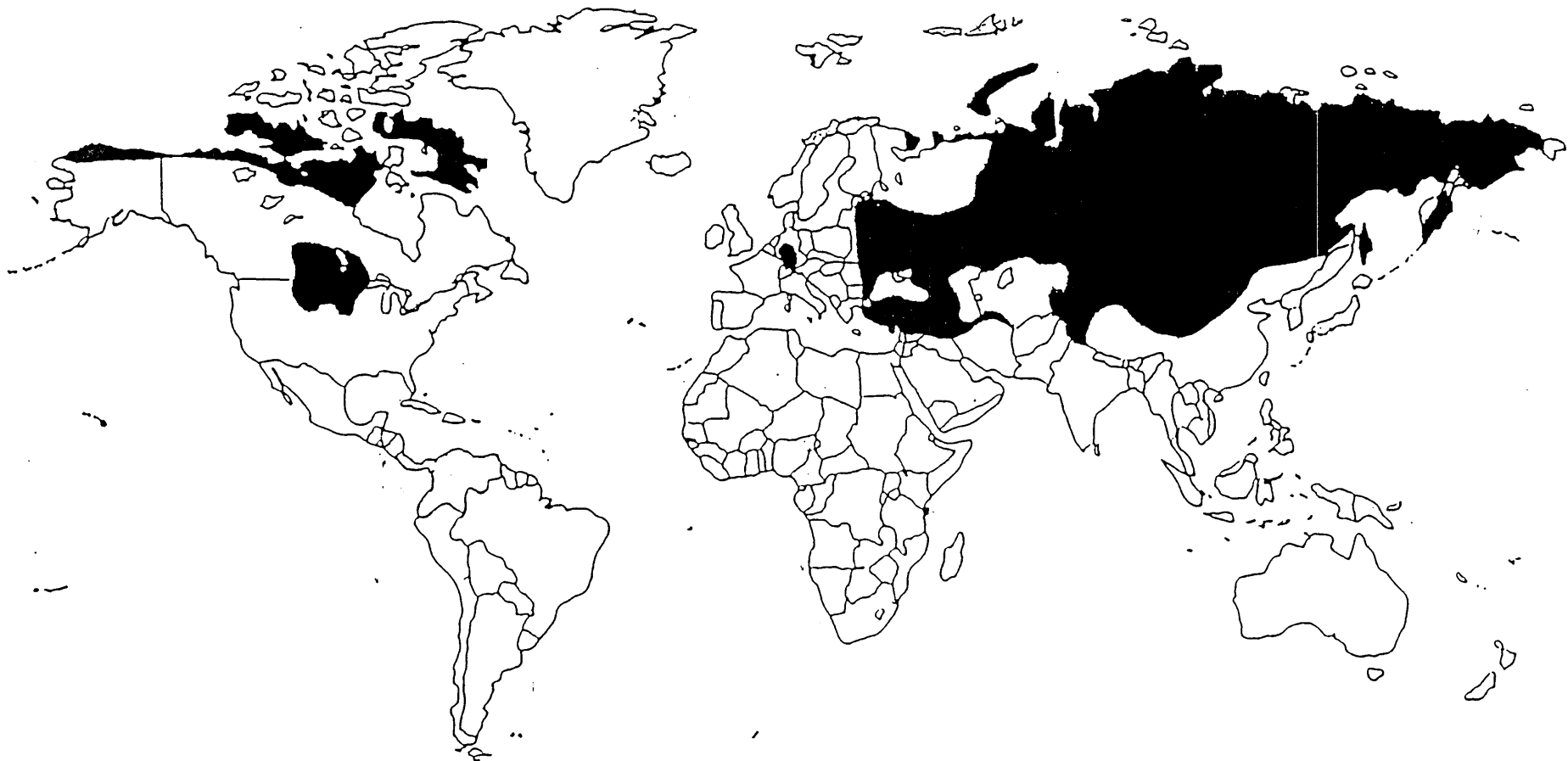
1.1.3 DISTRIBUTION

E. multilocularis is found only in the northern hemisphere (Fig. 1.2). It is responsible for cases of AE in parts of Russia, Alaska, northern Japan and central Europe (particularly western Austria, Switzerland, southern France and central/southern Germany). Reported findings of AE in China, U.S.A., India and Iran, countries which had previously been free of it, has indicated recent spread of *E. multilocularis* (McManus and Smyth, 1986). *E. granulosus* is often found within the same regions as *E. multilocularis* and this increases the need for species-specific reagents for use in routine diagnosis and sero-epidemiological surveys.

1.1.4 METACESTODE DEVELOPMENT IN MAN

The proliferation of the metacestode of *E. multilocularis* within certain vital organs of man precipitates the AE condition. The multivesicular cyst that develops infiltrates the host tissue and, due to the lack of a host-tissue barrier, forms an intimate association with the surrounding tissue. The cyst ramifies throughout the vital organ, usually the liver, often until the point is reached when organ dysfunction occurs, resulting in death. The larval mass is made up of numerous small vesicles which contain a semisolid matrix. Human cysts are usually sterile and contain few or no protoscoleces (Rausch and Wilson, 1973). Infiltrative

Figure 1.2 World distribution of *E. multilocularis* (After Schantz, 1984).
(■) areas where *E. multilocularis* is reported to occur.



growth is made possible by virtue of a network of filamentous, solid, cellular protrusions of the germinal layer, which transform into tube-like and cystic structures (Mehlhorn *et al.*, 1983). The proliferation of undifferentiated germinal layer cells results in simultaneous exogenous and endogenous growth. Germinal cells often detach from the infiltrating structures and are transported by blood or lymph to remote regions of the body e.g. the lungs or brain, where secondary cysts (metastatic foci), characteristic of AE, may develop (Eckert *et al.*, 1983).

1.1.5 THE INCIDENCE OF ALVEOLAR ECHINOCOCCOSIS

The occurrence of AE in predominantly rural areas makes reliable information regarding the number of cases difficult to obtain. However, in Switzerland, where AE is a well known public health problem, the incidence of AE has been well documented. Drolshammer *et al.* (1973) have reported an annual average of 8.7 new cases of AE diagnosed between 1956 and 1969. This amounted to a total of 122 cases, most of which were of Swiss nationality and within the 51 to 60 year old age group. More recently, 10 new cases of AE per annum, have been registered in Switzerland for the period 1970 to 1983 (Gottstein *et al.*, 1987 a).

1.1.6 THE CLINICAL MANIFESTATION AND TREATMENT OF ALVEOLAR ECHINOCOCCOSIS

The initial symptoms of AE are often non-specific, but epigastric pain with hepatomegaly which progresses to obstructive jaundice is a possible manifestation (Schantz and Gottstein, 1986). Diagnosis is usually only made when the condition is well advanced, often making the lesion inoperable. The consequence of this is that only 20 to 40% of cases are surgically resectable and, of the remaining non-resectable cases, 90% die within 10 years of diagnosis (Schantz and Gottstein, 1986). Drug therapy with mebendazole in human cases (Woodtli *et al.*, 1985; Morris, 1986) and experimental animals (Eckert, 1985) has been shown to have, at best, an inhibitory effect on metacestode growth. The use of currently available anthelmintics is, therefore, limited to prolonging the life of the patient.

The high degree of mortality resulting from *E. multilocularis* metacestode infection makes it one of the most lethal parasites known to man.

1.2 THE IMMUNOLOGY OF ALVEOLAR ECHINOCOCCOSIS

1.2.1 THE INTERMEDIATE HOST RESPONSE TO TAENIID INFECTION

The immune system of the intermediate host is exposed to a range of parasite antigens. The initial response is to the invading oncosphere, followed by a response to the developing metacestode. The oncosphere is a powerful immunogen (Schantz and Gottstein, 1986; Craig, 1988) and its early stimulation of the immune system would indicate the potential usefulness of oncospherical antigens for early diagnosis and, as studies on *Taenia* spp. have demonstrated (Rickard and Williams, 1982; Harrison and Parkhouse, 1986; Lightowlers *et al.*, 1986 a, b), for vaccination. The developing metacestode also stimulates the immune system, but, the degree to which it does so depends largely upon the species involved and the general characteristics and integrity of the cyst(s) (Schantz and Gottstein, 1986; Craig, 1988).

1.2.2 THE HUMORAL RESPONSE GENERATED BY *E. GRANULOSUS* AND *E. MULTILOCULARIS* METACESTODE INFECTIONS

E. granulosus human infection results in increased antibody production of the IgG, IgM, IgA and IgE classes (Craig, 1986). Also, circulating antigen (Gottstein, 1984; Craig, 1986) and immune complexes (Craig and Nelson, 1984) have been detected in the serum of CE patients. Similar immunological responses are found with *E. multilocularis* infections. An antibody response was apparent in laboratory gerbils which correlated with the size of the metacestode (Kroeze and Tanner, 1986). Furthermore, levels of IgM and IgG, *E. multilocularis*-specific antibody (Ali-Khan and Siboo, 1982) and circulating immune complexes (Ali-Khan and Siboo, 1983) were found to increase during the course of *E. multilocularis* infection in mice. It is well established that human *E. multilocularis* infection results in a powerful humoral immune response (Schantz and Gottstein, 1986) and a study of circulating and basophil-bound *E. multilocularis*-specific IgE

antibodies has been performed recently (Vuitton *et al.*, 1988).

Both AE and CE result in stimulation of the host immune system, but the metacestodes of the two species develop in dissimilar ways and each species possesses specific antigens. These differences may influence the exact nature of the immune response invoked in each case (Schantz and Gottstein, 1986; Craig, 1988). This can be exploited serologically to differentiate between the infections in man.

1.3 THE DIAGNOSIS OF ALVEOLAR ECHINOCOCCOSIS

1.3.1 NON-IMMUNOLOGICAL DIAGNOSIS

Several non-immunological methods have been used for the diagnosis of echinococcosis with varying degrees of success. These include clinical symptoms (carcinoma, cirrhosis), plain film roentgenography, angiography, computerised axial tomography (CAT), ultrasound scanning and the detection of larval elements. These techniques have been reviewed by Schantz (1984) and Schantz and Gottstein (1986). Their major limitations are late diagnosis, complexity, interpretation difficulties and cost. Immunological methods overcome many of these problems, and when fully developed, should provide more appropriate diagnosis.

1.3.2 IMMUNOLOGICAL DIAGNOSIS

1.3.2.1 Immunological tests

Extensive evaluation of all available serodiagnostic techniques has been performed with regard to echinococcosis diagnosis. The outcome of these evaluations has been reviewed by Schantz and Gottstein (1986). The enzyme-linked immunosorbent assay (ELISA) is one system which has been shown to demonstrate high levels of sensitivity. This test is based on the reaction of antigens or antibodies bound to a solid matrix with antibodies or circulating antigens, respectively, in patients' serum. The immune-complex formed is detected by a second antibody coupled to an enzyme which produces, in the presence of a suitable substrate, a colour reaction. The extent of this colour change is directly proportional to the level of

antibody or circulating antigen within the patient serum. This test is relatively simple to perform and the results are easy to interpret, important factors when considering the rural localities in which the test may have to be performed. A small amount of blood is all that is required from the patient and the reagents for the test are readily available and inexpensive. This technique also lends itself to the rapid, automated screening of large numbers of test samples. This technique could, therefore, provide an accurate means of early diagnosis of AE, when used in conjunction with suitable parasite antigens.

Despite the obvious advantages of this technique, such a system is at present beset by a number of problems which arise mainly from the type of antigen used with the test. When relatively crude antigen preparations are used, a large number of false-positive diagnoses are made, due to the high level of cross-reactivity demonstrated by such antigens. This is a particular problem when two or more parasitic diseases occur within the same geographical area, as is often the case with AE and CE. The identification and purification of species-specific antigens is, therefore, a necessary prerequisite for accurate diagnosis. A further problem is the poor availability of well characterised parasite material from which to extract these antigens. Both of these problems have to be surmounted before the full potential of immunologically based tests can be realised.

1.3.2.2 Species-specific antigens for immunodiagnosis

Attempts have been made to immunodiagnose various metacestode infections using crude antigen preparations and different assay techniques (see, for example, Dottorini *et al.*, 1983; Knobloch *et al.*, 1984; Yong and Heath, 1984; Pappas *et al.*, 1986; Rickard and Lightowlers, 1986; Hira *et al.*, 1987). Sensitivity has been achieved, but cross-reactivity of the antigens used has led to a low degree of specificity. In an effort to explain these effects, several workers have attempted to characterise the antigenic components (Gottstein *et al.*, 1986 a; Parkhouse and Harrison, 1987). Additional studies, discussed below, have led to the identification of species-specific antigens with potential for the immunodiagnosis of CE and AE. Problems exist, however, with regard to the large scale production of

these antigens, which can potentially be overcome by the use of recombinant DNA techniques.

1.3.2.3 Diagnostic antigens for cystic echinococcosis

Hydatid cyst fluid is a convenient source of antigen for *E. granulosus* as it contains a number of parasite components and is often present within cysts in large amounts which are easily accessible. Infected sheep are a useful source, and Gottstein *et al.* (1987 b) compared the protein constituents of fluid from various animal infections. For the detection of circulating antibody, antigen and immune complexes in human hydatid infections, relatively crude cyst fluid antigen has been used (d'Amelio *et al.*, 1983; Craig and Nelson, 1984; Gottstein, 1984; Craig, 1986). These tests were not on the whole intended to be diagnostic, thereby making the use of highly purified antigen unnecessary.

Lightowers *et al.* (1984), in studies on diagnosis of infected sheep, used cyst fluid depleted of certain components by affinity absorption as antigen. They found cross-reactions occurred despite removal of certain fluid components although non-specific background was reduced. Characterisation of cyst fluid components has been extensive (reviewed by Schantz and Gottstein, 1986), and more recent work includes that by d'Amelio *et al.* (1985), Gottstein *et al.* (1987 b) and Shepherd and McManus (1987). Two antigens, antigen 5 and antigen B, have been studied in particular with regard to immunodiagnostic potential. There has been, however, some confusion over which of these antigens is specific and which is conserved. This has now been resolved by Shepherd and McManus (1987) whose work indicates that antigen B is *E. granulosus*-specific and, due to the presence of an attached phosphorylcholine epitope, that antigen 5 is highly cross-reactive. Monoclonal antibodies have been raised against antigen 5 to aid in its characterisation by Di Felice *et al.* (1986) but their immunodiagnostic value has yet to be evaluated.

A monoclonal antibody has been produced against an *E. granulosus* oncospherical antigen (Craig *et al.*, 1986) which is genus-specific and hence may be used to identify, by immunofluorescence, *Echinococcus* eggs. These are morphologically identical to those of other taeniids. This monoclonal antibody may be of limited value, however, in

serodiagnosis where CE and AE overlap because distinguishing between *E. granulosus* and *E. multilocularis* infections would not be possible.

Work on *E. granulosus* antigens is, therefore, relatively advanced and potentially immunodiagnostic determinants have been identified. The application of these to serological diagnosis of CE is proving difficult, however, as the immune response generated by patients can vary widely depending upon several factors which include, 1) the viability, location and number of cysts (Schantz, 1984; Shepherd and McManus, 1987; Craig, 1988), 2) the presence of encapsulating cyst membranes which prevent antigen release into the host, resulting in a poor antibody response (Schantz and Gottstein, 1986; Craig, 1988), and 3) the titre of certain *E. granulosus*-specific antibodies may be reduced by immune-complex formation with circulating antigen (Craig, 1986; Shepherd and McManus, 1987).

Tests which can detect circulating antigen and immune-complex disruption techniques may help to overcome some of these problems (Gottstein, 1984; Craig, 1986).

1.3.2.4 Diagnostic antigens for alveolar echinococcosis

E. multilocularis infections, due to the intimate association of host and cystic material, without intervening membranes, generally produce high titres of parasite-specific antibodies in patient serum (Schantz and Gottstein, 1986; Craig, 1988). For this reason, the serological diagnosis of AE should present fewer sensitivity problems than that of CE.

The study of *E. multilocularis* antigens has, until recently, been limited, despite the serious consequences of the human infection. Preliminary work, using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, to identify the antigens which stimulate the immune response, was reported by Lingelbach and Hinz (1984). This work demonstrated that anti-*E. multilocularis* infection sera obtained from different host species recognised dissimilar parasite antigens. Four *E. multilocularis* antigens were shown to be recognised only by anti-*E. granulosus* and anti-*E. multilocularis* infection sera. They were, therefore, characteristic of *Echinococcus* spp. infection.

Perhaps the most significant advance towards producing an immunodiagnostic reagent for AE, to date, has been the isolation of a

species-specific antigen by affinity chromatography. Initial purification was achieved by passing a crude *E. multilocularis* extract through a rabbit anti-*E. granulosus* cyst fluid IgG column. The components which passed through the column were shown to demonstrate *E. multilocularis*-specificity when tested by ELISA (Gottstein *et al.*, 1983). The protein responsible for this specificity, designated Em 2a, was found to have a molecular weight of 54 kDa, an iso-electric point of 4.8 and its antigenicity was proven by immunoprecipitation and immunoblotting (Gottstein, 1985). The use of this antigen in an ELISA assay produced a 95% discrimination rate between AE and CE (Gottstein *et al.*, 1986 b) indicating its potential as an immuno-diagnostic reagent. Recently, it has also been shown (Rausch *et al.*, 1987) that this antigen can detect antibody in the sera of patients whose metacestodes have apparently undergone spontaneous death and which, consequently, present no further threat. This could have serious implications in terms of whether surgery or drug therapy would be necessary to remedy certain cases of AE. Consequently, additional tests would have to be performed in order to establish metacestode viability prior to treatment.

A species-specific antigen with diagnostic potential has, therefore, been successfully identified for *E. multilocularis*, but problems of antigen availability may be encountered when it is used routinely as a diagnostic reagent. This will necessitate consistent quality control of the antigen, a requirement which will be difficult to achieve using conventional purification techniques. The problems associated with classical purification methods can include, 1) lack of availability of well characterised parasite material, 2) achieving sufficient purity necessary to decrease the likelihood of false-positive reactions, 3) length of time required for purification, and 4) the technical difficulties encountered when large amounts of antigen are processed.

1.3.2.5 Recombinant DNA technology as a source of diagnostic antigens

An alternative approach for eliminating cross-reactivity and overcoming antigen availability problems has become available with the recent advances in molecular biology. The insertion of foreign genes into heterologous biological systems allows the synthesis of large quantities of defined proteins. The use of such systems can also facilitate the rapid

identification and isolation of the required protein and its corresponding gene.

The present study has exploited this technology in an effort to identify and synthesize *E. multilocularis*-specific antigens which may demonstrate immunodiagnostic potential in tests such as ELISA.

1.4 THE PROJECT AIMS

The primary objective of this project was to utilise molecular cloning techniques to allow the production of *E. multilocularis*-specific antigens which could be incorporated in immunodiagnostic tests, thereby eliminating the inherent problems associated with crude antigen preparations currently in use. A further aim was to characterise cloned antigens at both the protein and genetic level. This would provide useful information regarding the biology of the parasite and its interaction with the human host. In order to fulfil these aims the following steps were employed:

- 1) *E. multilocularis* antigen genes were cloned in a suitable expression system which allowed the preliminary identification and isolation of putatively *E. multilocularis*-specific antigen gene clones and their expressed peptides.
- 2) The immunodiagnostic potential of the expressed peptides was assessed with regard to their use in assays such as ELISA and methods for improving their efficacy as diagnostic reagents were explored.
- 3) Characterisation of the native parasite proteins corresponding to the cloned antigens was performed to allow their preliminary identification and localisation. The antigens were also characterised at the immunological, protein and genetic levels. This included studies regarding the genomic organisation of the antigen genes and nucleotide sequencing of a putatively diagnostic clone.

CHAPTER 2

2 THE CLONING AND EXPRESSION OF PUTATIVELY SPECIFIC E. MULTILOCULARIS ANTIGEN GENES

2.1 INTRODUCTION

2.1.1 THE EXPRESSION OF FOREIGN GENES USING MOLECULAR CLONING TECHNIQUES

2.1.1.1 General

The advent of molecular cloning technology has now made it possible to express desired gene sequences within either prokaryotic or eukaryotic systems (Shatzman and Rosenberg, 1987; Bitter, 1987; Cullen, 1987). This methodology can facilitate the production of unlimited amounts of a defined foreign protein which can be purified free of other protein contaminants. The characteristics of the protein produced will remain the same unless deliberate changes are introduced to the system. Such systems are, therefore, particularly suitable for the production of serodiagnostic reagents as they can overcome the problems of antigen availability and variability. Furthermore, as single defined genes are expressed, problems caused by cross-reactive components in crude extracts are often eliminated.

The choice of expression system used depends largely upon the objectives of the work being undertaken. Genes from an extremely diverse range of organisms have now been expressed successfully in *E. coli*, yeast and mammalian cells, the fundamentals of which have been described by Shatzman and Rosenberg (1987), Bitter (1987) and Cullen (1987), respectively.

2.1.1.2 Eukaryotic systems

Several factors have to be taken into account when deciding upon the most appropriate system to use. Eukaryotes, namely yeast and especially mammalian cells, are capable of expressing foreign peptides in such a way as to allow almost completely faithful reproduction of the

native protein as it would be synthesized within its native cell type (Bitter, 1987; Cullen, 1987). These systems, although appropriate for certain specialised applications, such as the functional analysis of cloned genes, are not well suited to some studies on account of their relative complexity. Prokaryotic systems, however, despite their shortcomings with regard to the quality of the protein produced, are more applicable to certain studies due to their simplicity and the fact that they have been extensively characterised. These systems also facilitate the initial cloning, identification and isolation of target genes, which can be expressed subsequently using more complex systems, if necessary.

2.1.1.3 Prokaryotic systems

Considering the objectives of the current study, it was not envisaged that extensive processing (glycosylation, correct folding, etc.) of the cloned antigen genes would be necessary initially. The antigen genes were to be isolated solely on the ability of their expressed products to react with human infection sera and this criterion could be met using the more basic prokaryotic systems. Consequently, this type of system was thought to be best suited to this work and, of the systems available, *E. coli* has been the most widely used and characterised (Shatzman and Rosenberg, 1987).

E. coli systems have been designed to allow the insertion of the DNA sequence of interest into a multicopy vector system, usually a plasmid, which directs efficient transcription and translation of the desired coding sequence. Most foreign genes do not contain the signals required for ribosome recognition and translation initiation in *E. coli*. These must be provided, therefore, by either linking the gene directly to an *E. coli* ribosome binding site, or by fusing the foreign sequence to that of an existing bacterial structural gene. The latter approach is the most common, and it results in the production of a chimeric protein, consisting of a portion of the *E. coli* protein fused to the foreign protein. This hybrid product is referred to as a fusion-peptide.

Many foreign protein products can be lethal to *E. coli*, the expression of the inserted gene must, therefore, be under tight regulatory control. Bacterial promoter/operator sequences which can direct high levels

of transcription and which can be readily turned on or off are utilised in order to overcome this problem. The most favoured systems are, 1) the leftward promoter of the lambda bacteriophage, which is controlled by the temperature-sensitive c1857 repressor, 2) the trp promoter, which can be induced with indoleacrylic acid (IAA), 3) the lac promoter, which can be induced with isopropylthiogalactopyranoside (IPTG), and 4) the tac promoter, constructed from the trp and lac promoters, which can direct very high levels of expression and is also induced by IPTG (McIntyre *et al*, 1986).

2.1.1.4 Parasite gene expression in *E. coli*

Several *E. coli* vectors have been used to clone and express parasite antigens (McIntyre *et al*, 1986). The use of immunological screening techniques has meant that antigen genes can be rapidly identified within gene expression libraries without the need for any preliminary characterisation or knowledge of the cloned DNA sequences (Shatzman and Rosenberg, 1987; Stahl *et al*, 1984). Because of the lack of data available for *E. multilocularis* antigens, such an approach was well suited to the isolation of cloned *E. multilocularis* antigen genes. These could then be expressed, using the same or a related system, providing preparative amounts of recombinant antigens for development as immunodiagnostic reagents.

Only one *E. multilocularis*-specific antigen (Em 2a) has been identified to date (Gottstein *et al*, 1983). It may be possible to isolate an antigen-gene clone corresponding to this protein from an expression library using specific antiserum but, for reasons that will be discussed later, this may not be feasible. In order, therefore, to identify at random uncharacterised, species-specific antigen genes, a large *E. multilocularis* gene expression library needed to be constructed which would contain as many parasite gene sequences as possible. Lambda bacteriophage is the vector of choice as the inherent efficiency of such a system, relative to that of plasmids, when introducing recombinant vector DNA molecules into *E. coli* cells, is much greater. Of the *E. coli* expression systems so far developed, the ones which satisfy this criterion best are bacteriophage lambda gt11 (λ gt11) and its derivative λ gt11-amp3.

2.1.2 THE λ gt11 EXPRESSION SYSTEM

The expression vector λ gt11 was constructed by Young and Davis (1983) to provide a system that would allow the use of antibody probes to detect certain sequences of cloned DNA. The *E. coli* β -galactosidase (β -gal) *lacZ* structural gene has been spliced into the lambda genome. This allows the insertion of foreign gene sequences at an Eco R1 cloning site in the C-terminal region of β -gal resulting in the production of a β -gal fusion-peptide. Gene expression is directed and controlled by the *lac* promoter/operator which controls normal β -gal expression in *E. coli*. Expression can, therefore, be stimulated by the addition of IPTG. The vector was designed to allow the production of temperature sensitive lysogens of *E. coli* using certain *hflA* (high-frequency lysogeny) and *lon*⁻ (*lon* protease-deficient) mutant cells to facilitate immunoscreening and allow synthesis of preparative amounts of fusion-peptide. Non-recombinant lysogens (*lac*⁺) produce active β -gal which can be detected using the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).

An apparent problem with this system, however, is the occurrence of *lac*⁻ residual non-lysogenic colonies which grow alongside genuine *lac*⁻ recombinant lysogens (Kemp *et al.*, 1983). These may arise due to the unstable nature of some recombinant lysogens (McIntyre *et al.*, 1986). In an effort to overcome this, Kemp *et al.* (1983) modified the λ gt11 vector by inserting an ampicillin-resistance (*Amp*^r) gene, excised from pBR322, to produce the vector λ gt11-amp3. This conferred *Amp*^r to genuine lysogenic colonies, allowing their differentiation from non-lysogenic ampicillin-sensitive colonies which died in the presence of ampicillin.

A drawback with both of these systems has been the repeated failure of certain recombinants to form stable lysogens. This has now been alleviated by the immuno-screening of plaques as opposed to lysogenic colonies, which removes the need for lysogen construction (Huynh *et al.*, 1985). This form of screening also allows a greater number of recombinants to be probed simultaneously (Maniatis *et al.*, 1982).

Any advantage the λ gt11-amp3 system has over that of the original λ gt11 vector is largely nullified when a plaque immunoscreening strategy is adopted. The λ gt11 system has also been the most widely used

and characterised and it has now become commercially available. These factors made this system the most appropriate for use in this study.

2.1.3 THE STUDY OF PARASITE ANTIGENS USING λ gt11 AND OTHER VECTORS

The λ gt11 expression system has been used extensively to study the antigens of many different parasite species with regard to vaccine candidates and diagnostic reagents. Most work has been on the former, examples of which are the cloning of protective antigens of: *Schistosoma mansoni* (Balloul *et al.*, 1987; Havercroft *et al.*, 1988), *Brugia malayi* (Nilsen *et al.*, 1988), *Plasmodium falciparum* (Hori *et al.*, 1988) and *Eimeria tenella* (Profous-Juchelka *et al.*, 1988), to name but a few. With regard to immunodiagnosis, fewer studies have been reported, examples of this type of work, where potentially diagnostic antigens have been cloned, are: *Schistosoma mansoni* (Klinkert *et al.*, 1987; Newport *et al.*, 1988), *Leishmania donovani* (Blaxter *et al.*, 1988) and, most recently *E. multilocularis* (Vogel *et al.*, 1988).

The application of molecular cloning techniques to the study of cestodes has been extremely limited. To date, a *Taenia taeniaeformis* λ gt11-amp3 cDNA library has been constructed (Bowtell *et al.*, 1984) and *T. ovis* genes have been cloned into the pEX1/2/3 series of expression plasmids (Howell and Hargreaves, 1988) in order to facilitate vaccine development.

With regards to *E. multilocularis*, a recent study has described the construction of a λ gt11 library and preliminary identification of potentially immunodiagnostic antigen gene clones (Vogel *et al.*, 1988). The objectives of that study, relating to the isolation of recombinant diagnostic reagents, closely paralleled several of those relevant to the current project.

2.1.4 THE CONSTRUCTION OF AN *E. MULTILOCULARIS* cDNA LIBRARY IN λ gt11

2.1.4.1 Source of parasite genes

Fragments of genomic DNA or cDNA synthesized from mRNA isolated from a particular *E. multilocularis* life-cycle stage were considered for library construction. The use of genomic DNA has the

advantage that all DNA sequences are represented. The major disadvantage of this type of library is that many cloned genes do not code for antigens, and those that do may only contain fragments of the entire gene. Intergenic gene sequences (introns and non-coding DNA) are also cloned along with the genes of interest. This may have deleterious effects upon the expression of certain genes and can complicate the characterisation process of the cloned sequences (McIntyre *et al.*, 1986). The advantages of cloning cDNA are, 1) entire gene sequences without extraneous fragments can be cloned, allowing expression of entire peptides in a form more closely resembling that of the native protein, 2) the characterisation of clones of interest is simplified, and 3) subsets of genes that are expressed at particular life-cycle stages can be selected (McIntyre *et al.*, 1986). The cloning of *E. multilocularis* cDNA was, therefore, thought to be the most suitable approach towards this work.

The choice of life-cycle stage from which to isolate mRNA depended upon which stages were immunogenic to the human host and the nature of the immune response generated by each stage. Following ingestion of *E. multilocularis* eggs, the human immune system is exposed sequentially to two life-cycle stages, the invading oncosphere, followed by the developing metacystode cyst mass (Schantz and Gottstein, 1986). In order to clone those antigens which stimulate an early immune response, which might be advantageous for immunodiagnosis, it would have been appropriate to construct an oncospherical cDNA library. Anti-oncospherical antibodies have been detected 1 week postinfection in sheep infected with *T. ovis* and *T. hydatigena* (Craig and Rickard, 1981; 1982). This work revealed, however, that the immune response, although rapid, was not species-specific. Certain oncospherical antigens may not, therefore, be suitable for use as diagnostic reagents. The use of oncospheres as a source of clonable material also has limitations of a technical nature. The inherent danger of working with infective oncospheres in the laboratory is a major drawback and the production of sufficient oncospherical material also presents additional problems. Adult worms, when allowed to reach sexual maturity, may provide a suitable source, but maintenance of this life-cycle stage would not be convenient. The most readily available and appropriate source of species-specific antigen genes is, therefore, the developing metacystode larval mass. This life-cycle stage may also possess some shared oncospherical antigens,

which could stimulate both an early and species-specific immune response in the human host, as it is known that some antigens are shared by different life-cycle stages (Schantz and Gottstein, 1986; Craig, 1988).

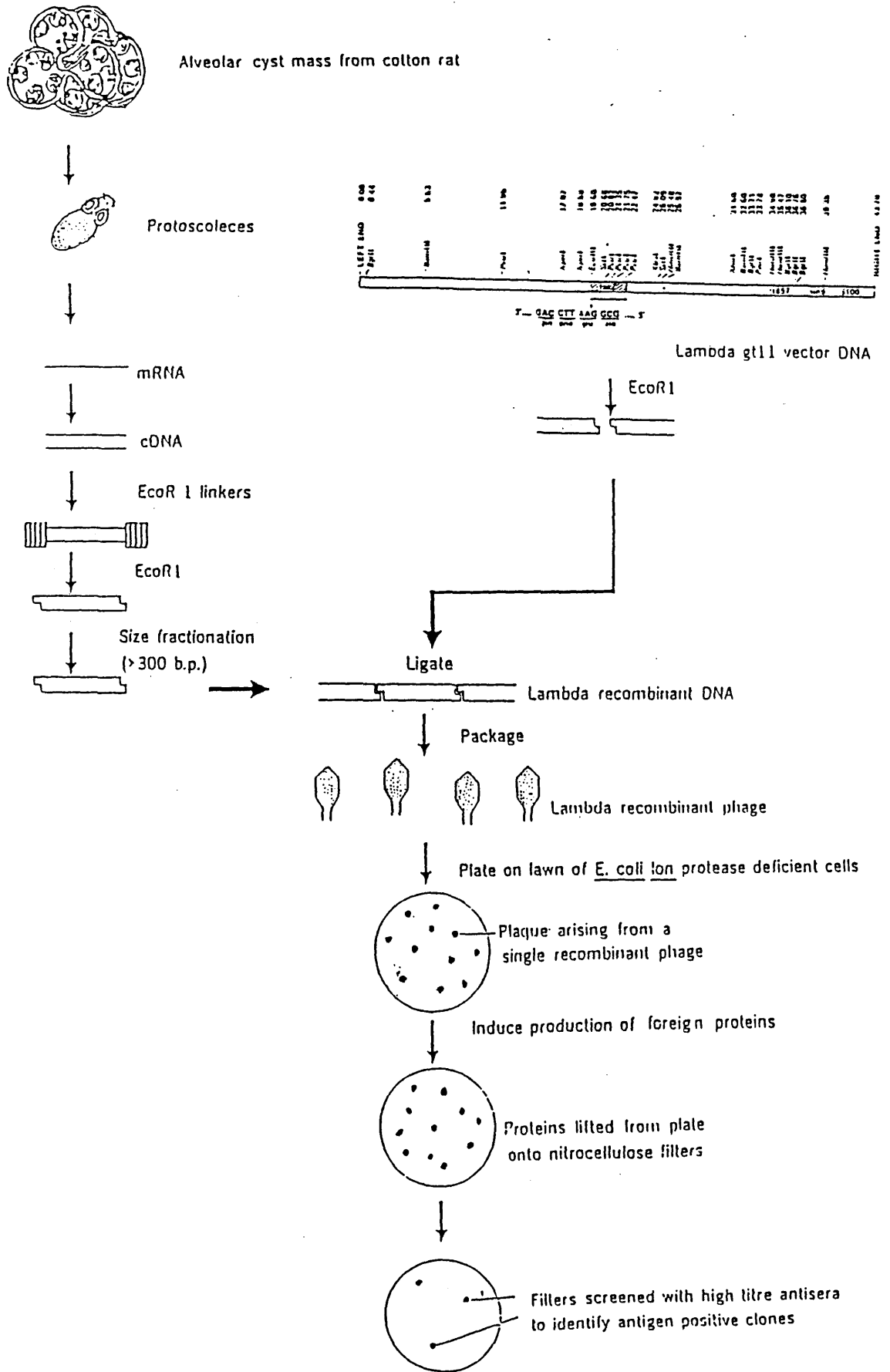
E. multilocularis metacestode material can be maintained in the laboratory by the intraperitoneal passage of cyst material in cotton rats (*Sigmodon hispidus*) (Lubinsky, 1960). The alveolar cyst material when removed, however, is heavily contaminated with host tissue and parasite RNA cannot, therefore, readily be extracted from this crude material. The protoscoleces present within the cyst matrix can, however, be isolated and purified by treatment with pepsin, to remove all host tissue contaminate (McManus and Smyth, 1978; McManus and Barrett, 1985). Purified protoscolex material is, therefore, the most convenient source from which to extract RNA for subsequent cDNA synthesis and was employed in this study.

2.1.4.2 cDNA cloning

Having available a suitable source of parasite material from which to extract RNA, the λ gt11 library was constructed. The strategy used is outlined in Figure 2.1. The first stage was the synthesis of cDNA. The classical method of cDNA synthesis, following synthesis of the 1st strand by reverse transcriptase and the 2nd strand by DNA polymerase 1, uses single-strand specific nuclease S1 digestion to remove the 3' hairpin loop joining the two strands of the cDNA molecule (Efstratiadis *et al.*, 1976). This procedure results in the removal of some of the 3' cDNA sequence, preventing the cloning of 'full-length' molecules. More recently a technique has been used which does not rely on S1 nuclease digestion. This procedure utilises partial digestion of the RNA in the DNA/RNA hybrid, produced by 1st strand synthesis, by ribonuclease H to create fragments which prime the synthesis of the 2nd DNA strand (Watson and Jackson, 1985). This process potentially allows the synthesis and cloning of full-length cDNA molecules and, hence, was adopted for this cloning work.

Following their synthesis, the cDNA molecules were processed by a number of standard molecular biological techniques, to allow their insertion into the λ gt11 vector. These procedures have been described by Huynh *et al.* (1985). In short, small oligonucleotide linker DNA sequences were placed at the ends of the cDNA molecules, which facilitated their

Figure 2.1 Outline of the *E. multilocularis* cDNA λ gt11 cloning strategy.



cloning into the Eco R1 site of λ gt11. The recombinant DNA was then packaged to form infective phage particles, which constituted the library. These DNA sequences were introduced into suitable *E. coli* cells by virtue of the infectivity of the phage, allowing expression of the *E. multilocularis* cDNA sequences.

2.1.5 THE ISOLATION OF PUTATIVELY *E. MULTILOCULARIS*-SPECIFIC ANTIGEN GENE CLONES

2.1.5.1 Screening of the library

The screening procedure for λ gt11 expression libraries, using plaques as opposed to lysogenic colonies, has now become well established and is described by Huynh *et al.* (1985). Briefly, recombinant λ gt11 phage are plated out on *lon* protease deficient host cells, where they are allowed to infect a sufficient number of cells prior to induction of expression by the addition of IPTG. Having induced expression of the foreign gene sequences, β -gal fusion-peptides are synthesized within the *E. coli* host cells which are liberated upon cell lysis. These fusion-peptides (along with other *E. coli* and λ gt11 proteins) are transferred to a solid support, usually a nitrocellulose filter. This filter is probed with antibody, which, if bound by the fusion-peptide, can be detected by a second antibody. This can either be radioactively labelled or coupled to an enzyme capable of producing a colour reaction in the presence of a suitable substrate. This reveals the location on the filter of phage plaques which are producing the desired fusion-peptide. Reorientation of the filter to the original plaque lawn facilitates isolation of the relevant recombinant clones.

The most important component of this technique is the antibody probe, which may be a highly-specific polyclonal or monoclonal antiserum raised to a single protein (or one of its' epitopes), or a polyspecific infection serum which contains a complex mixture of cross-reactive and specific antibodies. The type of antibody probe used is dictated by the objectives of the work and the constraints imposed by the heterologous expression of the desired protein(s).

2.1.5.2 The isolation of a recombinant Em 2a antigen with specific antiserum

To date, only one *E. multilocularis*-specific antigen (Em 2a) has been identified and tested for use as an immunodiagnostic reagent (Gottstein *et al.*, 1983; Gottstein, 1985; Gottstein *et al.*, 1986 b). This antigen has proved to be a good candidate for diagnosis, but it was isolated by classical methods, which are both time consuming and require the availability of well characterised *E. multilocularis* and *E. granulosus* parasite material. Expression of this antigen by *E. coli* would, therefore, be highly desirable. In order to accomplish this, an *E. multilocularis* expression library could be probed using a specific antiserum raised to Em 2a. Despite the apparent simplicity of such an approach, there are several reasons why this strategy may not be effective. Primarily, if an antigen is cloned and subsequently expressed in a prokaryotic system as a fusion-peptide, its antigenicity may be altered to such an extent as to make it unsuitable as a diagnostic tool and this may even render its isolation from the library impossible. These changes could be attributable to the inability of the system to recreate carbohydrate epitopes, incorrect folding and masking of certain epitopes by the fusion-peptide carrier protein or, the cloning of cDNA sequences which are not of full length and, therefore, do not code for the complete antigenic peptide. Some of these problems could be alleviated by the use of eukaryotic expression systems or the expression of the antigens without fusion to carrier proteins, but the drawbacks of this have been discussed earlier. Consequently, an alternative approach was sought which would allow the isolation of putatively *E. multilocularis*-specific antigen genes from a λ gt11 library without the need for an antigen-specific antiserum.

2.1.5.3 The isolation of putatively *E. multilocularis*-specific recombinant antigens by 'differential screening'

The *E. multilocularis*-specific fusion-peptide antigens synthesized using λ gt11 should be able, when incorporated into immunodiagnostic tests, to differentiate between infection sera from AE and CE patients. The most direct way, therefore, of ensuring the diagnostic species-specificity

and antigenicity of the fusion-peptides would be to identify the recombinant clones using human infection sera. Such an approach is known as 'differential screening' and was pioneered by Stahl *et al.* (1984) for the analysis of *Plasmodium falciparum* antigen genes cloned in λ gt11. This approach has since been used by Knight *et al.* (1986), resulting in the successful isolation of putatively species-specific *Schistosoma mansoni*, *S. japonicum* and *S. haematobium* λ gt11 cloned antigens. These workers used mouse infection sera, as opposed to human, for screening purposes.

Differential screening with human sera requires the availability of well characterised infection sera from patients suffering from the disease which is to be diagnosed and others with closely related infections. In regard to this work, sera from *E. multilocularis* and *E. granulosus* infected patients were used.

A potential drawback of this approach is the variation in antibody titre of individual patients to different parasite antigens. This results from variation in the location, integrity and viability of the larval cyst(s) (Schantz, 1984; Shepherd and McManus, 1987; Craig, 1988) and immune-complexing of antibody with circulating antigen (Craig, 1986; Shepherd and McManus, 1987). Patients undergoing drug therapy may also have a particularly atypical antigen recognition profile (Gottstein *et al.*, 1984). These effects could result in the isolation of a cloned species-specific antigen recognised by a small number of individuals infected with *E. multilocularis*. This would render the antigen, if used alone, unsuitable for use as a diagnostic reagent. In an effort to overcome this problem, two pools, each consisting of several individual infection sera, were used for screening. It was hoped that this would tend to dilute out minor individual variations in antibody repertoire while maintaining the titre of those antibodies directed towards consistently recognised antigens. Preliminary testing of the antigen recognition profile of individual sera, together with the analysis of patient medical histories with regard to surgery and drug treatment, prior to the formation of serum pools, was performed to further reduce the possibility of isolating inconsistently recognised antigens.

An approach, based on differential screening, potentially also allows several putatively specific clones to be isolated simultaneously. Several species-specific recombinant antigens could be used together in an immunodiagnostic test. This may be an important factor in overcoming the

problems of individual variation in antibody titre to particular antigens, which could lower the sensitivity of certain cloned immunodiagnostic reagents. Furthermore, differential screening allows rapid, preliminary identification of putatively species-specific antigens without the need for their prior purification or characterisation (Stahl *et al*, 1984).

2.1.5.4 The confirmation of diagnostic *E. multilocularis*-specificity of recombinant antigens

In order to confirm the diagnostic specificity of the cloned antigens identified by differential screening and to test their suitability as diagnostic reagents, they must be amenable to preparative synthesis by *E. coli*. This can be achieved using λ gt11 by constructing lysogens of *E. coli* with the recombinant phage. This allows large quantities of fusion-peptide to be produced which can then be subjected to immunological testing. Specificity of the cloned antigens can be assessed by testing with pools of human sera from diverse parasitic infections. The immunodiagnostic potential of those antigens which demonstrate putative species-specificity can then be determined by extensive testing with panels of individual human infection sera from a range of parasitic infections. This particular aspect of the work is considered largely in the following chapter.

For the reasons given above, the differential screening of a λ gt11 *E. multilocularis* cDNA library with human infection serum pools was the approach taken for the initial identification and production of putatively species-specific recombinant antigens.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

2.2.1.1 Parasite material

E. multilocularis was maintained in laboratory-reared cotton rats by intraperitoneal passage (Lubinsky, 1960).

Crude *E. multilocularis* antigen extract (EmAgA) (cotton rat cysts) was prepared from whole cyst material by one freeze-thaw cycle, sonication for 2 x 30 seconds at maximum amplitude (MSE Soniprep 150) and 5 strokes in a dounce homogeniser.

E. granulosus sheep cyst fluid antigen (EgSCF) (aspirated from abattoir-collected unilocular hydatid cysts) was clarified by centrifugation at 4 000 g for 15 minutes at 4 °C and concentrated in an Amicon ultrafiltration cell (Amicon Corp., USA) over a YM2 (1 kDa cut-off) membrane.

2.2.1.2 Antisera

Human anti-*E. multilocularis* sera were obtained from 13 confirmed French AE patients. No clinical information was available for these patients, although all sera were known to be of high antibody titre. The human anti-*E. granulosus* sera were obtained from Kenyan (9 individual sera and a pool), U.K. (3 individual sera) and Spanish (a pool of 15 sera) confirmed CE patients. None of the Kenyan patients had been drug-treated but some had undergone surgery to remove cysts. One U.K. patient was known to have started albendazole chemotherapy and another had undergone surgery prior to collection of the serum. The other U.K. patient had also undergone surgery, but the status regarding chemotherapy was unknown. No information regarding the individual sera in the Spanish pool was available.

The human anti-*T. solium* serum pool was made up from confirmed, high titre, cysticercosis patient sera (19 Zimbabwean and 4 Mexican sera). The human anti-*Ascaris lumbricoides*, anti-*Brugia malayi*, anti-*Onchocerca volvulus*, anti-*Schistosoma haematobium* and anti-*S. mansoni* serum pools consisted of individual sera from various endemic regions. Normal human serum (NHS) was a pool of 4 sera from U.K. laboratory workers.

Rabbit anti-*E. multilocularis* (EmAgA) serum was raised by subcutaneous injection of 250 µg EmAgA protein in Freund's complete adjuvant as primary inoculation, followed, after 14 days, by a secondary boost in Freund's incomplete adjuvant. Subsequent booster inoculations of 5 mg parasite protein in PBS [150 mM NaCl/16 mM Na₂HPO₄/4 mM NaH₂PO₄/pH 7.3] were administered at 14 day intervals (Johnstone and Thorpe, 1982). Antibody titre was tested by ELISA using EgSCF as antigen.

Mouse antisera were raised to native β-gal (Sigma) and electroeluted β-gal fusion-peptides as above using 50 µg protein for each inoculation at 7 day intervals. Antibody titre was tested by ELISA using native β-gal as antigen.

Protein concentrations were estimated by the Bradford method (Bradford, 1976).

2.2.2 METHODS

2.2.2.1 The construction of the *E. multilocularis* λgt11 cDNA expression library

2.2.2.1.1 Extraction of total RNA

Prior to all procedures involving RNA, stringent precautions were taken to remove the possibility of RNA degradation by contaminating ribonucleases. This involved the treatment of solutions and equipment with diethylpyrocarbonate (Maniatis *et al.*, 1982), the baking of glassware for 9 h at 180 °C and the wearing of disposable gloves.

Total RNA was isolated from *E. multilocularis* protoscoleces using a variation of the method described by Chirgwin *et al.* (1979). Metacestode material was removed aseptically from an infected cotton rat and homogenised in a blender (2 × 10 seconds, max r.p.m). The resulting homogenate was sieved through a fine mesh by washing repeatedly with Hanks' saline to separate protoscoleces from the cystic tissue. Host tissue was removed from the protoscoleces by digestion at 37 °C for 1 h with 0.5% (w/v) pepsin/Hanks'/pH 2.0 followed by further 4 × 10 minute rinses in Hanks' medium (McManus and Smyth, 1978; McManus and Barrett, 1985). The viability of protoscoleces was monitored throughout by observing motility under a light microscope. Many protoscoleces were seen to evaginate, this

may have been accompanied by a corresponding transcriptional activation of some genes. Therefore, the repertoire of mRNA present in pepsinised protoscoleces may differ from that found in unactivated protoscoleces within the alveolar cyst. 1 ml aliquots of packed protoscoleces were either stored under liquid nitrogen or processed immediately.

4-6 volumes of lysis solution [5 M guanidine thiocyanate (Fluka)/0.01 M ethylenediaminetetraacetic acid (EDTA)/0.05 M Tris-HCl pH 7.6/5% (v/v) 2-mercaptoethanol (2-me)] was added to 1 ml of protoscoleces in a dounce homogeniser and the tissue was rapidly disrupted with 20 strokes of the probe. The resulting mixture was sonicated for 5 × 5 seconds at maximum amplitude. The sonicate was centrifuged at 8 000 g for 20 minutes at ambient temperature, the supernatant made to 4% (w/v) sarkosyl/0.5 g ml⁻¹ caesium chloride and layered onto a 1.5 ml cushion of 5.7 M CsCl₂/0.1 M EDTA in Beckman SW 50.1 ultracentrifuge tubes. The gradients were centrifuged at 130 000 g for 15 h at 20 °C in an SW 50.1 swing-out rotor. The supernatant was removed and the RNA pellet resuspended in sterile distilled water (SDW). This was extracted with an equal volume of butanol: chloroform (4 : 1) followed by an ether extraction, any remaining insoluble material in the aqueous phase being removed by centrifugation at 15 000 g for 10 minutes in a microcentrifuge (MSE). The RNA was ethanol precipitated with 0.3 M sodium acetate and 2.5 × vol absolute ethanol at -70 °C for at least 30 minutes. Centrifugation at 15 000 g for 20 minutes at ambient temperature recovered the RNA as a pellet which was dried briefly under vacuum and resuspended in SDW. The concentration and purity was estimated by OD_{260/280nm} absorbance readings (an OD_{260nm} absorbance value of 1.0 was taken to be equivalent to a nucleic acid concentration of 50 µg ml⁻¹. An OD_{260nm}/OD_{280nm} ratio of approximately 2.0 was indicative of sufficient purity). The yield from 1 ml of packed protoscoleces was approximately 500 µg of RNA.

2.2.2.1.2 Partial purification of mRNA

This was performed in order to reduce the number of recombinant clones encoding ribosomal genes. One cycle of poly(A)⁺ RNA enrichment was achieved using 'Hybond' messenger affinity paper (Amersham Int. code no: RPN.1513) and the conditions recommended by the manufacturer. 50 µg of message-enriched RNA was recovered from 500 µg of total RNA.

2.2.2.1.3 cDNA synthesis

The protocol and reagents for cDNA synthesis using the ribonuclease H method (Watson and Jackson, 1985) were supplied by Amersham International (cDNA synthesis kit, code no: RPN.1256). 1 µg of message-enriched RNA was used as substrate for the 1st strand reaction. 1st strand synthesis by reverse transcriptase was primed using oligo dT. 5 µCi of [α - ^{32} P] dCTP (Amersham Int.) was included in the reaction, this allowed radiolabel to become incorporated into the cDNA strand, enabling the reaction process to be monitored. Following removal of an aliquot of the completed 1st strand reaction for gel electrophoresis, the 2nd strand reaction was performed. Ribonuclease H was used to partially degrade the RNA within the RNA/DNA hybrid product of the preceding reaction. The short fragments of undegraded RNA annealed to the 1st strand DNA then primed the synthesis of the 2nd strand by DNA polymerase I. 50 µCi of [α - ^{32} P] dCTP, a 10 fold increase in the amount added to the 1st strand reaction, was included to facilitate monitoring of the reaction. Following 2nd strand synthesis, an aliquot, equivalent to that removed from the first strand reaction, was taken for analysis. The samples of 1st and 2nd strand products were electrophoresed under denaturing conditions using an alkaline agarose gel (Maniatis *et al.*, 1982) and autoradiographed. The autoradiograph reveals, if the reactions are successful, 2nd strand products containing a greater amount of radiolabel relative to the 1st strand products. It is also important that the 1st and 2nd strand products are of equal size as this ensures that no priming of 2nd strand synthesis has occurred due to hairpin loops that arise at the 3' ends of the 1st strand DNA products. If this occurs, the two strands synthesized would be joined, increasing their size relative to the 1st strand products, and this discrepancy would be apparent upon electrophoresis (Watson and Jackson, 1985).

The double stranded cDNA produced was 'blunt-ended' with T4 DNA polymerase using the manufacturers specified conditions. The entire yield of cDNA from 1 µg of message-enriched RNA was then cloned into λ gt11.

2.2.2.1.4 Cloning into λ gt11

The protocols and reagents for λ gt11 cloning, as described by Huynh *et al.* (1985), were supplied by Amersham International (λ gt10 cloning

kit, code no: RPN.1257) and the λ gt11 vector and *E. coli* host cells were supplied by Stratagene (code no: 04 GT11).

Free nucleotides were removed from blunt-ended cDNA products by repeated ammonium acetate/ethanol precipitation. Methylation of cDNA Eco R1 restriction sites, linker ligation, Eco R1 digestion and column size fractionation to remove excess, unligated linkers, were performed as recommended by the manufacturer. Linkered cDNA was ligated with 1 μ g of Eco R1 cut and phosphatase treated λ gt11 vector arms in a total reaction volume of 5 μ l and the products were packaged directly *in vitro* to produce viable phage particles.

Dilutions of the resulting library phage were absorbed for 15 minutes at 37 °C to *E. coli* Y1088 plating cells and plated out with 10 mM IPTG and 0.5 mg ml⁻¹ Xgal in LB [1% (w/v) Bacto-tryptone (Difco)/0.5% (w/v) Bacto-yeast extract (Difco)/1% (w/v) NaCl/pH 7.2] top agar [10 mM MgSO₄/0.7% (w/v) Bacto-agar (Difco)/LB] onto LB agar [1.5% (w/v) Bacto-agar/LB] as described by Huynh *et al.* (1985). The library titre and percentage of recombination was determined by the number of clear plaques (recombinant phage) and blue plaques (non-recombinant phage). The library was amplified once using Y1088 cells (Huynh *et al.*, 1985) prior to screening.

2.2.2.2 The analysis of library phage by *in situ* plaque hybridisation with a cDNA probe

1st strand cDNA was synthesized as described previously (section 2.2.2.1.3) using 0.5 μ g message-enriched RNA as substrate and incorporating 50 μ Ci [α -³²P] dCTP radioisotope in the reaction. Following completion of the 1st strand reaction, the products were processed as described by Cowman *et al.* (1984). The RNA template was hydrolysed with 0.3 M potassium hydroxide. This was neutralised with 0.3 M HCl and equilibrated with approximately 3 volumes of 2 M Tris-HCl pH 7.5. The single stranded cDNA was phenol: chloroform (1 : 1) and ether extracted. An aliquot was taken to determine activity of the probe by trichloroacetic acid (TCA) precipitation (Maniatis *et al.*, 1982). Activity of the probe was estimated at 2 $\times$ 10⁵ cpm μ g⁻¹.

The labelled probe was used to screen a representative portion

of the amplified library by *in-situ* plaque hybridisation (Maniatis *et al.*, 1982).

2.2.2.3 SDS-PAGE, immunoblotting and ELISA

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using a vertical LKB electrophoresis apparatus. Samples were loaded in the buffer described by Studier (1973) with 5 minutes boiling prior to loading with or without 2-me, using reducing or non-reducing conditions, respectively.

Transfer of proteins to nitrocellulose (Schleicher and Schuell) (Towbin *et al.*, 1979) was performed in a Bio-Rad transfer cell using 80 mA overnight. Non-specific binding was blocked by incubation of nitrocellulose strips with 5% (w/v) skimmed milk (blotto)/PBS for 1 h at ambient temperature. Unbound primary and secondary antibody was removed using 3 × 10 minute washes with wash solution [0.1% (v/v) Triton-X100/0.05% (v/v) Tween-20/PBS], a final wash with PBS alone was used prior to the addition of chromogenic substrate. Antisera and horse radish peroxidase (HRP) conjugated secondary antibody (Sigma, 1/1000 dilution) were diluted in 1% (w/v) blotto/wash solution. Incubations with primary antibody were at 4 °C overnight and those with secondary conjugate antibody were at ambient temperature for 2 h. Bound antibody was detected by incubation for 30 minutes at ambient temperature with DAB substrate [0.5 mg ml⁻¹ 3, 3'-diaminobenzidine tetrahydrochloride/0.003% (w/v) hydrogen peroxide/50 mM Tris-HCl pH 7.6]. The colour reaction was terminated by washing with water and the nitrocellulose strips were air dried.

The ELISA procedure was an adaptation of that described by Voller *et al.* (1978). The ELISA plate (Nunc Immunoplate) wells were coated with antigen overnight in carbonate buffer [50 mM NaHCO₃/20 mM Na₂CO₃] at 4 °C. The optimum antigen concentration was determined using a 'checkerboard' titration technique, varying antigen concentration against serial dilutions of antiserum. Incubation for 30 minutes at 37 °C with 1% (w/v) bovine serum albumin (BSA)/PBS was used to block non-specific binding sites and these incubation conditions were also used for primary and secondary antibody binding. Test antisera and HRP-conjugated second

antibody (Sigma, 1/1000 dilution) were diluted in 1% (w/v) BSA/0.5% (v/v) Tween-20/PBS and 3 × 3 minute washes with 0.5% (v/v) Tween-20/PBS at ambient temperature were used to remove unbound antibody. Following additional 2 × 3 minute washes with PBS alone at ambient temperature, the chromogenic OPD substrate [0.1 mg ml⁻¹ orthophenylene diamine/0.003% (w/v) hydrogen peroxide/100 mM sodium acetate/10 mM citric acid/pH 5.0] was added and incubated for 30 minutes at ambient temperature in the dark. The reaction was terminated by the addition of 0.125 × vol 4 M sulphuric acid per well. Absorbance readings were taken at OD_{492nm} using a Bio-Rad ELISA reader.

2.2.2.4 Antibody screening

Plaques were screened using a variation of the protocol of Huynh *et al.* (1985). Library phage were plated on Y1090, *lon* protease deficient host cells at the desired density and incubated at 42 °C until the plaques were just visible (3-4 h). Nitrocellulose filters (Schleicher and Schuell), previously soaked in 10 mM IPTG, were laid over the plaque lawn and orientated with ink dots. The plates were then incubated for a further 2-3 h at 37 °C. Filters were removed and placed in 5% (w/v) blotto/TBS [150 mM NaCl/50 mM Tris-HCl pH 8.0] to block for 1 h at ambient temperature and the plates were stored at 4 °C. If replica plaque lifts were taken, a second IPTG soaked filter was positioned on the plate, orientated and incubated for a further 2-3 h at 37 °C and blocked as before.

Filters were transferred to the probe antiserum suitably diluted in 1% (w/v) blotto/TNT [0.05% (v/v) Tween-20/0.05% (v/v) Nonidet P40/TBS], the optimum dilution having previously been determined by immunoblotting, and incubated overnight at 4 °C. Unbound antibody was removed with 3 × 10 minute washes in TNT at ambient temperature. Following incubation for 2 h at ambient temperature with HRP-conjugated second antibody (Sigma, 1/1000 dilution) in 1% (w/v) blotto/TNT, and washing as before (together with an additional final wash of TBS alone), bound antibody was detected by incubation for 30 minutes at ambient temperature with DAB substrate. The colour reaction was terminated by washing with water and the filters were air dried.

Positive plaques were identified by reorientating filters to

the plates. The plaques were picked using sterile Pasteur pipettes, the agar plugs being placed in 1 ml of SM [0.01% (w/v) gelatin/10 mM MgSO₄/10 mM Tris-HCl pH 7.5] with 10 µl of chloroform. The phage were left to diffuse into the buffer overnight at 4 °C. Plaque purification was achieved by several cycles of re-screening using progressively greater dilutions of phage until single plaques could be isolated. Phage stocks were made using the plate lysate method (Maniatis *et al.*, 1982).

Sera used for screening were subjected to several cycles of absorption against lysed, λgt11 infected *E. coli* cells bound to nitrocellulose filters prior to use, in order to reduce background signal levels. These filters could be reused several times by removing bound antibody with strip solution [0.1 M glycine/0.15 M NaCl/pH 2.6] and re-equilibrating with TBS. 0.05% (w/v) sodium azide was added to preabsorbed sera as a preservative to allow the reuse of one batch of serum for several screening cycles, this helped to further decrease background signal levels.

Spot-lysis arrays of purified clones were produced by spotting 1 µl dots of phage stock onto a lawn of Y1090 cells containing 10 mM IPTG. Following diffusion of the phage into the lawn, the incubation of plates and processing of filters was identical to that described above.

2.2.2.5 **The affinity purification of specific anti-fusion-peptide antibody**

The procedure used was a modification of that described by Saint *et al.* (1986). 10⁵ phage of the clone of interest were plated out using Y1090 cells in the presence of 10 mM IPTG. Following growth for 4 h at 42 °C, a nitrocellulose disc was placed on the lawn and, after a further 2 h incubation at 37 °C, the disc was removed and blocked with 5% (w/v) blotto/TBS as described previously. The filter was then incubated as before with preabsorbed rabbit anti-EmAgA serum or a preabsorbed pool of human anti-*E. multilocularis* sera. Unbound antibody was washed off as before followed by a 15 minute wash in 0.1 M boric acid/0.5 M NaCl/0.05% (v/v) Tween-20/pH 8.0 and a 15 minute wash in PBS. The remaining bound antibody was released from the filter by incubation with strip solution for 1 minute at ambient temperature. The eluate was collected and immediately re-equilibrated with 0.04 × vol 2 M Tris-HCl pH 8.0.

2.2.2.6 **Restriction digest analysis of putatively *E. multilocularis*-specific recombinant clones**

Mini liquid lysates of recombinant phage were produced using a variation of the protocol provided by Amersham International. A volume of phage stock equivalent to 10^7 phage was added to 0.5 ml of Y1090 plating cells and allowed to adsorb for 15 minutes at 37 °C. 5 ml of LB/5 mM CaCl_2 was added and the culture was incubated with shaking at 42 °C overnight. 10 μl of chloroform was added and the culture was agitated for a further 5 minutes. The lysate was centrifuged at 4 500 g for 10 minutes to remove bacterial debris and the supernatant was removed by aspiration.

To 5 ml of supernatant was added an equal volume of 20% (w/v) polyethyleneglycol-6000 (PEG)/2 M NaCl/SM and the mixture was allowed to incubate for 1 h on ice. The PEG precipitated phage were collected by centrifugation at 4 500 g for 20 minutes. The pellet was drained and all traces of PEG were removed. Following resuspension of the pellet in 750 μl of LB, an equal volume of DEAE-cellulose (DE52, Whatman), equilibrated in LB according to the Amersham protocol, was added, and the suspension mixed by inversion 20-30 times at ambient temperature. The DE52 resin was removed by centrifugation at 15 000 g for 5 minutes, the supernatant transferred to a fresh tube and the step repeated. To the supernatant was added 13 μl of 0.1 mg ml^{-1} proteinase K and 32 μl of 10% (w/v) SDS and this mixture was incubated at ambient temperature for 5 minutes. 130 μl of 3 M potassium acetate was added and the solution incubated at 88 °C for 20 minutes followed by cooling on ice for 10 minutes. Precipitated material was removed by centrifugation at 15 000 g for 10 minutes. The supernatant was transferred to a fresh tube and the DNA precipitated at -70 °C for 10 minutes following the addition of an equal volume of cold isopropanol. The precipitate was recovered by centrifugation at 15 000 g for 10 minutes. The pellet was drained, dried under vacuum and resuspended in 20 μl of TE [1 mM EDTA/10 mM Tris-HCl pH 8.0].

The entire yield of phage DNA was digested with Eco RI (Amersham Int.) using the conditions recommended by the manufacturer. Following heating of the digest to 70 °C for 10 minutes, the restriction fragments were 'end-labelled' with [α - ^{32}P] dATP (Amersham Int.) using DNA polymerase I Klenow fragment (Maniatis *et al.*, 1982). The labelled products

were run on an agarose gel (Maniatis *et al.*, 1982) which was subsequently vacuum dried on a Bio-Rad gel drier at ambient temperature for 1 h and autoradiographed at -70 °C (Maniatis *et al.*, 1982).

2.2.2.7 Lysogen construction and fusion-peptide purification

Lysogens of recombinant clones were produced (Huynh *et al.*, 1985) by infecting exponentially growing Y1089 cells with recombinant λ gt11 phage. Lysogenic colonies were selected according to their temperature sensitivity.

Fusion-peptides were produced (Huynh *et al.*, 1985), by inducing exponentially growing lysogens at 42 °C for 20 minutes and, following the addition of IPTG to 10 mM, incubating for a further 2 h at 37 °C. Cells were harvested by centrifugation at 4 000 g for 5 minutes at ambient temperature. The pellet was resuspended in 0.01 $\times$ the original culture volume of 1 mM EDTA/1 mM phenylmethylsulphonylfluoride (PMSF)/PBS. One freeze-thaw cycle plus sonication for 5 $\times$ 10 seconds at maximum amplitude at 4 °C was used to lyse the cells and decrease chromosomal DNA viscosity.

To purify recombinant peptides, crude lysate, equivalent to 100 ml of culture volume, was loaded under reducing conditions on a 7.5% preparative SDS-PAGE gel. Following electrophoresis, protein bands were visualised by precipitating unbound SDS with 4 M sodium acetate at ambient temperature. The fusion-peptide band was excised and the protein electroeluted at 100 V overnight using a modified LKB tube-gel apparatus consisting of dialysis tubing fixed to the base of a Pasteur pipette containing the gel slice. Extensive dialysis against PBS at ambient temperature was used to remove excess SDS and the sample was concentrated to a final volume of 1 ml in a Minicon-B (Amicon) ultrafiltration cell (15 kDa cut-off). The approximate yield and purity of the fusion-peptides were estimated by SDS-PAGE of aliquots alongside known concentrations of commercially available β -gal (Sigma). The yield of pure fusion-peptide was in the region of 250 μ g per 100 ml of lysogen culture volume. The fusion-peptides were then tested by immunoblotting and ELISA.

2.3 RESULTS

2.3.1 THE CONSTRUCTION AND ANALYSIS OF THE λ gt11 LIBRARY

2.3.1.1 cDNA analysis

The quality of the cDNA synthesized from *E. multilocularis* protoscolex mRNA was assessed by electrophoresis (Fig. 2.2) prior to cloning. The 1st strand reaction products (lane 1) could be visualised upon autoradiography, proving radiolabel had been incorporated with synthesis having taken place. The incorporation of a greater amount of radiolabel in the 2nd strand products (lane 2) relative to the 1st strand, proved that the 2nd strand had been synthesized.

A band of approximately 1 000 base pairs (bp) was observed within the 1st strand products. The most likely explanation for this was the presence of ribosomal RNA within the message-enriched RNA. This would be supported by the fact that the mRNA purification yielded greater levels of message than might have been expected, the discrepancy being due to the presence of contaminating rRNA.

The size of products from both reactions was approximately 300-1 500 bp, the lack of high molecular weight 2nd strand products indicated that no 2nd strand synthesis had been primed by 3' 'hairpins' originating from the 1st strand reaction. The double stranded cDNA produced appeared to be suitable for cloning.

2.3.1.2 The determination of the percentage of recombinant phage within the library

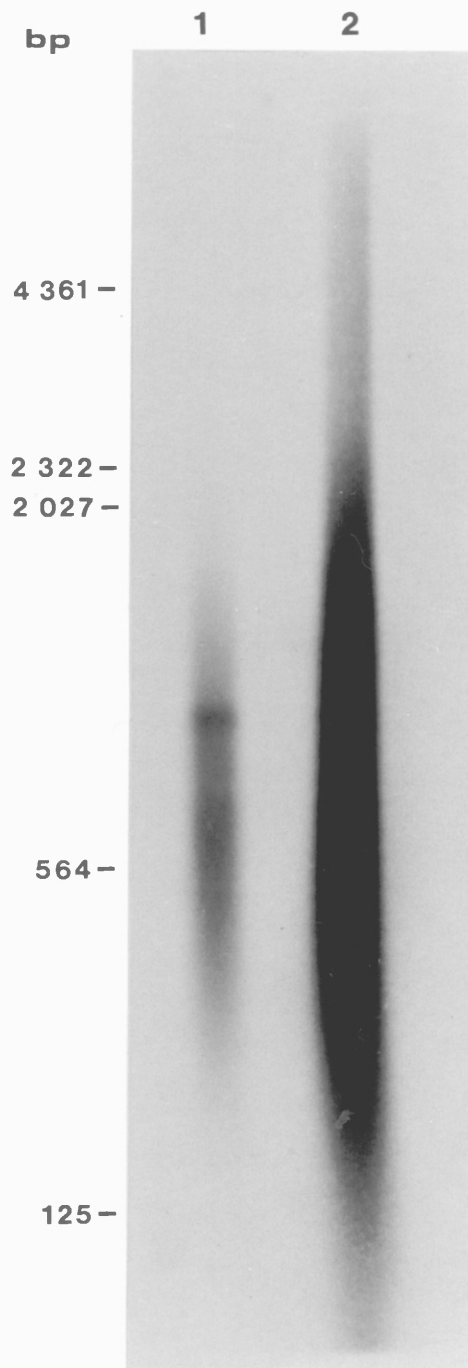
The library was titred by plating out phage in the presence of the chromogenic substrate Xgal and the β -gal gene inducer IPTG. The total number of viable phage in the library, blue 'non-recombinant' plaques plus clear 'recombinant' plaques, was estimated at 1.3×10^6 plaque forming units (pfu). The percentage of recombination, [no. recombinant plaques $\div$ no. total plaques] $\times$ 100, was 95% and, therefore, the number of recombinant phage was approximately 1.2×10^6 pfu.

This method of assessing library complexity can be misleading

Figure 2.2 Alkaline agarose gel electrophoresis of cDNA synthesized from protoscolex message-enriched RNA.

Lane 1, first strand reaction products.

Lane 2, second strand reaction products.



as some recombinant phage may produce a small amount of blue colour (Huynh *et al.*, 1985) and some non-recombinant phage may produce clear plaques if the β -gal gene has been damaged during the cloning process or by alkaline phosphatase treatment of the vector. In order to make a more accurate assessment of the number of phage containing cDNA inserts, it was decided to screen a number of library phage with radiolabelled cDNA, as performed by Knight *et al.* (1986).

Approximately 6×10^8 phage from the amplified library were plated out and *in situ* hybridised with the cDNA probe (Fig. 2.3). 13% of plaques appeared to hybridise with cDNA, indicating that at least 10^5 recombinant phage in the original unamplified library contained genuine cDNA inserts.

2.3.1.3 The determination of the percentage of clones expressing *E. multilocularis* peptides

To ensure that a significant number of phage were capable of expressing fusion-peptides which could be recognised by anti-*E. multilocularis* antibodies, a proportion of the library was screened with rabbit anti-EmAgA serum (Fig. 2.4).

10^5 phage from the amplified library were screened with the serum, diluted 1 in 50 (determined previously by immunoblotting, data not shown), and 52 antigen expressing clones were detected. Therefore, approximately 0.05% of library phage synthesized fusion-peptides which were capable of being detected by antibodies specific to native parasite proteins.

2.3.2 THE IDENTIFICATION OF PUTATIVELY SPECIES-SPECIFIC CLONES

2.3.2.1 The characterisation of human infection sera

The individual human anti-*E. multilocularis* and anti-*E. granulosus* infection sera, intended for differential screening of the library, were first tested for antibody titre and antigen recognition profiles by ELISA (data not shown) and immunoblotting (Fig. 2.5), respectively. The antibody titre for each of these sera was determined to

Figure 2.3 *In situ* hybridisation of amplified recombinant *E. multilocularis* λ gt11 library phage with single stranded protoscolex cDNA.

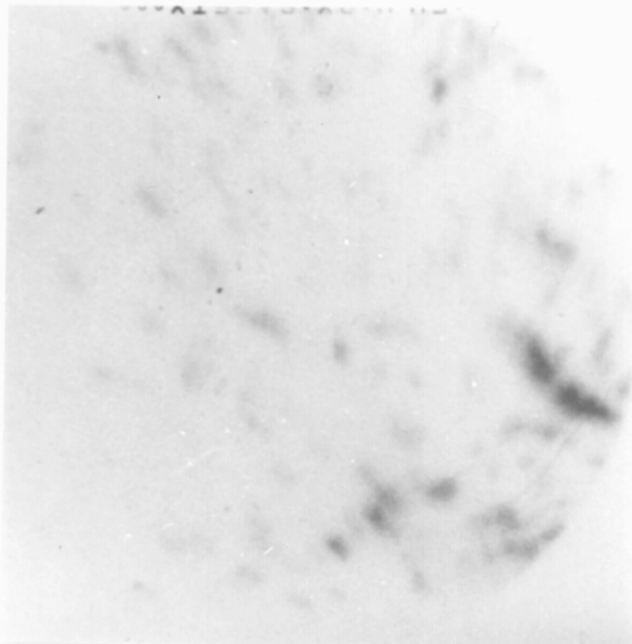
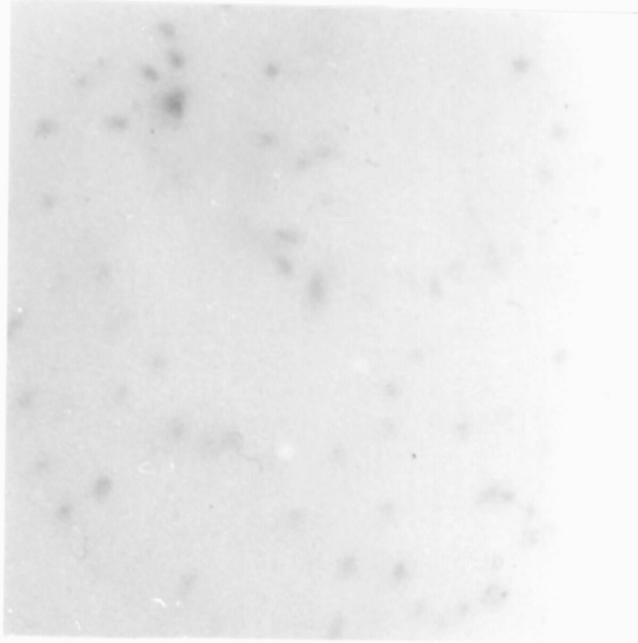


Figure 2.4 Screening of amplified recombinant *E. multilocularis* λ gt11 library phage with rabbit anti-EmAgA serum. Two antigen expressing clones are arrowed.

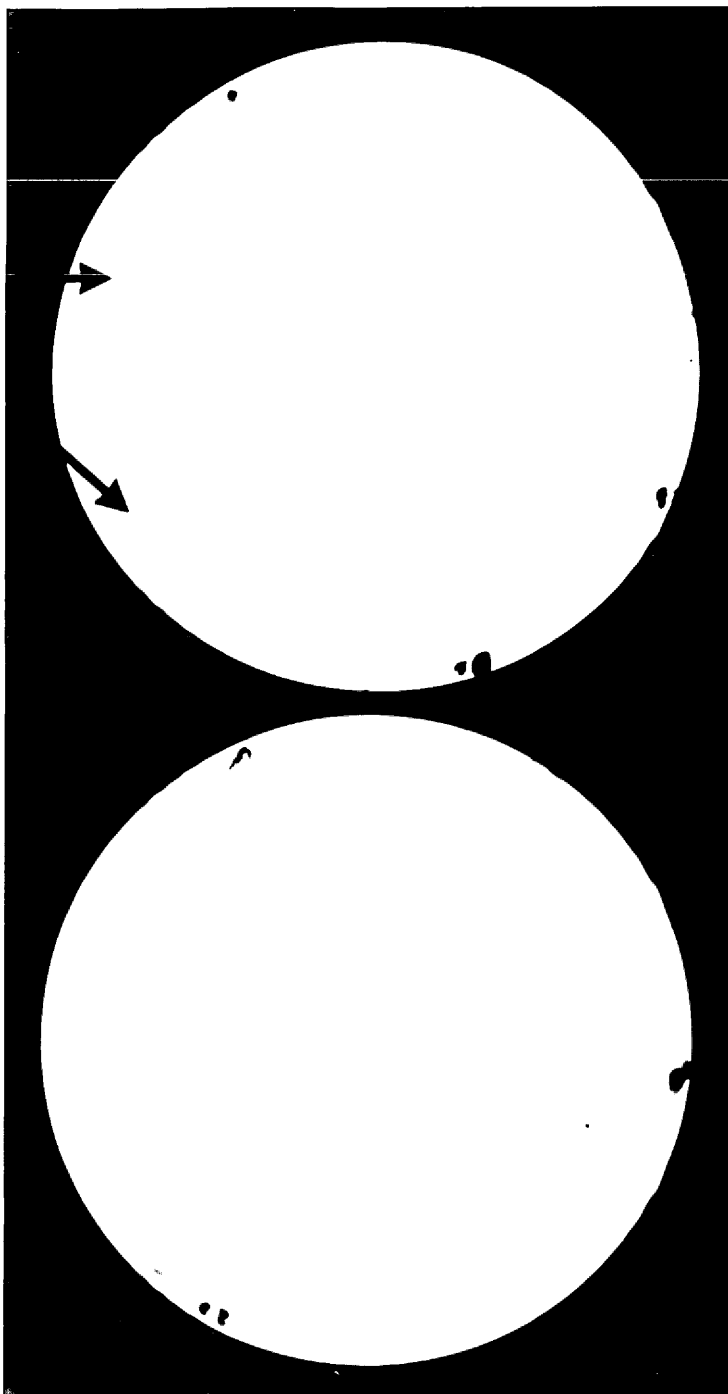


Figure 2.5 SDS-PAGE and immunoblotting of human infection sera.

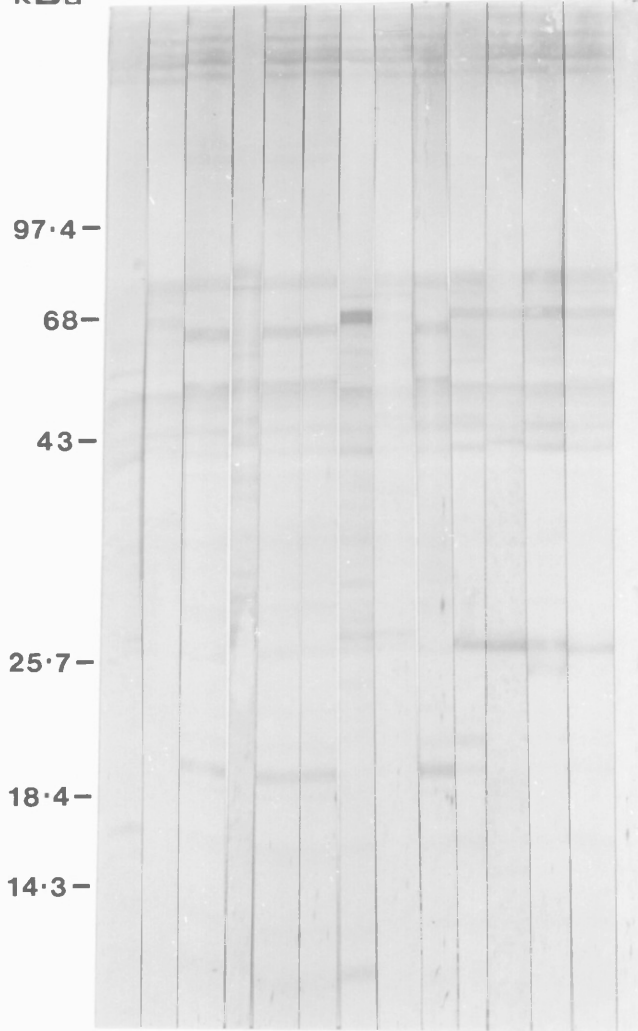
(A) EmAgA tested with: lanes 1 to 13, individual human anti-*E. multilocularis* sera (French origin); lane 14, normal human serum.

(B) EgSCF tested with: lanes 1 to 14, human anti-*E. granulosus* sera; lanes 1 to 3, U.K. individual sera; lane 4, Spanish pool; lanes 5 to 13, Kenyan individual sera; lane 14, Kenyan pool; lane 15, normal human serum.

10 µg of protein was used per strip for both antigens. Proteins were reduced prior to electrophoresis.

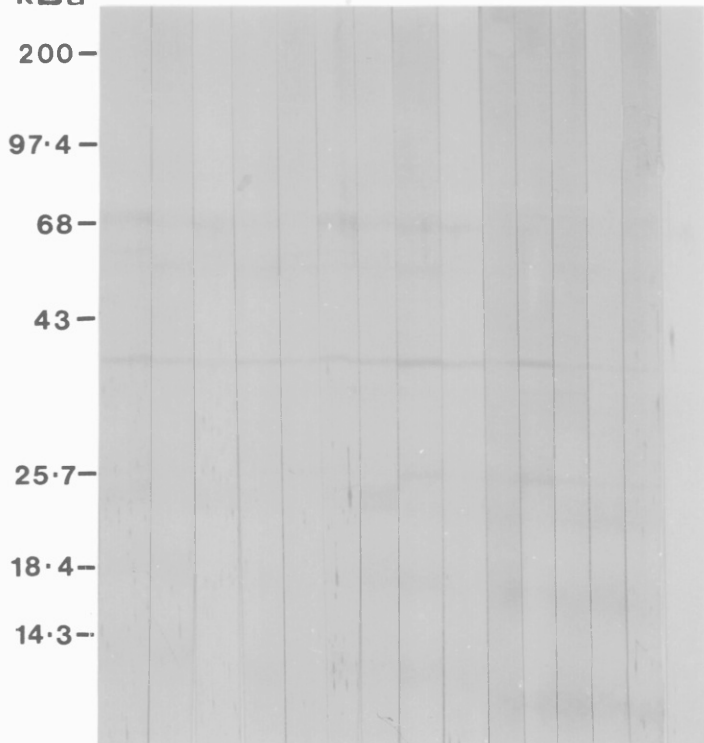
A

kDa 1 2 3 4 5 6 7 8 9 10 11 12 13 14



B

kDa 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



ensure that they were sufficiently high and to allow appropriate amounts of each to be included in the serum pools used for screening. For the ELISA, EgSCF was used as antigen as it was hoped that this extract, less crude than EmAgA, would help to overcome problems associated with high background produced by non-specific antibody binding.

Testing of individual anti-*E. multilocularis* sera, each diluted to equivalent antibody titre (using ELISA data) with immunoblots of EmAgA (Fig. 2.5 A) showed all sera, apart from number 4 (lane 4), recognised the same major antigens. All of the individual anti-*E. granulosus* sera, diluted to equivalent antibody titre and tested with immunoblots of EgSCF (Fig. 2.5 B), appeared to recognise the same major antigens, although some heterogeneity was observed regarding the minor antigens.

2.3.2.2 Differential screening with human infection sera

2.3.2.2.1 Human infection serum pools

Using the ELISA and immunoblotting data, pools of human anti-*E. multilocularis* and anti-*E. granulosus* infection sera were constructed for differential screening of the library. All of the anti-*E. multilocularis* individual sera, except number 4, were pooled using differing volumes of each serum depending on their respective antibody titres, in order to represent each serum equally within the pool. All of the anti-*E. granulosus* sera, without exception, were pooled in the same manner.

To ensure the validity of the differential screening, probing with equivalent antibody concentrations in both serum pools was necessary in order to prevent putatively *E. multilocularis*-specific clones being detected as a result of low anti-*E. granulosus* serum pool antibody titre. The optimum dilutions of the human anti-*E. multilocularis* and anti-*E. granulosus* serum pools to be used for screening were, therefore, first determined by immunoblotting (Fig. 2.6). This technique was used as it has the equivalent degree of sensitivity as the plaque immunoassay procedure. Dilutions of anti-*E. multilocularis* and anti-*E. granulosus* pooled sera of 1 in 100, 1 in 50 and 1 in 25 were tested using EmAgA as antigen. The dilutions which appeared to produce equivalent signal strengths were 1 in 100 for the anti-*E. multilocularis* serum pool (lane 4) and 1 in 25 for the anti-*E. granulosus* serum pool (lane 5).

Figure 2.6 SDS-PAGE and immunoblotting of human infection serum pools to determine appropriate dilutions for library screening.

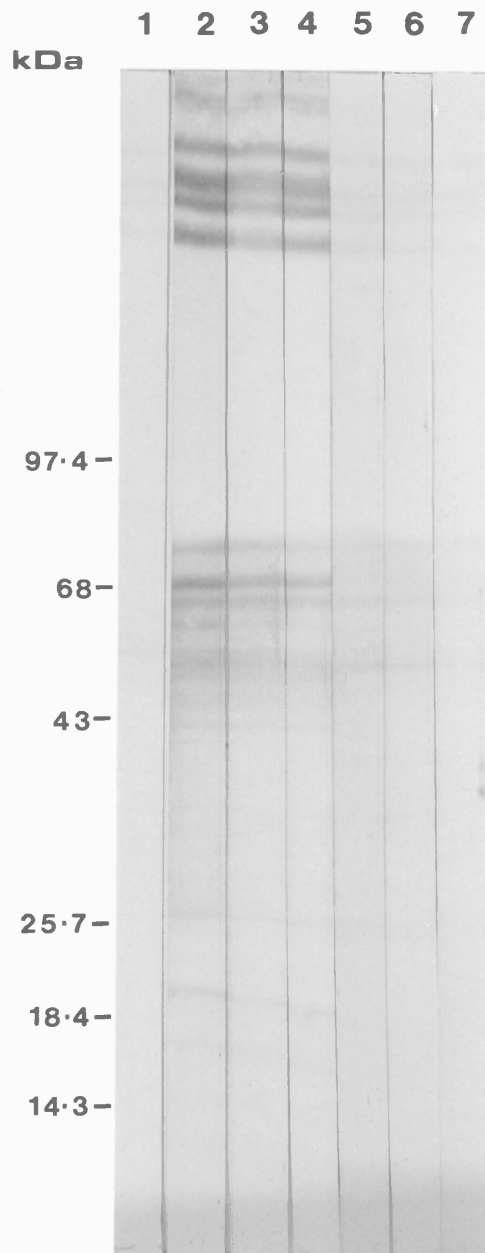
EmAgA tested with:

Lane 1, normal human serum diluted 1 in 25.

Lanes 2-4, human anti-*E. multilocularis* serum pool; lane 2, diluted 1 in 25; lane 3, diluted 1 in 50; lane 4, diluted 1 in 100.

Lanes 5-7, human anti-*E. granulosus* serum pool; lane 5, diluted 1 in 25; lane 6, diluted 1 in 50; lane 7, diluted 1 in 100.

10 µg of protein was used per strip having been reduced prior to electrophoresis.



2.3.2.2.2 Differential screening

Initially, 10^6 phage from the amplified library were screened with the human anti-*E. multilocularis* serum pool and 364 positive clones were detected. These plaques were picked and pooled. The pooled phage were plated at a density of approximately 10^4 and two replica nitrocellulose filter lifts were taken. The first lift was probed with the anti-*E. granulosus* serum pool at 1 in 25 dilution and the second lift with the anti-*E. multilocularis* serum pool at 1 in 100 dilution. This was to ensure that no loss in signal would occur with the anti-*E. granulosus* serum pool due to lack of protein transfer. The developed filters (data not shown) were compared and the clones which appeared to react with anti-*E. multilocularis* serum pool only, were picked. The replica screening procedure was repeated a total of 7 times until 13 putatively *E. multilocularis*-specific clones had been identified. These were then plaque purified and a phage stock made of each. The differential screen was performed repeatedly to allow sufficient clones to be isolated to compensate for screening artifacts and biases.

To confirm putative *E. multilocularis*-specificity of these clones, a spot-lysis array of the clones was made on separate plates and a filter taken from each was probed, as before, with the serum pools (Fig. 2.7). The colour intensity varied significantly depending on the degree of antibody binding and, using this criterion, three levels of reactivity could be observed. With the anti-*E. multilocularis* serum pool (filter A), a strong signal was produced by clone 4, moderate signals by clones 1, 2, 3 and 6 and weak signals by clones 5, 7, 8, 9, 10, 11, 12 and 13, equivalent to the background reactivity of the λ gt11 control. With the anti-*E. granulosus* serum pool (filter B), weak signals were produced by all clones, except clone 1 and the λ gt11 control, similar to the signals produced by the λ gt11 control on the *E. multilocularis* filter. The strong signals produced by clone 1 and λ gt11 with the anti-*E. granulosus* serum pool were probably artifacts produced by uneven protein transfer to the nitrocellulose filter. From these data, therefore, it was apparent that a significant level of antibody to clones 1, 2, 3, 4 and 6 was present in the anti-*E. multilocularis* serum pool only, indicating that these clones coded for immunogenic peptides which may be species-specific.

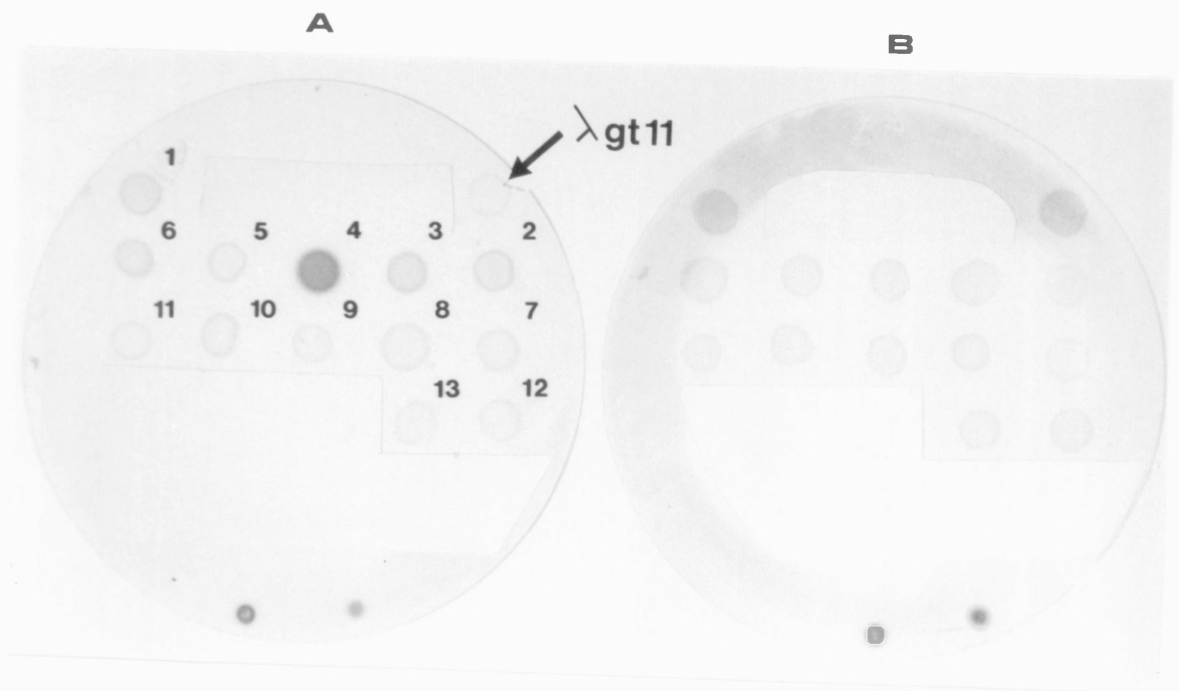
Figure 2.7 Differential screening of putatively species-specific clone replica plaque spot-lysis arrays with human infection serum pools.

Filters were probed with:

(A) a preabsorbed pool of human anti-*E. multilocularis* sera.

(B) a preabsorbed pool of human anti-*E. granulosus* sera.

Recombinant clones are numbered 1-13 and the λ gt11 negative control is arrowed.



2.3.3 THE ANALYSIS OF PUTATIVELY SPECIES-SPECIFIC CLONES

2.3.3.1 Sibling analysis

Although plaque immunoassay demonstrated variable levels of antibody binding, this was not sufficient to reveal which clones contained unique cDNA sequences, particularly as the cloning of cDNAs of varying length which may represent the same antigen, could alter the antigenicity of the recombinant peptide. In order to assess which clones coded for the same or different antigens, spot-lysis arrays of the putatively specific clones were probed with affinity purified antibody specific for each clone (Fig. 2.8). Affinity purification of antibody from rabbit anti-EmAgA serum proved to be possible only with clones 3, 4, 9, 11 and 13. Purification of antibody from the human anti-*E. multilocularis* serum pool was also attempted for the remaining clones but this proved to be unsuccessful, possibly due to the relatively low levels of clone-specific antibody, within this serum pool.

Probing of the arrays with specific antibody to clones 3, 4, 9, 11 and 13 showed clones 3 (filter B), 4 (filter C) and 13 (filter F) expressed fusion-peptides which may possess unique epitopes. These clones, may contain cDNA sequences which correspond to different parasite antigens. Clones 9 (filter D) and 11 (filter E) appeared to express recombinant peptides which possess shared epitopes and these clones may, therefore, contain cDNA inserts which represent the same antigen.

2.3.3.2 cDNA insert analysis

Detailed molecular characterisation of λ gt11 recombinants requires excision of the cDNA inserts. In order to establish whether this was possible with the putatively specific clones isolated and to assess the size of the cloned cDNA molecules, the inserts of several clones were excised with Eco R1 and visualised (Fig. 2.9). The clones were chosen at random and numbers 4, 7, 9 and 13 were analysed. Clone 4 contained an insert of approximately 1 000 bp (lane 2), clone 9 an insert of approximately 500 bp (lane 4) and clones 7 (lane 3) and 13 (lane 5) inserts of approximately 300 bp. The appearance of only one restriction fragment in

Figure 2.8 Sibling analysis of putatively species-specific clones. Spot-lysis arrays of recombinant phage were probed with clone-specific affinity purified antibody.

(A) layout of array.

Arrays were probed with affinity purified antibody specific for:

(B) clone 3.

(C) clone 4.

(D) clone 9.

(E) clone 11.

(F) clone 13.

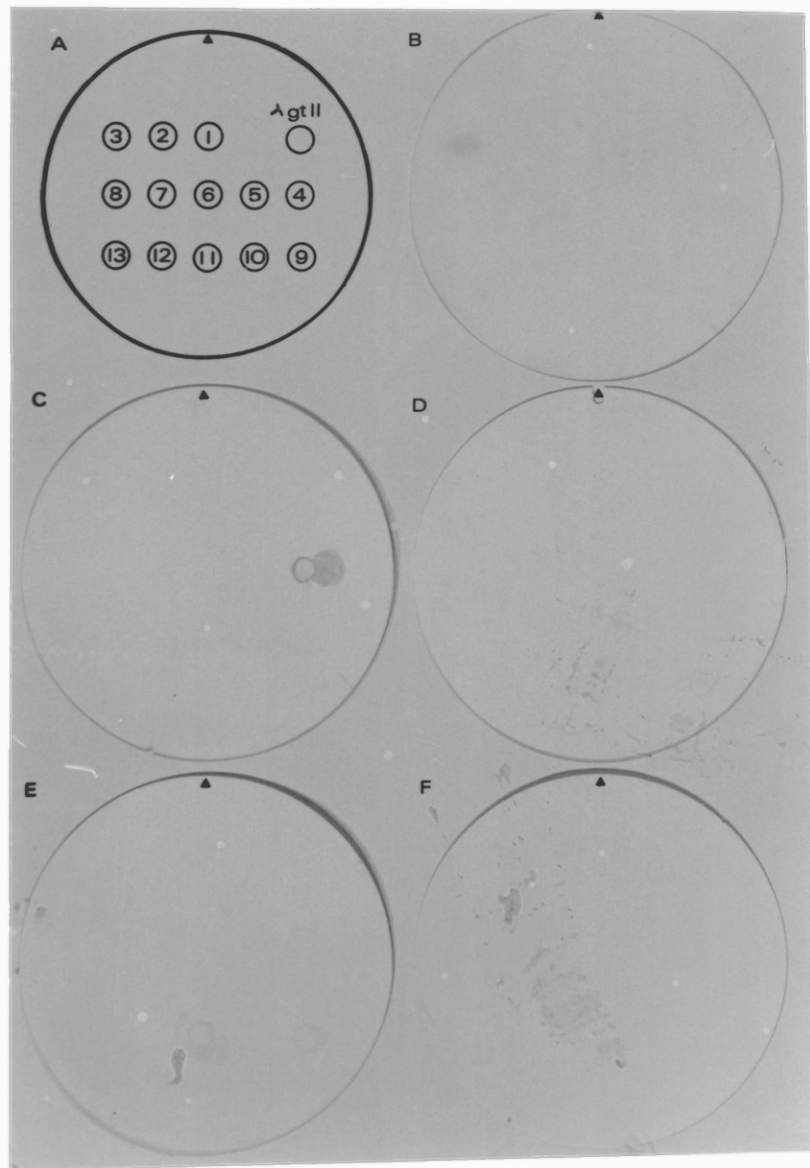


Figure 2.9 cDNA insert analysis of recombinant clones. Eco R1 digestion products of phage DNA were end-labelled with [α - ^{32} P] dCTP, electrophoresed and autoradiographed.

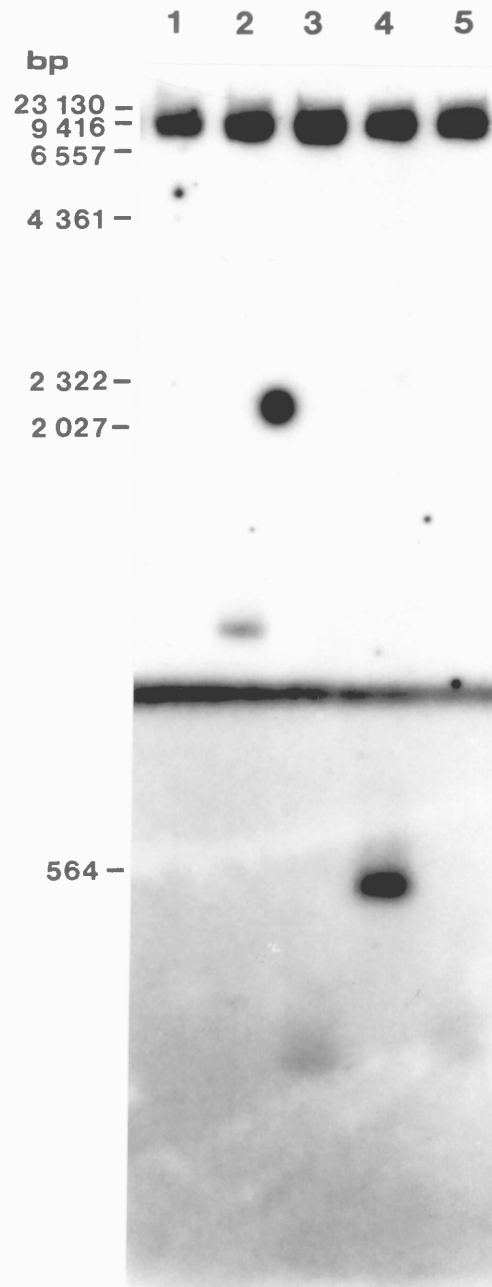
Lane 1, non-recombinant λ gt11.

Lane 2, clone 4.

Lane 3, clone 7.

Lane 4, clone 9.

Lane 5, clone 13.



all of the lanes suggests that none of the cDNAs excised contained internal Eco R1 restriction sites. The ability to excise the cDNA inserts proved that the library construction strategy had been successful and the presence of a 1 000 bp insert in clone 4 demonstrated that relatively large cDNA inserts had been cloned.

2.3.3.3 Fusion-peptide production and analysis

Lysogens of all 13 recombinant clones were prepared, together with non-recombinant λ gt11. Preparative quantities of β -gal and the fusion-peptides were produced and aliquots of the crude bacterial lysates run on 5-20% gradient SDS-PAGE gels. Coomassie blue staining was used to reveal the constituent protein bands (Fig. 2.10). 5-20% gels were used in preference to single concentration gels to allow the resolution of any peptide products which may have arisen from the breakdown of unstable fusion-peptides, a phenomenon previously described by Kemp *et al.* (1983).

The β -gal band of 116 kDa could be clearly seen in the λ gt11 lysogen lanes (lanes 8 and 17). The change in motility of this band in the recombinant lysogen lanes was attributable to the increase in size caused by the addition of the foreign peptide. Stable fusion-peptides, with M_r 's greater than that of β -gal, were produced by all recombinant lysogens. The yield of fusion-peptide appeared to vary between clones. This may have been influenced by a number of factors which included the size of the fusion-peptide, the toxicity of the peptide and its' stability within *E. coli*. The size variation of the fusion-peptides produced by clones 4, 7, 9 and 13 (lanes 5, 9, 11 and 16, respectively) correlated with the size of their respective cDNA inserts (Fig. 2.9).

From the spot-lysis immunoassay data (Fig. 2.7), it was apparent that clones 1, 2, 3, 4 and 6 coded for the most immunogenic, putatively specific, peptides. Their corresponding lysogens were also capable of synthesizing sufficient quantities of fusion-peptide for further analysis. In view of the amount of work that would be required to fully characterise all five clones, it was decided to initially select two recombinants for further studies. Clone 4, designated EM4, was chosen as it appeared to synthesize the most immunogenic fusion-peptide antigen. Of the moderately immunogenic clones, number 2, designated EM2, was selected as

Figure 2.10 Analysis of lysogen lysates, after induction with IPTG, by SDS-PAGE and Coomassie blue staining. Y1089 *E. coli* cells were lysogenised with:

Lane 1, clone 1.

Lane 2, clone 2.

Lane 4, clone 3.

Lane 5, clone 4.

Lane 6, clone 5.

Lane 7, clone 6.

Lane 9, clone 7.

Lane 10, clone 8.

Lane 11, clone 9.

Lane 13, clone 10.

Lane 14, clone 11.

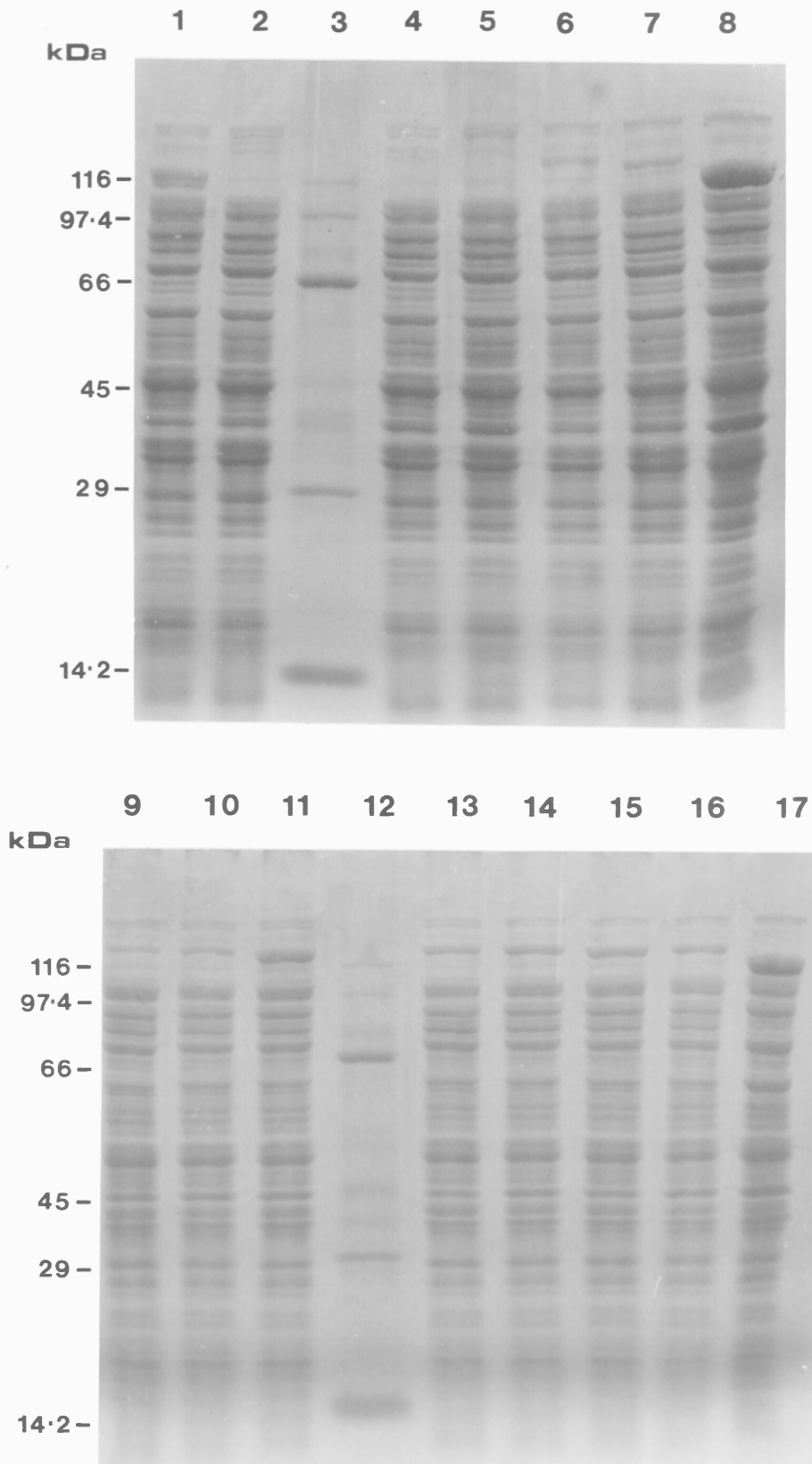
Lane 15, clone 12.

Lane 16, clone 13.

Lanes 8 and 17, non-recombinant λ gt11.

Lanes 3 and 12, molecular weight markers.

Extracts were reduced prior to electrophoresis.



its' fusion-peptide appeared to be of the highest M_r relative to those produced by the other clones. This suggested the increased likelihood of a full-length cDNA having been cloned. In addition, EM2 and EM4 did not appear to be related by sibling analysis (Fig. 2.8).

2.3.4 THE PUTATIVE SPECIES-SPECIFICITY OF RECOMBINANT PEPTIDES

2.3.4.1 The purification of the fusion-peptides coded for by EM2 and EM4

The use of fusion-peptides in immunodiagnostic tests such as ELISA requires their initial purification from *E. coli* lysates as antibodies to *E. coli* are found in human sera (Klinkert *et al.*, 1988). The small amount of recombinant peptide present in lysogen lysates, relative to *E. coli* proteins, would further necessitate purification to prevent 'swamping' of the fusion-peptide in ELISA assays.

Various methods have been employed for the purification of β -gal fusion-peptides from lysogen lysates and these have been reviewed by Carroll and Laughon (1987). Of the methods available, it was decided that, for preliminary work, the direct, simple technique of electroelution of fusion-peptides from preparative SDS-PAGE gels would be suitable. This technique is well suited for purification of β -gal fusion-peptides as these proteins are among the largest found within the *E. coli* lysate and are, therefore, separated well away from the majority of *E. coli* proteins.

The β -gal fusion-peptides produced by EM2 and EM4, designated EM- β galFP2 and EM- β galFP4 respectively, were purified by electroelution following separation on 7.5% preparative SDS-PAGE gels (Fig. 2.11). The crude lysate protein components are evident in lanes 2 (EM2) and 3 (EM4). EM- β galFP2 has an apparent M_r of 155 kDa, which corresponds, when compared to the 116 kDa of β -gal (lane 1), to approximately 39 kDa of *E. multilocularis* peptide. EM- β galFP4 has an apparent size of 162 kDa which corresponds to approximately 46 kDa of parasite peptide.

The purity of electroeluted EM- β galFP2 and EM- β galFP4 was assessed by SDS-PAGE (Fig. 2.11), lanes 4 and 5 respectively. EM- β galFP2 contained no visible contaminants but EM- β galFP4 appeared to contain some additional protein bands which may have been *E. coli* contamination. However,

Figure 2.11 Analysis of electroelution purified EM2 and EM4 recombinant fusion-peptides, by SDS-PAGE and Coomassie blue staining.

Lane 1, non-recombinant λ gt11 lysogen lysate.

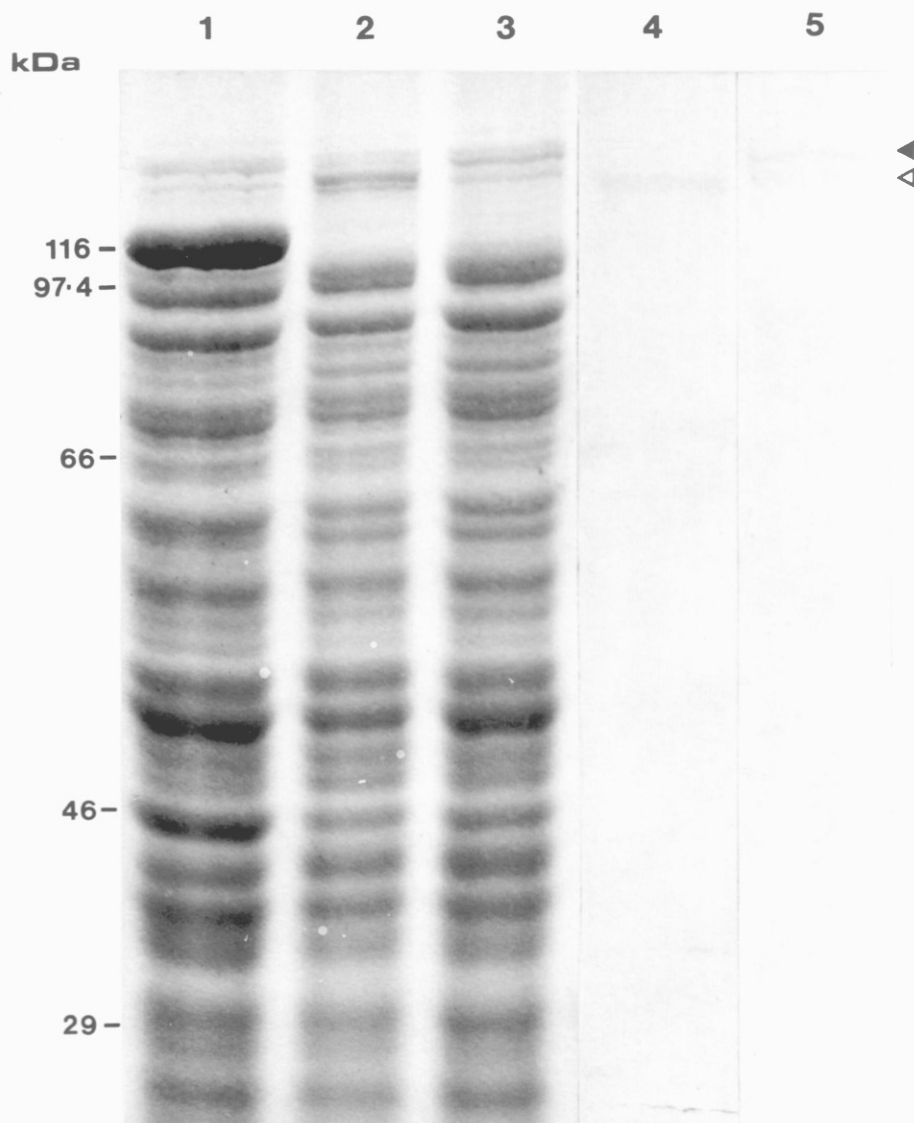
Lane 2, EM2 lysogen lysate.

Lane 3, EM4 lysogen lysate.

Lane 4, purified EM- β galFP2.

Lane 5, purified EM- β galFP4.

Extracts were reduced prior to electrophoresis. The fusion-peptide bands are arrowed: EM- β galFP2 ($\blacktriangleleft$); EM- β galFP4 ($\blacktriangleleft$).



a more probable explanation would have been the presence of proteolytic breakdown products of the fusion-peptide.

2.3.4.2 **The immunological species-specificity of EM- β galFP2 and EM- β galFP4**

A preliminary experiment, to ascertain whether purified EM- β galFP2 and EM- β galFP4 retained their putative *E. multilocularis*-specificity demonstrated by plaque immunoassay (Fig. 2.7), was performed. The purified fusion-peptides were subjected to SDS-PAGE and immunoblotting. Each was tested with the pools of human anti-*E. multilocularis* and anti-*E. granulosus* sera to confirm whether a relatively high level of antibody towards these two peptides was present only in the anti-*E. multilocularis* serum pool (Fig. 2.12). The dilution used for each serum pool was the same as that used for differential screening (section 2.3.2.2.2). NHS (lanes 1 and 4), used at the same dilution as the anti-*E. granulosus* serum pool, showed no significant antibody binding. The human anti-*E. multilocularis* serum pool (lanes 2 and 5) produced a strong band at the expected M_r of both fusion-peptides. Additional bands of lower M_r were also present in both cases, the most likely explanation for this being the proteolytic degradation of the fusion-peptides. The human anti-*E. granulosus* serum pool (lanes 3 and 6) did not appear to react with either fusion-peptide.

The incorporation of the purified fusion-peptides into an ELISA assay was attempted, to establish whether they would be compatible with this technique, and to allow further assessment of the degree of diagnostic species-specificity of the recombinant peptides. EM- β galFP4 proved to be compatible with ELISA but EM- β galFP2 did not bind antibody when used with this technique. Pools of human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* sera and NHS were tested by ELISA against EM- β galFP4 (Fig. 2.13 A) and native β -gal (Fig. 2.13 B). EM- β galFP4 bound high levels of antibody only with the human anti-*E. multilocularis* serum pool. Human anti-*E. granulosus* and anti-*T. solium* serum pools produced levels of binding similar to that of NHS. β -gal produced a background level of binding with all serum pools similar to that of NHS. Table 2.1 shows the ELISA values obtained when various, diverse human infection serum pools were tested against EM- β galFP4 and β -gal. No significant level of anti-EM- β galFP4

Figure 2.12 SDS-PAGE and immunoblotting of purified fusion-peptides with human infection serum pools.

Lanes 1 to 3, EM- β galFP2 probed with: lane 1, normal human serum; lane 2, pool of human anti-*E. multilocularis* sera; lane 3, pool of human anti-*E. granulosus* sera.

Lanes 4 to 6, EM- β galFP4 probed with: lane 4, normal human serum; lane 5, pool of human anti-*E. multilocularis* sera; lane 6, pool of human anti-*E. granulosus* sera.

2 μ g of purified fusion-peptide was used per strip. Proteins were reduced prior to electrophoresis. The undegraded fusion-peptide bands are arrowed.

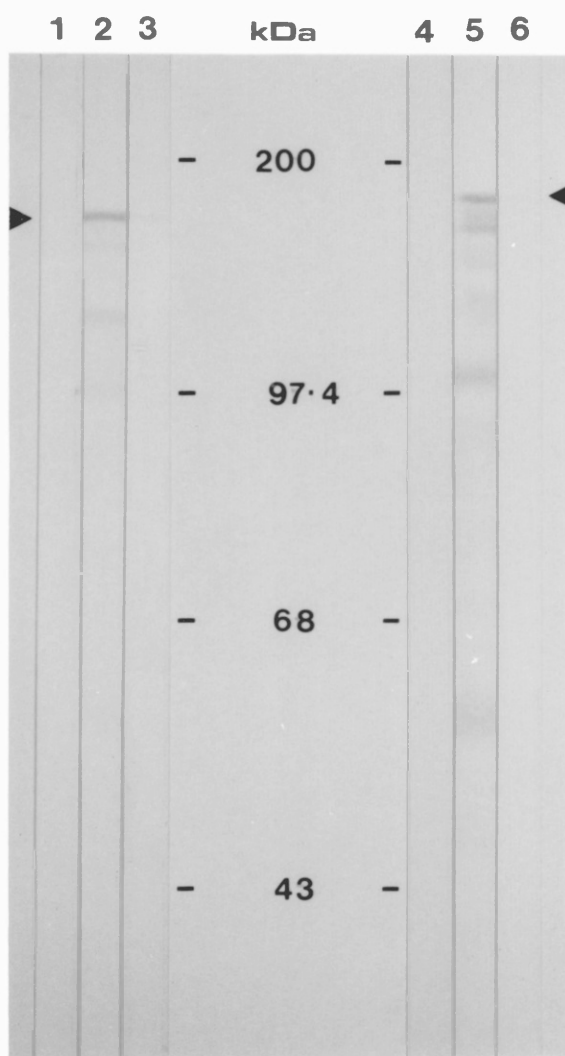
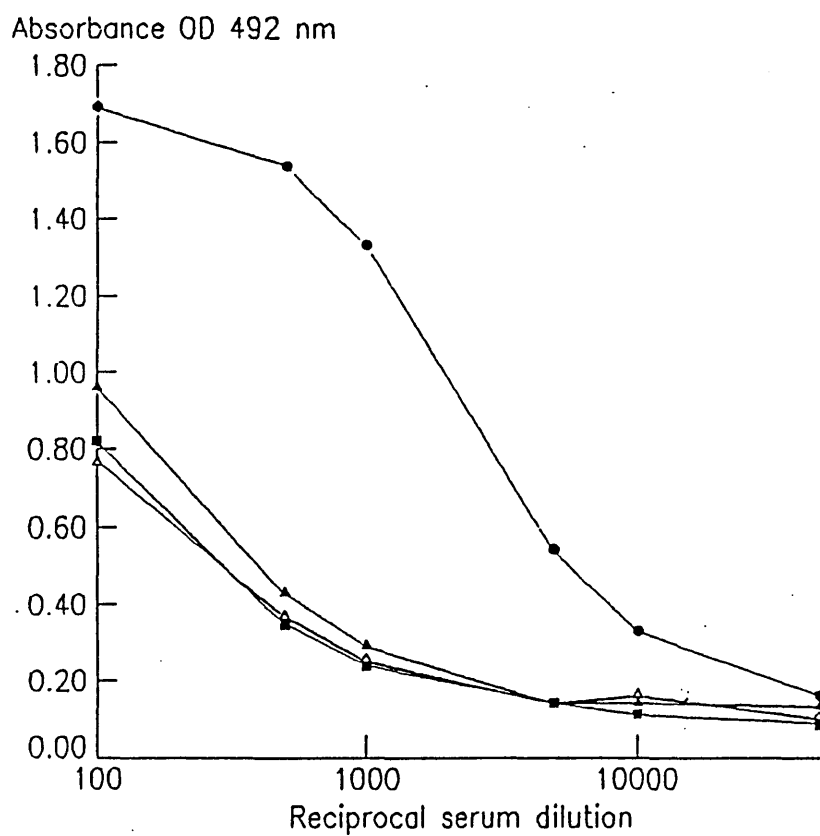


Figure 2.13 ELISA using EM- β galFP4 (A) and β -gal (B) as antigens ($2 \mu\text{g ml}^{-1}$) tested with various human infection serum pools. Pool of human anti-*E. multilocularis* sera (●); pool of human anti-*E. granulosus* sera (■); pool of human anti-*T. solium* sera (▲); pool of normal human sera (Δ).

A



B

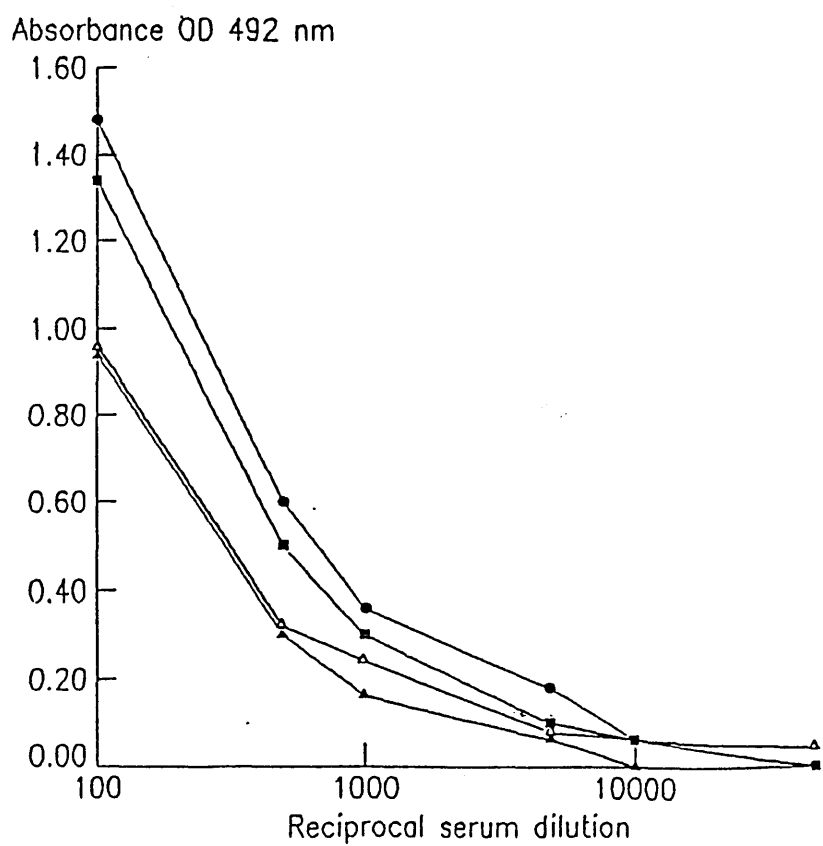


TABLE 2.1

The diagnostic specificity of EM- β galFP4, tested using diverse human infection serum pools in ELISA

1 in 500 pooled human antiserum to:	Antigen	
	EM- β -galFP4	β -gal
<i>E. multilocularis</i>	1.54 (1.53, 1.55)	0.60 (0.56, 0.64)
<i>E. granulosus</i>	0.35 (0.34, 0.35)	0.50 (0.28, 0.72)
<i>T. solium</i>	0.43 (0.42, 0.44)	0.30 (0.24, 0.36)
<i>Ascaris lumbricoides</i>	0.61 (0.58, 0.64)	0.42 (0.36, 0.48)
<i>Brugia malayi</i>	0.26 (0.26, 0.26)	0.34 (0.36, 0.32)
<i>Onchocerca volvulus</i>	0.34 (0.37, 0.30)	0.56 (0.56, 0.56)
<i>Schistosoma haematobium</i>	0.27 (0.27, 0.27)	0.46 (0.44, 0.48)
<i>Schistosoma mansoni</i>	0.30 (0.32, 0.28)	0.50 (0.52, 0.48)
Normal Human Serum	0.37 (0.37, 0.36)	0.32 (0.36, 0.28)

Figures represent OD_{492nm} absorbance readings in an ELISA and are the means of the 2 determinations given in brackets

Antigens were used at 2 μ g ml⁻¹

antibody was bound with any of the serum pools apart from the human anti-*E. multilocularis* serum pool. All serum pools produced similar levels of binding to β -gal. Substantial levels of anti- β -gal antibody were present at low serum pool dilution due to the inherent human response to β -gal produced by, for example, gut microflora (Klinkert *et al*, 1988).

The failure to incorporate electroeluted EM- β galFP2 into ELISA meant that an alternative approach had to be taken for testing of this fusion-peptide with diverse human infection serum pools. The previous success of immunoblotting with this peptide (Fig. 2.12) made this the most obvious technique to use. The ability to distinguish the fusion-peptide from *E. coli* proteins on the basis of M_r using this method, eliminated the need for fusion-peptide purification prior to testing. Crude EM2 lysogen lysate was electrophoresed, immunoblotted and subjected to testing with diverse human infection serum pools (Fig. 2.14 A). Significant antibody binding was only observed with the human anti-*E. multilocularis* serum pool (lane 3), producing a band at the expected M_r of EM- β galFP2. The location of this band was confirmed by the mouse anti-EM- β galFP2 serum (lane 1). The additional bands observed of lower M_r in this lane, were the result of antibody binding to *E. coli* proteins and fusion-peptide breakdown products. No significant anti-*E. coli* or anti- β -gal antibody binding was apparent at the dilution used (1 in 100) in the other human infection serum pool lanes. To ensure that the anti-*E. multilocularis* serum pool reactivity was not the result of high levels of anti- β -gal antibody binding to the β -gal portion of the fusion-peptide, the crude lysate products of an induced, non-recombinant λ gt11 lysogen were electrophoresed, immunoblotted and tested as before (Fig. 2.14 B). The location of the β -gal protein band was seen in the mouse anti- β -gal serum lane (lane 1). There was no apparent band of this M_r in any of the human serum pool lanes, confirming that the EM- β galFP2 band detected by the human anti-*E. multilocularis* serum pool was the result of anti-parasite antibody binding only.

Figure 2.14 SDS-PAGE and immunoblotting of crude EM2 and λ gt11 lysogen lysates with diverse human infection serum pools.

(A) EM2 lysogen lysate.

(B) λ gt11 lysogen lysate.

Lane 1 (A), mouse anti-EM- β galFP2 serum.

Lane 1 (B), mouse anti- β -gal serum.

Lane 2 (A & B), pool of normal human sera.

Lane 3 (A & B), pool of human anti-*E. multilocularis* sera.

Lane 4 (A & B), pool of human anti-*E. granulosus* sera.

Lane 5 (A & B), pool of human anti-*T. solium* sera.

Lane 6 (A & B), pool of human anti-*A. lumbricoides* sera.

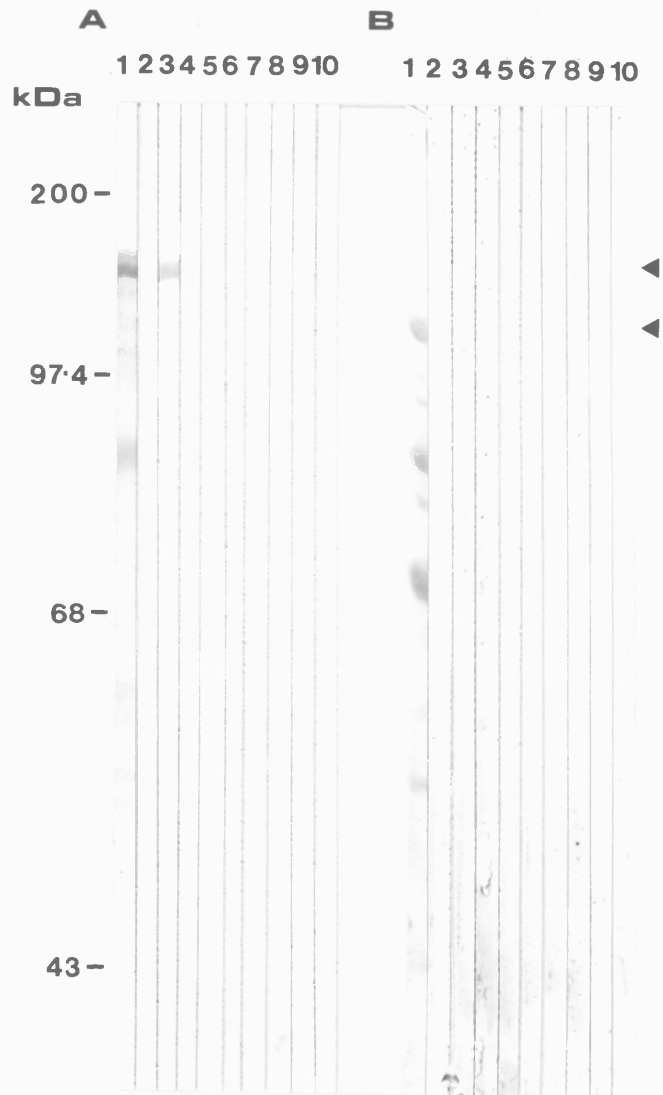
Lane 7 (A & B), pool of human anti-*B. malayi* sera.

Lane 8 (A & B), pool of human anti-*O. volvulus* sera.

Lane 9 (A & B), pool of human anti-*S. haematobium* sera.

Lane 10 (A & B), pool of human anti-*S. mansoni* sera.

50 μ g of crude extract was used per strip. Proteins were reduced prior to electrophoresis. The undegraded EM- β galFP2 and β -gal bands are arrowed.



2.4 DISCUSSION

2.4.1 λ gt11 LIBRARY CONSTRUCTION AND ANALYSIS

cDNA was successfully synthesized from message-enriched RNA isolated from the protoscolex life-cycle stage of *E. multilocularis*. This cDNA was cloned into the *E. coli* expression vector λ gt11 to yield a gene library containing at least one million recombinant clones. A library of this size should have contained cDNAs representing most, if not all, genes expressed by this life-cycle stage (Maniatis *et al.*, 1982). Colorimetric assay showed the library contained 95% recombinant clones, however, cDNA hybridisation showed only 13% of the amplified clones to contain cDNA inserts. The large discrepancy between these figures may have been the result of the relatively low specific activity of the probe (2×10^5 cpm μg^{-1}). This would have allowed detection by autoradiography only of those clones with cDNA sequences which corresponded to abundant parasite mRNA species i.e. clones containing cDNA molecules corresponding to rare parasite mRNAs went undetected. Other contributory factors may have been the greater incorporation of label into large cDNA molecules and the bias towards certain clones created when the library was amplified. Despite the difference in values obtained, this experiment confirmed that a large number of library phage did contain cDNA inserts.

Antibody screening with rabbit anti-EmAgA serum revealed 0.05% of amplified library phage to be expressing *E. multilocularis* peptides. It was considered that, as the antiserum was raised against whole parasite metacestode material, a higher proportion of clones expressing *E. multilocularis* peptides should have been detected. Numerous explanations could account for the result obtained, these include, 1) poor specific antibody titre in the probe serum to low abundance parasite proteins and to highly glycosylated parasite proteins whose protein epitopes are masked by carbohydrate moieties, 2) the inability of the prokaryotic expression system to recreate conformational epitopes, and 3) low levels of expression of certain fusion-peptides which were highly toxic to the *E. coli* host cells.

Similar figures for recombination and numbers of expressing clones have been obtained by Knight *et al.* (1986), with regard to the construction of adult *Schistosoma* spp. cDNA libraries using commercially

available λ gt11. These workers appeared, however, to have obtained a higher percentage of expressing clones, despite the fact that their figures were derived from screening with infection sera as opposed to a serum raised against total parasite proteins. This relatively high level of antigen expression was also obtained despite their use of total RNA as the substrate for cDNA synthesis. This strategy would presumably have resulted in the cloning of a greater number of non-antigenic ribosomal gene clones. Possible reasons for the greater levels of antigen gene expression achieved by this group are, 1) significantly, as they make no mention of amplifying their libraries prior to screening, it must be assumed that their data related to unamplified libraries, 2) there may be a higher level of antigen-encoding mRNAs within the schistosome adult life-cycle stage, compared with the protoscolex of *E. multilocularis*, 3) there may have been variation in cDNA synthesis and cloning efficiency i.e. small cDNA molecules would have been cloned with greater efficiency, 4) variation in the number of distinct antigens possessed by the respective parasite life-cycle stages, and 5) quality and specificity of the antiserum used for screening.

2.4.2 HUMAN INFECTION SERA

Immunoblotting analysis of the individual human anti-*E. multilocularis* and anti-*E. granulosus* infection sera intended for screening, produced results consistent with previous findings.

The work of Lingelbach and Hinz (1984), where the serum from one AE patient was tested by immunoblotting against *E. multilocularis* metacestode antigens, revealed major bands at approximate M_r s of >94, 87, 86, 80, 70, 67, 61, 55, 44, 42, 41, 39, 38, 37, 36, 27 and 20 kDa. None of the human anti-*E. multilocularis* infection sera tested, produced a major 94 kDa band but a very weak signal at this approximate M_r was, however, produced by four sera. The 87 and 86 kDa bands were produced by all sera, but the 80 and 70 kDa antigens were only recognised by seven sera. Four sera produced a strong signal in the region of the 67 kDa antigen. Several weak bands were produced by most sera in the region of the 61 and 55 kDa antigens. Distinct bands corresponding to the 44, 42 and 41 kDa antigens were observed with all sera, except for serum number 4. Conversely, a strong 39 kDa band was produced by this serum alone. Minor bands in the 38,

37 and 36 kDa regions could be seen in most serum lanes, but they were quite distinct with serum number 7. The level of antibody specific to the 27 and 20 kDa antigens appeared to be highly variable among the sera tested. Four sera produced a major 27 kDa band, the remainder producing either a weak or negative response at this M_r . Five sera produced a major 20 kDa band, the remainder producing no detectable response at this M_r .

Generally, most of the anti-*E. multilocularis* sera tested produced antigen recognition profiles which correlated well with previous findings and could, therefore, be used for screening. Serum number 4 was the only one to deviate significantly, producing a largely unique antigen recognition profile. The reason for this may have been chemotherapeutic treatment of this patient, the serum was not, therefore, used. The individual minor discrepancies apparent in the remaining sera highlighted the large degree of variability in specific antibody titre that can occur between different patients infected with the same parasite. Inconsistencies between this data and that obtained previously could have resulted from differences in the experimental systems used and any strain-related differences in the parasite material used as the antigen source. It is now well recognised that *E. granulosus* exists as a complex of genetically distinct strains (McManus and Smyth, 1986) and *E. multilocularis* from Europe, Alaska and central North America has been shown to differ in a variety of characteristics such as morphology, pathogenicity, developmental characteristics and host specificity (Thompson, 1986). However, genetic variation has yet to be reported for *E. multilocularis*.

Of the individual human anti-*E. granulosus* infection sera tested, all produced the antigen recognition profile that had been reported by the two independent laboratories, Gottstein *et al.* (1987 b) and Shepherd and McManus (1987). Both groups tested human anti-*E. granulosus* infection sera with immunoblotted sheep hydatid fluid antigen. The resulting antigen recognition profile, in both cases, consisted of major bands at 67, 55, 41, 38, 26, 20, 16 and 12 kDa. Shepherd and McManus (1987) have suggested that the 20, 16 and 12 kDa components are the subunits of antigen B and the 20 and 38 kDa components are the subunits of antigen 5. Antigen B was shown to be *E. granulosus*-specific by these workers, making it a potential candidate for the immunodiagnosis of CE. The consistent antigen recognition profiles of the human anti-*E. granulosus* sera enabled all of them to be

used for screening.

Following pooling of the individual human infection sera, both anti-*E. multilocularis* and anti-*E. granulosus* serum pools were tested by immunoblotting with EmAgA to ascertain the appropriate dilution of each serum pool for screening. From the intensities of the bands it could be seen that signals of equivalent strength were obtained with the anti-*E. multilocularis* serum pool diluted 1 in 100 and the anti-*E. granulosus* serum pool diluted 1 in 25. This discrepancy substantiated previous findings that a generally lower immune response is produced by CE patients relative to AE patients (Schantz and Gottstein, 1986; Craig, 1988). Cross-reactive antigens, not recognised by NHS, of 88, 70, 68, 55, 27 and 12 kDa were visible at these serum dilutions. Lingelbach and Hinz (1984) found using a similar approach, antigens of the same M_r s to be cross-reactive. They also found, however, that the 39, 37, 36 and 20 kDa *E. multilocularis* antigens demonstrated cross-reactivity with a pool of human anti-*E. granulosus* infection sera. This was not confirmed for one or more of the following possible reasons, 1) variation in experimental technique and antigen source, 2) low antibody titre of infection sera, and 3) differences in patient immune response producing variable antigen recognition. From the immunoblot data obtained in this study, putatively *E. multilocularis*-specific antigens of 44, 42, 41, 20 and 16 kDa could be seen. Putatively *E. multilocularis*-specific antigens of 42, 41 and 39 kDa were revealed by the work of Lingelbach and Hinz (1984). The ability of the serum pools, at the appropriate dilutions, to recognise previously documented conserved and unique *E. multilocularis* antigens established their suitability for library screening.

2.4.3 DIFFERENTIAL SCREENING

The differential screening technique was used by Stahl *et al.* (1984) to isolate λ gt11-amp3 *Plasmodium falciparum* recombinant cDNA clones which coded for the S antigen of strain FC27. Human infection sera with positive or negative reactivity to this antigen were used to differentially screen lysogenic colonies, bound antibody being detected by ^{125} I-labelled protein A. These workers found large variations in the consistency of recombinant antigen recognition, which resulted from numerous factors. These

included the screening conditions, the dilution of the antisera, the antibody titre of antisera with regard to other antigens and the reversion of some lysogenic colonies to non-producing mutants. Despite these problems, by extensive optimisation of the assay procedure, these workers successfully isolated clones encoding the S antigen without the prior need for purification of the native antigen or production of specific anti-S antigen serum by conventional methods.

A variation of this approach has been used by Knight *et al.* (1986) to isolate species-specific *S. mansoni* and *S. japonicum* λ gt11 cDNA clones. These workers differentially screened plaques, as opposed to lysogenic colonies, using mouse anti-*S. mansoni* and anti-*S. japonicum* infection sera and detected bound antibody using HRP-conjugated second antibody. They successfully identified 6 putatively specific *S. mansoni* clones and 4 putatively specific *S. japonicum* clones using this procedure.

For the present study, it was hoped that by using plaque instead of colony immunoassay, that the problems associated with high anti-*E. coli* background could be avoided, together with the problem of loss of expressing colonies due to mutation. The use of less sensitive HRP-conjugated second antibody, as opposed to radiolabelled protein A, should, it was hoped, also decrease the likelihood of ambiguous signals being generated. A great deal of work would have been required to test all antigenic clones with a range of individual patient sera to determine their putative species-specificity. For this reason, differential screening of plaques with human serum pools was adopted as a preliminary screen to identify potentially *E. multilocularis*-specific clones. These were then further characterised using pooled and individual infection sera, by standard immunological techniques, to establish their full immunodiagnostic potential.

The differential screening approach resulted in the identification, at the immunological level, of 13 putatively *E. multilocularis*-specific clones. Antibody binding reactivity of the 13 clones was confirmed using a spot-lysis technique. This demonstrated that three levels of reactivity were represented within the 13 clones. With the anti-*E. multilocularis* serum pool, the strongest signal was produced by one clone, moderate signals by four clones and weak signals (equivalent to the background reactivity of λ gt11) by the remaining eight clones. With the

anti-*E. granulosus* serum pool, 12 clones produced weak signals equivalent to that of λ gt11 and one clone a strong signal that was thought to be artefactual. Five immunodominant, putatively *E. multilocularis*-specific clones were, therefore, identified.

2.4.4 THE ANALYSIS OF PUTATIVELY SPECIFIC CLONES

Sibling analysis showed that 5 of the 13 clones might represent 4 different antigen genes. Absolute confirmation of this was not obtained using this technique, however, as different fragments of the same antigen gene could have been present in several clones. These clones may have appeared to be unique by immunoassay but each may have been expressing a different set of epitopes on the same native antigen. The probability of this being the case would have been small, however, as the clones would have contained the same 3' end of the antigen gene but the length of the fragments may have varied depending on the efficiency of the original cDNA synthesis reaction. This would have produced varying degrees of reactivity, as opposed to an 'all or nothing' response.

Insert analysis demonstrated that cDNA molecules could be excised from clones, indicating that no damage to the Eco R1 cloning site had occurred during library construction. The insert sizes proved that cDNA sequences from approximately 300 bp to at least 1 000 bp had been cloned.

2.4.5 FUSION-PEPTIDE ANALYSIS

All 13 putatively specific clones synthesized fusion-peptides with M_r s greater than that of β -gal, ranging from the smallest produced by clone 1 to the largest produced by clone 4. No degradation was observed as reported for certain *P. falciparum* β -gal fusions (Kemp *et al.*, 1983).

By virtue of its' antigenicity, the fusion-peptide (EM- β galFP4) produced by clone 4 (EM4) was chosen for further analysis. A second clone, clone 2 (EM2), was also selected for further study from the moderately immunogenic clones (numbers 1, 2, 3 and 6), on the basis of the relatively large size of its' fusion-peptide (EM- β galFP2). Sibling analysis showed these clones were probably not related.

ELISA was used as the basis for immunodiagnosis. This

technique had proved successful in conjunction with the *E. multilocularis*-specific native antigen Em 2a, identified, characterised and tested by Gottstein *et al.* (1983) and Gottstein (1985). To facilitate testing of EM- β galFP2 and EM- β galFP4 by ELISA, these fusion-peptides had first to be purified from *E. coli* proteins. Electroelution from SDS-PAGE gels has been shown to be a rapid, simple technique facilitating fusion-peptide purification with little *E. coli* protein contamination (Carroll and Laughon, 1987). Recombinant *Brugia pahangi* β -gal fusion-peptide antigens purified in this manner have been successfully used with ELISA (Selkirk *et al.*, 1986; 1987). EM- β galFP2 and EM- β galFP4 were purified using this technique and their putative *E. multilocularis*-specificity appeared to be retained when tested by immunoblotting with human anti-*E. multilocularis* and anti-*E. granulosus* infection serum pools.

EM- β galFP4 was incorporated into ELISA and appeared to retain putative *E. multilocularis*-specificity when tested with a range of human infection serum pools. EM- β galFP2 could not be incorporated successfully into ELISA, possibly because of its' relatively weak antigenicity combined with inefficient binding to the ELISA plate in the presence of residual SDS. Immunoblotting was used to assess its' putative *E. multilocularis*-specificity with human infection serum pools. This technique had been used by Blaxter *et al.* (1988) and Vogel *et al.* (1988) to identify potentially immunodiagnostic *Leishmania donovani* and *E. multilocularis* recombinant β -gal fusion-peptide antigens, respectively. A significant level of antibody directed towards EM- β galFP2 was only present in the human anti-*E. multilocularis* infection serum pool.

CHAPTER 3

3 THE DEVELOPMENT OF EM2 AND EM4 CLONED ANTIGENS FOR IMMUNODIAGNOSIS

3.1 INTRODUCTION

3.1.1 DIAGNOSTIC ANTIGENS

Antigens which are suitable for immunodiagnostic use must satisfy certain criteria. These can be summarised as follows: 1) The antigen must be species-specific to eliminate cross-reactivity, which could lead to a false-positive diagnosis, 2) The antigen must demonstrate high sensitivity whereby it must be recognised by, ideally, all infection sera, eliminating the possibility of false-negative reactions, 3) The antigen should stimulate a host immune response rapidly following infection and this response should be sustained throughout the course of infection. This would facilitate early diagnosis, allowing appropriate action to be taken prior to the onset of complications brought about by prolonged growth of the parasite, and would also allow monitoring of the condition following treatment, 4) The antigen should be compatible with simple and sensitive immunological assays such as ELISA, and 5) The antigen must be amenable to purification in bulk quantities by simple, inexpensive procedures, to yield large quantities of pure, well characterised reagent.

3.1.2 THE DIAGNOSTIC POTENTIAL OF CLONED ANTIGENS

The diagnostic antigen Em 2a, isolated by Gottstein *et al* (1983) and characterised by Gottstein (1985), has been shown to demonstrate a high level of species-specificity and sensitivity (both approximately 95%) when used in a diagnostic ELISA test (Gottstein *et al*, 1987 a). This antigen, therefore, satisfies several of the criteria mentioned above.

The immunodiagnostic potential of this antigen was unknown following its original isolation, although, by virtue of the purification procedure employed, it was known to be a putatively specific molecule. Its diagnostic efficacy had, therefore, to be evaluated by extensive testing

with human infection sera from various confirmed parasitic disease patients. This evaluation proved this antigen to demonstrate high specificity and sensitivity, making it a good candidate for diagnosis.

The major drawback associated with this antigen relates to its production. Classical affinity chromatography, used to purify this antigen (Gottstein *et al.*, 1983), although amenable to large scale production, would require constant monitoring to ensure consistent levels of purity. A constant supply of large amounts of well characterised *E. multilocularis* material would also be required.

Cloned antigens can largely circumvent the problems associated with production but expression in heterologous systems can result in altered antigenicity which, in turn, can affect specificity and sensitivity. Carrier proteins fused to antigens, in the case of fusion-peptides, can also confer undesirable characteristics to expressed peptides when they are used in immunological assays. These problems prompted our decision not to attempt to clone the gene corresponding to Em 2a. Consequently, the immunodiagnostic value of any putatively specific cloned antigens isolated, would require assessment. In addition, even if an antigen clone corresponding to Em 2a were isolated, this recombinant antigen would still require extensive diagnostic evaluation on account of the changes that may have taken place in the heterologous expression system.

The testing of any cloned antigens intended for use in immunodiagnosis would, therefore, have to be carried out using the same strategy as that employed for the classically purified Em 2a antigen. The diagnostic potential of the two putatively specific recombinant clones EM2 and EM4, was assessed using this approach and was made feasible because of the availability of a large number of characterised human sera. Recently, this strategy has been used to confirm the diagnostic potential of cloned antigens of *Leishmania donovani* (Blaxter *et al.*, 1988) and *E. multilocularis* (Vogel *et al.*, 1988).

3.1.3 THE USE OF λ gt11 RECOMBINANT ANTIGENS FOR DIAGNOSIS

Relatively little work has been performed, to date, regarding the direct application of recombinant antigens to immunodiagnostic tests. Recent studies by Blaxter *et al.* (1988) and Vogel *et al.* (1988) have used

immunoblotting in conjunction with β -gal fusion-peptides, synthesized by λ gt11 recombinants, as the basis for testing potentially diagnostic antigens with human infection sera. Selkirk *et al.* (1987) have successfully used a purified *Brugia pahangi* β -gal fusion-peptide antigen in ELISA, to demonstrate variations in antibody titre with differing clinical conditions. One group of workers have suggested that diagnostic *S. mansoni* β -gal fusion-peptides would not be suitable for this type of assay (Klinkert *et al.*, 1988), the major reason being the presence of cross-reacting anti- β -gal antibodies within human sera, which would require removal by preabsorption, prior to testing.

The level of synthesis of fusion-peptides by λ gt11 lysogens is generally lower than that achieved using plasmid expression systems (Shatzman and Rosenberg, 1987). Also, the purification of these peptides from lysogen lysates can often be difficult as a result of insolubility problems created by the presence of the large β -gal carrier protein (Bitter, 1987). The lack of a convenient method whereby large quantities of non-denatured fusion-peptide, completely free of *E. coli* contaminants, can be obtained, is a significant drawback. The majority of β -gal fusion-peptide purification procedures, reviewed by Carroll and Laughon (1987), result in denaturation of the product. This may have deleterious effects upon the antigenicity of recombinant peptides as secondary structure epitopes would be lost, reducing their efficacy as immunodiagnostic reagents.

Gel filtration chromatography is a procedure which enables the effective purification of soluble, non-denatured β -gal fusion-peptide (Carroll and Laughon, 1987). This technique takes advantage of the large size of β -gal fusion-peptides to facilitate their purification from smaller *E. coli* proteins. The fusion-peptides must, however, be soluble in aqueous solution. This is not always the case, on account of high M_r and the nature of the foreign peptide. Should the recombinant peptide prove to be soluble, however, this technique will allow its purification from the majority of *E. coli* proteins, although, some contamination is often apparent. This technique allows secondary structure epitopes to be maintained, possibly enhancing the diagnostic properties of fusion-peptide antigens. Also, SDS is not used in the purification process, and this may alleviate the ELISA plate binding problem associated with EM- β galFP2. Problems relating to yield and β -gal/*E. coli* cross-reactivity, would still, however, have to be surmounted.

The λ gt11 expression system and the resultant β -gal fusion-peptides, whether purified in the denatured or non-denatured state, retain certain inherent characteristics that make the system and its products unfavourable for use as a source of immunodiagnostic recombinant antigens. In an attempt to overcome these problems, one group of workers (Felleisen *et al*, 1988) have identified using deletion mutants generated by Bal31 exonuclease treatment, the antigenic regions on a recombinant *S. mansoni* diagnostic antigen for subsequent chemical synthesis as oligopeptides, thereby eliminating the need for carrier proteins and efficient purification techniques. However, detailed characterisation of this type is time consuming and may not yield diagnostic reagents with the desired properties. Consequently, the approach taken in the current study to alleviate the problems associated with β -gal fusion-peptides was to synthesize the candidate antigens, following identification and isolation in λ gt11, using an alternative *E. coli* expression system.

3.1.4 ALTERNATIVE *E. COLI* EXPRESSION SYSTEMS SUITABLE FOR THE PRODUCTION OF IMMUNODIAGNOSTIC ANTIGENS

The problem of poor expression of β -gal fusion-peptides associated with λ gt11 has been rectified by R  ther and M  ller-Hill (1983). These workers constructed a set of six plasmid expression vectors, pUR 278, 288, 289, 290, 291 and 292. These produce β -gal fusion-peptides identical to those synthesized by λ gt11, but the higher levels of expression obtained make this system more suitable for producing large quantities of fusion-peptides. This expression system does not, however, overcome any of the immunodiagnostic problems associated with β -gal fusion-peptides.

Other vectors, such as the pEX1-3 family (Stanley and Luzio, 1984), and the pTTQ series (Stark, 1987), have been developed. These plasmids produce a *cro-lacZ* gene fusion product and a *lacZ* α fusion product, respectively. Unfortunately, the presence of the *LacZ* gene may result in anti- β -gal antibody binding to the fusion-peptides. The *lacZ* region of pTTQ hybrid peptides is relatively small, however, which may help to reduce this problem. The fusion-peptides synthesized would offer no improvement with regard to purification.

Vectors constructed recently, pNGS18(+), pNGS20(+), pNGS20(-)

and pNGS21 (Stoker *et al.*, 1989), express high levels of foreign peptide fused to a short 2 kDa leader sequence, which contains a run of six consecutive asparagine residues followed by the start of the *lacZ* gene. The recombinant proteins produced have no apparent background reactivity with human sera. This vector does not, however, provide any convenient method by which to separate the recombinant peptide from bacterial lysates. Gel filtration may be possible, but this is prone to *E. coli* contamination.

One vector, pKK233-2, has been used to express chicken triosephosphate isomerase (Straus and Gilbert, 1985). This protein was expressed in its native form without fusion to a carrier peptide, and was shown to be active. The ability to express diagnostic antigens without fusion to other peptides such as β -gal would remove any possibility of background reactivity with human sera. Once again, however, isolation of the antigen from *E. coli* lysates, could prove difficult.

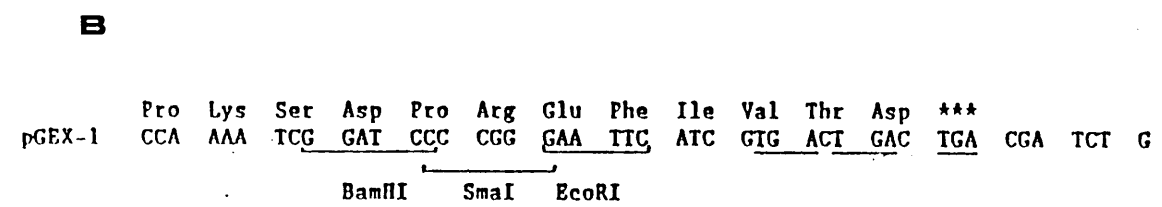
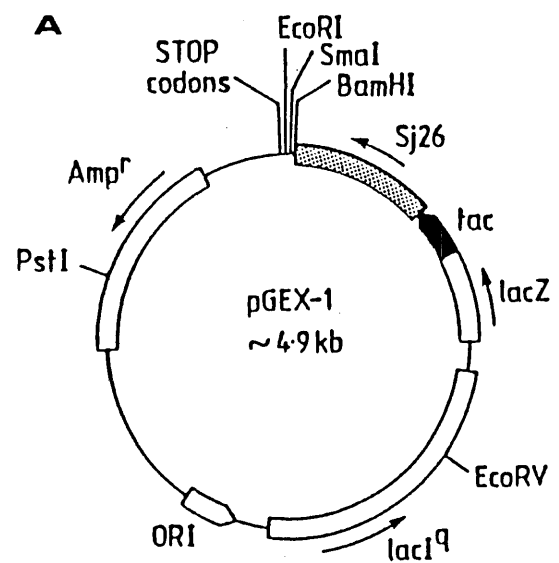
Another vector, pEx34b, constructed by Klinkert *et al.* (1988) in an effort to reduce anti- β -gal background binding, produces recombinant peptides fused to bacteriophage MS2 RNA polymerase. The hybrid proteins have been used in ELISA as diagnostic reagents and have shown no anti- β -gal cross-reactivity with human sera. The purification procedure used to obtain these fusion-peptides was very laborious, however, resulting in poor yields of denatured peptide. This aspect has led these workers to adopt a different strategy for the production of immunodiagnostic reagents (Felleisen *et al.*, 1988).

Perhaps the most promising vector system, to date, for this particular application, has been developed by Smith and Johnson (1988). These workers have constructed a series of vectors, pGEX1-3, which allow the synthesis of foreign polypeptides in *E. coli* as fusions with the C-terminus of a 26 kDa glutathione S-transferase (GST) derived from *S. japonicum*. The structure of the pGEX-1 vector is shown in Figure 3.1. The majority of fusion-peptides produced by these vectors are soluble and by virtue of the GST carrier protein, can be purified from bacterial lysates by a very rapid, simple and inexpensive affinity chromatography step using immobilised glutathione. This purification uses non-denaturing conditions and hence, no significant reduction in antigenicity should occur. The high level of expression, initiated by a *tac* promoter, coupled with the efficient

Figure 3.1 Structure of the pGEX-1 vector (After Smith and Johnson, 1988).

(A) Schematic representation of the plasmid vector.

(B) Nucleotide sequence at the C-terminus of the 26 kDa *S. japonicum* GST (Sj26) cDNA showing the reading frame of the Eco R1 cloning site.



purification procedure, allow high yields of pure, non-denatured recombinant peptides to be produced.

An important aspect of two of the pGEX vectors is that pGEX-2 and 3 contain specific protease cleavage sites at the junction of the GST carrier protein and the foreign peptide. The carrier protein and any uncleaved fusion-peptide can be removed, following protease digestion, by absorption on immobilised glutathione. Pure, native foreign peptide can, therefore, be recovered. This may be an important facility should the *S. japonicum* GST carrier molecule interfere with immunodiagnostic assays. The Eco R1 cloning site in these two vectors is, however, out of frame with λ gt11, several additional DNA manipulations would be required, therefore, to allow the correct antigenic peptide to be expressed.

The small GST carrier protein (26 kDa), relative to β -gal (116 kDa), may have less influence on the folding patterns of antigenic proteins and would not obscure epitopes to the same extent. It has also been shown that despite the amino-acid sequence identity of 42% between the 26 kDa *S. japonicum* GST and rabbit GST Yb, isozyme, no immunological cross-reactivity has been observed between *S. japonicum* GST and rabbit GST isozymes (Smith *et al.*, 1986). This may also hold true for human GST isozymes as it has been reported (Taylor *et al.*, 1988) that there is no significant immunological cross-reactivity between a 28 kDa *S. mansoni* GST and human GST. The use of a GST carrier protein may, therefore, reduce the background problem associated with β -gal fusion-peptides. Problems of cross-reactivity leading to false positive diagnoses may be experienced, however, if antibodies to GST are produced on account of other parasitic infections, particularly schistosomiasis (Taylor *et al.*, 1988). It is unlikely, however, that such zoonoses would be extensively found in the areas where

AE is a problem. No evidence has yet been forthcoming suggesting that taeniid infections stimulate an anti-GST response. GST fusion-peptides may, therefore, be suitable for diagnosis of these parasites. For these reasons, and the fact that pGEX-1 offers an Eco R1 cloning site in frame with that of λ gt11, this vector was used to synthesize one of the potentially immunodiagnostic *E. multilocularis* antigens.

3.1.5 THE IMMUNE RECOGNITION OF RECOMBINANT ANTIGENS

The importance of early and sustained recognition of diagnostic antigens by the host immune system has been discussed earlier. In order to fully evaluate the immunodiagnostic potential of the cloned antigens, it was necessary to establish the time of onset of their immune recognition following infection, and to determine the length of time for which this recognition was sustained.

Specific data regarding the onset of host recognition of the Em 2a antigen is not available. This is because most AE patient sera tested, to date, have been obtained from confirmed AE cases, which could only be diagnosed by conventional methods when the condition was well advanced. Human sera obtained soon after infection are, therefore, not easily found. In order to make an accurate estimation of the time taken between infection and onset of immune response, sera taken at the time of infection and at regular intervals thereafter is required. This is not always possible with human sera as the precise time of infection may be impossible to obtain. Furthermore, positive diagnosis may only be possible after a long time period, by which time the metacystode is well established. The ethical implications of using humans as a regular source of infection sera presents a further problem.

The most convenient source of well characterised infection sera is an animal model maintained in the laboratory. An *E. multilocularis* metacystode stage infection can be produced in cotton rats by intraperitoneal passage of protoscolex material (Rau and Tanner, 1972). The infection establishes and matures within a relatively short time (Rausch, 1975) and forms the basis of a suitable model with which to follow the time course of immune response to infection. It has been established (Ali-Khan, 1974; Ali-Khan and Siboo, 1982) that a strong humoral immune response is generated by mice experimentally infected with *E. multilocularis*. Lingelbach and Hinz (1984) have also shown by immunoblotting, that sera obtained 90 days postinfection from intraperitoneally *E. multilocularis* infected NMRI-mice were capable of recognising some of the major metacystode antigens detected by a human anti-*E. multilocularis* infection serum. Mouse infection is, therefore, a potentially useful model for the study of immune recognition over time, particularly as it may closely parallel some aspects

of human infection. However, this type of model system can not mimic all aspects of the immunological situation found in human infections for several reasons, 1) the introduction of parasite material into the peritoneal cavity does not allow true reproduction of the immune responses stimulated by the invading oncosphere and the initial development of the larval mass, 2) the metacestode develops generally in the peritoneal cavity of the animal and not the liver, the usual site of primary human infection, 3) cyst development within the natural intermediate rodent host differs from that in man; human cysts, for example, are usually sterile, with no protoscoleces (Rausch and Wilson, 1973) but rodent cysts, on the other hand, are usually fertile, containing many protoscoleces (Rausch, 1975), and 4) the time course of metacestode development differs between rodent and human hosts. Development occurs rapidly in the natural rodent host, protoscoleces being produced within 2-4 months (Rausch, 1975), with a following cessation in cyst growth (Rausch and Wilson, 1973). In man, however, cyst growth is slow, but can continue indefinitely for many years (Rausch and Wilson, 1973).

Despite the discrepancies between the human infection and the animal model, this was the only convenient method of obtaining the required characterised sera.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

3.2.1.1 Antigens

EmAgA and EgSCF antigens were prepared as described previously (section 2.2.1.1).

E. multilocularis pure protoscolex antigen (EmAgPP) was prepared from pepsinised protoscoleces (section 2.2.2.1.1). The purified protoscoleces were boiled for 5 minutes in SDS-PAGE sample buffer under reducing conditions (5% (v/v) 2-me). Following removal of insoluble debris by centrifugation at 15 000 g for 2 minutes, the supernatant was loaded directly onto SDS-PAGE gels. Protein concentration was estimated previously by the Bradford method (Bradford, 1976), 1 ml of packed protoscoleces containing approximately 100 mg of protein.

Fusion-peptide antigens were prepared and in the case of EM- β galFP4, purified by electroelution from SDS-PAGE gels as described previously (section 2.2.2.7).

3.2.1.2 Antisera

Individual human infection sera were obtained from,

E. multilocularis: 37 European and 12 Alaskan patients. Total 49.

E. granulosus: 8 Kenyan and 3 U.K. patients. Total 11.

T. solium: 8 Zimbabwean and 3 Mexican patients. Total 11.

A summary of the case histories of individual patients, is presented in Table 3.1.

Mouse anti- β -gal, anti-EM- β galFP2 and anti-EM- β galFP4 sera were prepared as described previously (section 2.2.1.2).

TABLE 3.1

Summary of patient case histories

Serum No:	Origin	Clinical manifestations and treatment
<i>E. multilocularis</i> infection sera		
1 ^a to 17	Europe	No data available
18 & 19	"	No chemotherapy
20 to 25	"	Mebendazole chemotherapy
26	"	No chemotherapy
27	"	Mebendazole chemotherapy
28	"	No chemotherapy
29 to 34	"	Mebendazole chemotherapy
35 to 37	"	No chemotherapy
38 to 49	Alaska	No data available
<i>E. granulosus</i> ^b infection sera		
1	Kenya*	12cm liver cyst & DC. No surgery
2	"	Liver & abd. cav. cysts. DC. Previous operation
3	"	No cyst detected by ultrasound
4	"	Liver & abd. cav. cysts. 1 cyst >16cm. DC. 3 ops
5	"	No cyst detected by U/S, possibly lung cyst
6	"	14cm liver cyst. DC. Previous operation
7	"	Liver cysts. 1 cyst >12cm. DC. Previous operation
8	"	Liver & abd. cav. cysts. DC? Previous operation
9	U.K.	Recently started on albendazole. No further data
10	"	2 liver cysts. Previous operation
11	"	No data available
<i>T. solium</i> infection sera		
1 to 8	Zimbabwe	No data available
9 to 11	Mexico	No data available

DC Daughter cysts. Presence of DC confirmed that cysts were viable

^a Sera 1 to 11 were included in the pool used for differential screening of the cDNA library

^b Sera used for differential screening of the cDNA library

* None of these patients were drug treated

3.2.2 METHODS

3.2.2.1 SDS-PAGE, immunoblotting and ELISA

SDS-PAGE, immunoblotting and ELISA were performed as described previously (section 2.2.2.3). For interpretation of seropositivity by ELISA when using individual human infection sera, a threshold value was calculated by taking the mean OD_{492nm} absorbance value plus 2 standard deviations (S.D.), obtained by testing the normal human serum pool of 4 U.K. sera a total of 8 times against the relevant antigen. Test sera mean OD_{492nm} absorbance values equal to, or greater than the threshold, were considered as positive.

3.2.2.2 The purification of EM- β galFP4 by gel filtration chromatography

The technique used was as described by Carroll and Laughon (1987). In order to maximise yield of EM- β galFP4 peptide, the λ gt11-EM4 lysogen was 'cured' of the pMC9 (*lacP*) ampicillin resistance plasmid. Complete removal of the *lac* repression results in a 3 to 4 fold increase in yield, although, over long time periods this may lead to selective pressure for mutations of the fusion protein. A permanent stock of pMC9-containing lysogen was retained in the event of loss of the lysogen through negative selection. The removal of the pMC9 plasmid was accomplished by the repeated growing of the lysogen on normal LB plates without ampicillin. Loss of drug resistance was monitored by replica plating on LB plates with or without ampicillin. Approximately 8 to 12 cycles of growth were required to achieve loss of the plasmid.

1 litre cultures of the cured lysogen were grown as before (section 2.2.2.6) but, following heat shock at 42 °C, no IPTG was added prior to incubation at 37 °C. The cells were harvested and lysed as before and the lysate was made 0.5 M with respect to NaCl. Following mixing, the extract was centrifuged at 15 000 g for 10 minutes at 4 °C to clarify. 0.1 volumes of 30% (w/v) streptomycin sulphate solution were added to the supernatant and the solution was mixed for 15 minutes at 4 °C followed by centrifugation at 15 000 g for 10 minutes at 4 °C. To the supernatant was added 25% (v/v) saturated $(NH_4)_2SO_4$ pH 8.0 dropwise while gently stirring

at 4 °C. The percentage of $(\text{NH}_4)_2\text{SO}_4$ pH 8.0 required to precipitate the fusion-peptide was determined previously by SDS-PAGE analysis of aliquots removed while adding between 25% and 40% (v/v) $(\text{NH}_4)_2\text{SO}_4$ pH 8.0. (data not shown). Stirring was continued for a further 30 minutes followed by centrifugation at 15 000 g for 30 minutes at 4 °C. The supernatant was discarded and the residual $(\text{NH}_4)_2\text{SO}_4$ was removed by centrifuging the pellet at 4 000 g for 1 minute. The pellet was dissolved in 2 ml of column buffer [150 mM NaCl/20 mM 2-me/1 mM PMSF/0.1% (v/v) Triton X-100/20 mM Tris-HCl pH 7.5]. The solution was centrifuged at 15 000 g for 5 minutes at 4 °C before applying the supernatant to the gel filtration column.

The column used was 2 cm × 54 cm (LKB) containing Sephacryl S-300 (Pharmacia), equilibrated in column buffer, as the separation medium. The column was constructed according to the manufacturer's instructions and was calibrated using molecular weight markers (Sigma) (data not shown). The flow rate was adjusted to 1 ml min⁻¹ and following application of the sample, the elution profile was monitored by absorbance at OD_{280nm} (LKB Uvicord). The fractions were collected using an LKB fraction collector. The first major elution peak was found to contain the fusion protein following analysis of fraction aliquots by SDS-PAGE (data not shown). The peak fractions were pooled and the fusion-peptide recovered by precipitation with 25% (v/v) $(\text{NH}_4)_2\text{SO}_4$ pH 8.0. The pellet was redissolved in PBS and the protein concentration estimated by the Bradford method (Bradford, 1976). The yield of fusion-peptide was approximately 250 µg per litre of culture volume.

3.2.2.3 The subcloning of EM4 cDNA into pGEX-1

3.2.2.3.1 λgt11/EM4 phage DNA preparation

A fresh recombinant λgt11/EM4 plaque, picked from a lawn of Y1090 cells, was used to inoculate 50 ml of 10 mM MgSO₄/LB and the culture was grown overnight with shaking at 37 °C. Several drops of chloroform were added and shaking resumed for 10 minutes to ensure lysis of all cells. The lysate was centrifuged at 4 000 g for 10 minutes at ambient temperature. To the supernatant was added 1 µg ml⁻¹ DNase 1 and RNase A (Sigma) followed by incubation for 30 minutes at ambient temperature. Debris was pelleted at 8 000 g for 10 minutes at 4 °C and the supernatant was

centrifuged further at 35 000 g for 2 h at 4 °C to pellet the phage particles. Phage were resuspended in 0.5 ml SM overnight at 4 °C. DNase I and RNase A were added to 1 µg ml⁻¹ and the mixture was incubated for 30 minutes at ambient temperature. The phage heads were disrupted at 65 °C for 10 minutes and proteinase K was added to 1 µg ml⁻¹, followed by incubation for 30 minutes at ambient temperature. The solution was deproteinised by chloroform, phenol/chloroform and chloroform extractions. The DNA was precipitated overnight at -20 °C following the addition of 0.1 volumes of 3 M NaAc and 2.5 volumes of cold absolute ethanol. The DNA was recovered by centrifugation at 15 000 g for 10 minutes and the pellet was drained and dried under vacuum. Following resuspension in 10 µl of TE the DNA concentration and purity was estimated from OD_{260/280nm} absorbance readings.

3.2.2.3.2 EM4 cDNA subcloning into M13mp19

Prior to the pGEX-1 vector becoming available, the EM4 cDNA insert had been subcloned into M13mp19 to facilitate nucleotide sequencing. Reagents and protocols for M13 subcloning were supplied by BRL. M13mp19 RF DNA was cut with Eco RI and treated with bacterial alkaline phosphatase as described by the manufacturer. The λgt11/EM4 DNA was cut with Eco RI and prepared for ligation by phenol/chloroform extraction and ethanol precipitation. The entire λgt11/EM4 digest was 'shotgun' ligated into Eco RI cut and phosphatased M13mp19 RF DNA as described by the manufacturer. A molar ratio of cDNA insert to M13 vector of 2 : 1 was used in a total ligation reaction volume of 5 µl. Ligation products were used to transform competent JM101 *E. coli* host cells and these were plated out in the presence of IPTG and Xgal as described by the manufacturer. Following incubation of the plates at 37 °C overnight, the recombinant M13mp19 phage were identified according to colour selection. White turbid plaques were recombinant, blue turbid plaques were non-recombinant. Two recombinants were selected and grown according to the manufacturer's recommendations. Culture supernatants were retained for the preparation of single strand template DNA for sequencing and the pelleted cells were used to prepare M13mp19/EM4 double stranded RF DNA.

3.2.2.3.3 EM4 cDNA subcloning into pGEX-1

Small-scale plasmid preparations were used to produce sufficient quantities of M13mp19/EM4 RF DNA as described by Miller (1987). The DNA was restricted with Eco R1 and the products analysed on a 1% agarose gel (Maniatis *et al.*, 1982). A single DNA fragment of approximately 1 000 bp was produced by both recombinant clones (data not shown). This confirmed the presence of the EM4 cDNA insert within the M13mp19 subclones. A preparative amount of M13mp19/EM4 RF DNA was prepared from one of the recombinant clones as described by Miller (1987) and following restriction with Eco R1, the entire digest was electrophoresed on a 1% agarose gel. The position of the 1 000 bp EM4 insert was determined by visualisation under ultra violet light using a transilluminator, and the band was excised from the gel. The insert DNA was recovered by electroelution into a dialysis bag as described by Maniatis *et al.* (1982). Impurities were removed by phenol, phenol/chloroform and chloroform extractions and ethanol precipitation, followed by gel filtration through a sephadex G-50 column (Maniatis *et al.*, 1982).

pGEX-1 plasmid DNA was prepared (Miller, 1987) and Eco R1 cut and phosphatased as described previously. Ligation of EM4 insert with pGEX-1 was performed as described above. A molar ratio of purified EM4 insert to pGEX-1 vector of 2 : 1 was used in a ligation reaction volume of 5 μ l. The ligation products were used to transform competent TG2 *E. coli* host cells according to the procedure described by Miller (1987). The cells were made competent by CaCl_2 treatment (Miller, 1987). Transformed cells were plated out on 100 $\mu\text{g ml}^{-1}$ ampicillin/LB agar and incubated overnight at 37 °C.

Recombinant clones were selected by a colony immunoassay technique based on that used by Stahl *et al.* (1984) for screening lysogens. Following overnight growth, nitrocellulose filters soaked in 10 mM IPTG were laid momentarily over the surface of the colonies and orientated. They were removed carefully onto fresh Amp/LB plates with the transferred bacteria uppermost. The original plates were incubated overnight and constituted master plates of the transformants. The nitrocellulose filters were incubated for 3 h at 37 °C and the colonies lysed by placing filters on blotting paper saturated with 1% (w/v) SDS for 15 minutes, followed by exposure to a chloroform saturated atmosphere for 15 minutes. Bacterial

debris was removed by washing for 2 h at ambient temperature with TNT. All subsequent steps were as described previously for plaque immunoassay (section 2.2.2.4). Affinity purified rabbit anti-EM4 antibody was used to probe the filters, avoiding the excessive background produced when screening with whole serum. Colonies expressing the EM4 antigen were picked and glycerol stocks made (Maniatis *et al*, 1982).

Plasmid DNA was extracted from recombinant colonies and cut with Eco R1. Digestion products were analysed by agarose gel electrophoresis and visualisation of the 1 000 bp EM4 insert was indicative of genuine recombinants (data not shown).

3.2.2.4 **The production and purification of GST and GST/EM4 fusion-peptide**

pGEX-1 and recombinant pGEX-1/EM4 were used to produce GST and GST/EM4 fusion-peptide, respectively, according to the protocol of Smith and Johnson (1988). The growth medium used was SOB [2% (w/v) Bacto tryptone/0.5% (w/v) Bacto yeast extract/10 mM NaCl/2.5 mM KCl/ 10 mM MgCl₂/10 mM MgSO₄/pH 7.0] supplemented with 100 µg ml⁻¹ ampicillin. An overnight culture was diluted 1 in 10 in fresh medium and incubated for 1 h at 37 °C. Following the addition of 0.1 mM IPTG, the culture was incubated for a further 3 h at 37 °C. Cells were harvested by centrifugation and lysed by mild sonication at 4 °C. After the addition of Triton X-100 to 0.1% (v/v), insoluble material was removed by centrifugation at 15 000 g for 5 minutes at 4 °C. The supernatant was mixed with glutathione-agarose beads (sulphur linkage, Sigma) and following absorption for 2 minutes, the beads were recovered by centrifugation at 500 g for 1 minute at 4 °C. Unbound material was removed using 3 × 50 ml washes with PBS. Bound GST or GST/EM4 fusion-peptide was eluted with 5 mM reduced glutathione (Sigma)/50 mM Tris-HCl pH 8.0. Protein yield was estimated from OD_{280nm} absorbance readings using known concentrations of BSA as a standard. From a 100 ml culture, approximately 250 µg of fusion-peptide could be recovered. In the case of GST, approximately double this amount could be recovered from an equivalent culture volume. Aliquots of the protein preparations were analysed by SDS-PAGE to ensure sufficient purity.

3.2.2.5 **Mouse *E. multilocularis* infections and serial bleeding**

The mouse strain chosen for this study was BALB/c as this had been shown by Alkarmi and Ali-Khan (1984) to consistently produce higher levels of parasite biomass than certain other mouse strains following intraperitoneal inoculation with cystic material. Cotton rats were not used on account of the difficulties experienced in obtaining them inbred. Inbred animals were required to reduce the possibility of individual variability that may have led to inconsistent immune responses.

7 inbred female BALB/c mice were each inoculated intraperitoneally with approximately 6 000 purified protoscoleces suspended in 0.5 ml Hanks' saline. 3 control mice were inoculated with Hanks' alone. Protoscoleces were obtained after lightly homogenised cystic material, obtained from an infected cotton rat, had been sieved repeatedly through a fine mesh. The mice were bled immediately pre-infection and every 14 days thereafter for a total of 168 days. This length of infection was thought to be sufficient to allow fully mature, fertile cysts to develop, as large cyst masses have previously been found in BALB/c mice within 84 days of infection (Alkarmi and Ali-Khan, 1984).

On termination of the infection, the mice were killed by cervical dislocation and dissected. The location, number, size, mass and fertility of the cysts was assessed. The sera obtained were used to probe immunoblots of EmAgPP and crude lysogen lysates.

3.3 RESULTS

3.3.1 THE CHARACTERISATION OF HUMAN INFECTION SERA

The human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* infection sera were tested individually by ELISA prior to use, in order to confirm that they were of sufficient antibody titre to allow positive diagnosis using a crude parasite antigen extract. For interpretation of seropositivity, a threshold value was calculated using the mean OD_{492nm} absorbance value plus 2 S.D., obtained from testing of the NHS pool, diluted 1 in 500, a total of 8 times against the EgSCF antigen (2 µg ml⁻¹). Values equal to or greater than the threshold value were considered as positive. A mean OD_{492nm} absorbance value of $\bar{x} = 0.441$ (S.D. = 0.096) was obtained. The discrimination value was calculated using $\bar{x} + 2 \text{ S.D.} = 0.633$ (OD_{492nm}). This diagnostic criterion was adapted from that used by Gottstein *et al.* (1983). The individual sera were each diluted 1 in 500 and tested in duplicate against EgSCF (2 µg ml⁻¹) (data not shown). 49 anti-*E. multilocularis* sera, 11 anti-*E. granulosus* sera and 11 anti-*T. solium* sera (Table 3.1) were found to be positive and only these were used for subsequent testing of recombinant antigens.

3.3.2 THE IMMUNOLOGICAL CHARACTERISATION OF EM-βgalFP2

EM-βgalFP2 purified by electroelution did not bind to ELISA plates and hence could not be tested using this technique. Immunoblotting of this fusion-peptide, following separation from *E. coli* proteins by SDS-PAGE, appeared to be an alternative method which would facilitate immunological characterisation of this recombinant antigen (Fig. 2.12). In order to fully evaluate diagnostic sensitivity and specificity, immunoblots of crude EM2 lysogen lysate were probed with individual human infection sera.

3.3.2.1 The diagnostic sensitivity of EM-βgalFP2

To evaluate immunodiagnostic sensitivity, individual anti-*E. multilocularis* sera were tested against immunoblots of crude EM2 lysogen

lysate (Fig. 3.2). Of the 49 sera, 26 produced detectable levels of antibody binding greater than the background signal produced by NHS (lane 2), at the expected M_r of EM- β galFP2 (this data is summarised in Table 3.4). This antigen was, therefore, recognised by 53% of the sera tested. Signals were produced by serum numbers 7, 28, 41 and 44 (lanes 9, 30, 43 and 46, respectively), of similar or greater intensity than that produced by the anti-*E. multilocularis* pool of positive control sera (lane 1). Other positive sera produced a range of band intensities reflecting the variations in anti-EM- β galFP2 antibody titre present in different individual sera. From this data, it could be seen that approximately half of the anti-*E. multilocularis* sera tested contained anti-EM- β galFP2 antibody, resulting in relatively poor diagnostic sensitivity of this antigen.

Table 3.4 shows that recognition of this antigen was independent of serum origin. All sera obtained from patients who had not received drug treatment recognised this antigen. Recognition by sera from drug-treated patients was variable.

3.3.2.2 The diagnostic specificity of EM- β galFP2

To confirm diagnostic specificity, crude EM2 lysogen lysate immunoblots were tested with individual human anti-*E. granulosus* and anti-*T. solium* infection sera (Figures 3.3 A and 3.3 B, respectively). These results are summarised in Table 3.3. 8 of the 11 anti-*E. granulosus* sera (Fig. 3.3 A) produced a band at the M_r corresponding to EM- β galFP2, of greater intensity than that produced by NHS (lane 2). Serum numbers 1 (lane 3) and 6 (lane 8) produced signals equivalent to that detected with the anti-*E. multilocularis* pool of positive control sera (lane 1). The remaining positive sera (lanes 4, 5, 6, 7, 10 and 12) produced bands of reduced intensity. Of the anti-*T. solium* sera (Fig. 3.3 B), 4 of the 11 tested produced significant levels of anti-EM- β galFP2 antibody binding (lanes 4, 6, 7 and 11) which was greater than that produced by NHS (panel A, lane 2). Serum number 11 (lane 11) produced the strongest signal, although this was not as intense as that produced by the anti-*E. multilocularis* pool of positive control sera (panel A, lane 1). In total, therefore, 10 (45%) of the 22 heterologous sera tested did not cross-react with this antigen. It was observed (Table 3.3) that the cross-reactivity produced by the remaining

Figure 3.2 SDS-PAGE and immunoblotting of crude EM2 lysogen lysate with individual human anti-*E. multilocularis* infection sera.

Lane 1, pool of anti-*E. multilocularis* sera.

Lane 2, pool of normal human sera.

Lanes 3-51, individual anti-*E. multilocularis* sera numbers 1-49.

50 µg of crude lysate was used per strip. The extract was reduced prior to electrophoresis. The undegraded EM-βgalFP2 band is arrowed.

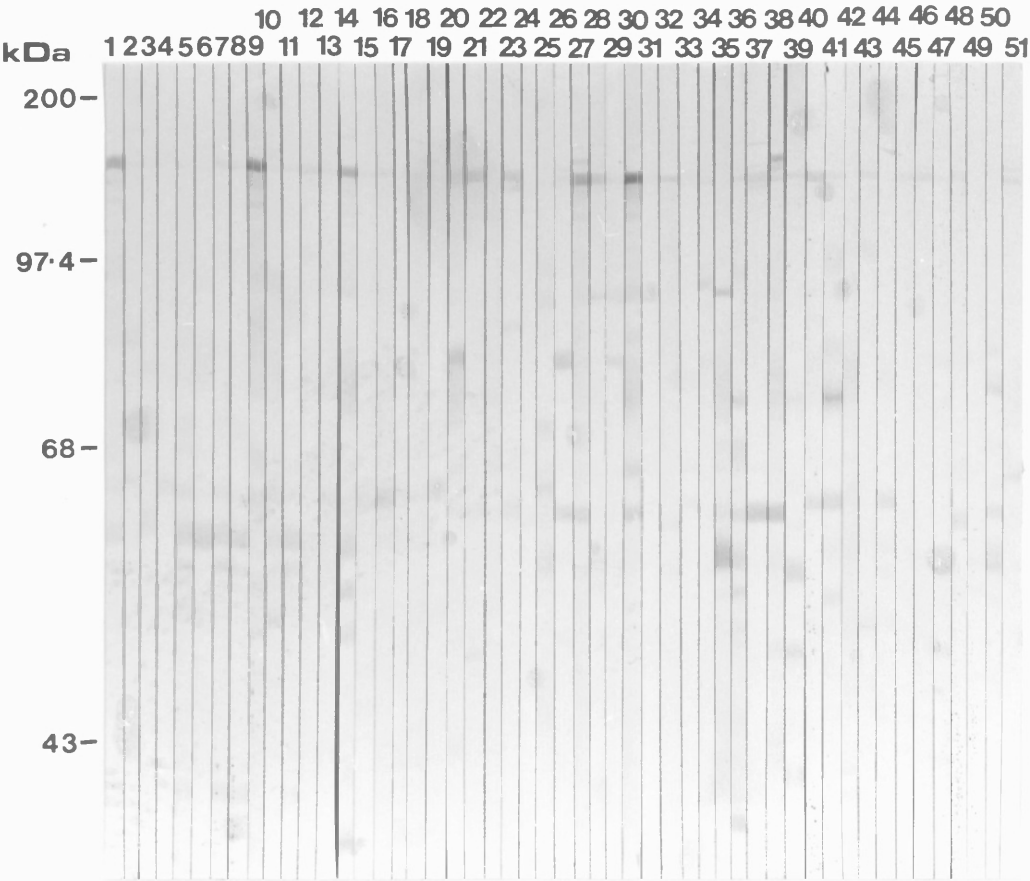
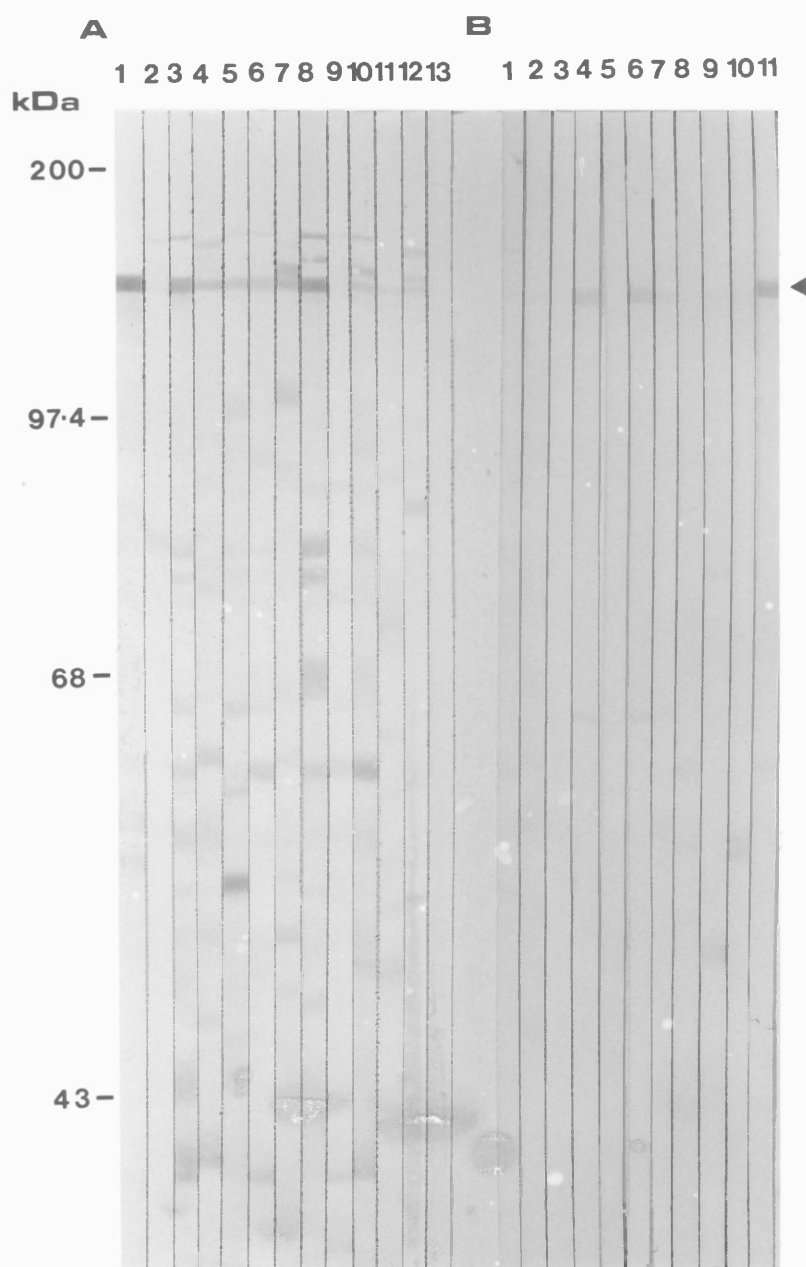


Figure 3.3 SDS-PAGE and immunoblotting of crude EM2 lysogen lysate with individual human anti-*E. granulosus* and anti-*T. solium* infection sera.

(A) anti-*E. granulosus* sera: lane 1, pool of anti-*E. multilocularis* sera; lane 2, pool of normal human sera; lanes 3-13, individual anti-*E. granulosus* sera numbers 1-11.

(B) anti-*T. solium* sera: lanes 1-11, individual anti-*T. solium* sera numbers 1-11.

50 µg of crude lysate was used per strip. The extract was reduced prior to electrophoresis. The undegraded EM-βgalFP2 band is arrowed.



sera was independent of the country of origin, as at least one serum from each of the four regions produced a positive reaction. No obvious correlation was evident between recognition of this antigen and the clinical characteristics of the *E. granulosus* infected patients.

The cross-reactivity produced with certain individual anti-*E. granulosus* and anti-*T. solium* sera revealed that this antigen was not *E. multilocularis*-specific at the immunological level, as was first demonstrated using pools of human infection sera (Fig. 2.14 A). Only 5 of the individual anti-*E. multilocularis* sera incorporated in the serum pool used to screen the library, recognised this antigen. This indicated a potential disadvantage to the use of pools of human infection sera for screening expression libraries. The poor levels of sensitivity and specificity produced by EM- β galFP2 would prohibit its use as a diagnostic reagent and thus further work was concentrated upon the EM4 antigen clone.

3.3.3 THE IMMUNODIAGNOSTIC VALUE OF THE EM4 RECOMBINANT ANTIGEN

3.3.3.1 The diagnostic sensitivity of EM- β galFP4

It was possible to use purified EM- β galFP4 in ELISA but because of the denatured state of this antigen, its diagnostic value may have been reduced. To verify this EM- β galFP4 was purified under non-denaturing conditions, to allow comparison of its sensitivity by ELISA, with its denatured counterpart. Purity of the antigen, following gel filtration chromatography, was assessed by SDS-PAGE (Fig. 3.4). Contamination of the fusion-peptide with *E. coli* proteins and/or fusion-peptide degradation products was evident (lane 2). The majority of contaminants appeared to correspond to major *E. coli* protein bands, evident in the soluble fraction (lane 1).

Native β -gal and denatured and non-denatured EM- β galFP4 were tested by ELISA using the 12 Alaskan human anti-*E. multilocularis* infection sera (Fig. 3.5 A, B and C, respectively). A higher concentration of the gel filtration purified antigen was used ($5 \mu\text{g ml}^{-1}$), relative to SDS-PAGE purified ($2 \mu\text{g ml}^{-1}$), in order to compensate for the increased protein concentration value resulting from *E. coli* contamination. 7 of the sera tested reacted with the denatured antigen to give mean $\text{OD}_{492\text{nm}}$ absorbance

Figure 3.4 Analysis of gel filtration purified EM- β galFP4 by SDS-PAGE and Coomassie blue staining.

Lane 1, EM4 lysogen lysate soluble fraction.

Lane 2, gel filtration purified EM- β galFP4.

Samples were reduced prior to electrophoresis. The undegraded EM- β galFP4 band is arrowed.

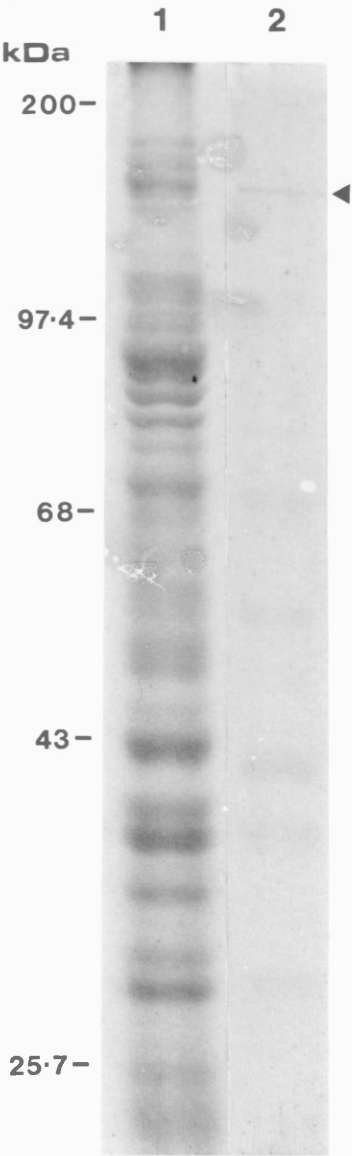
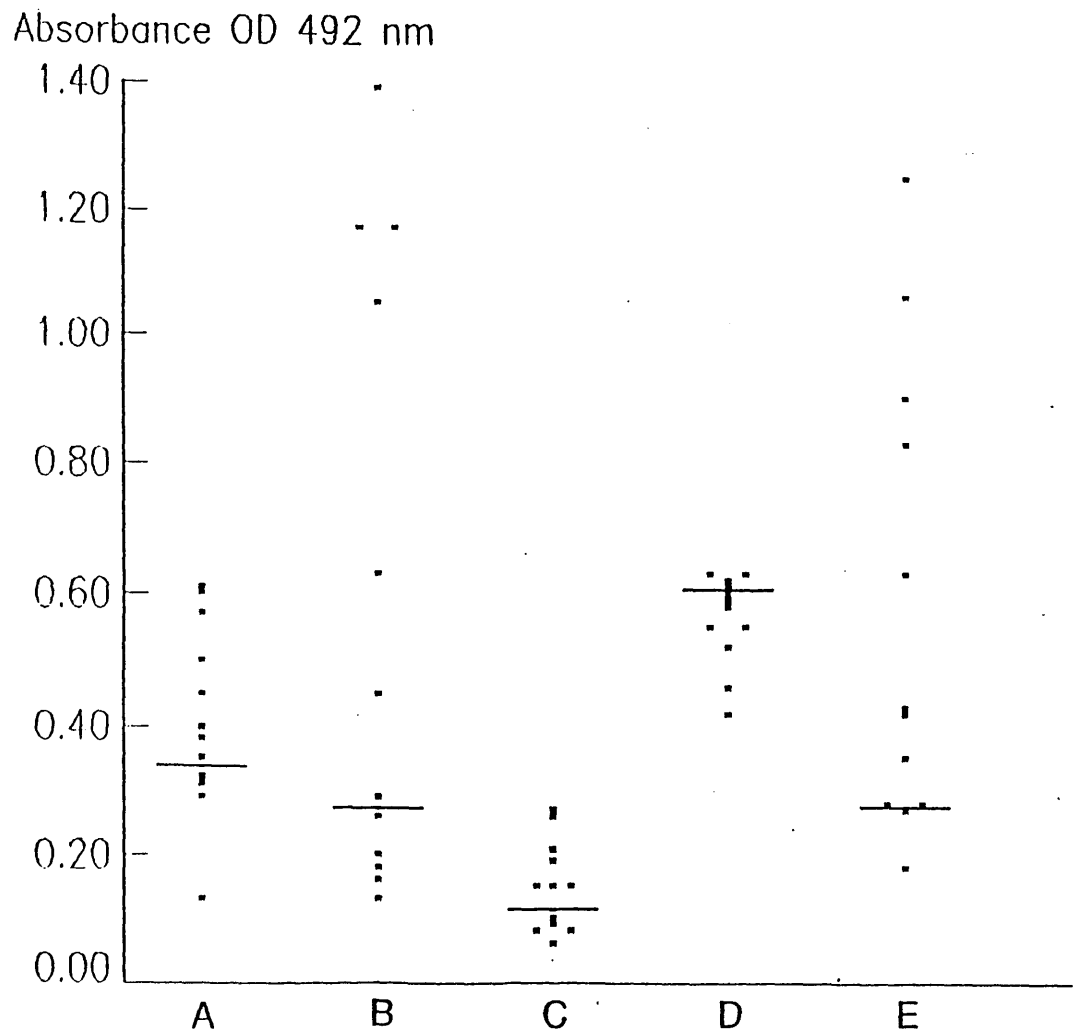


Figure 3.5 ELISA using non-denatured β -gal (A), denatured EM- β galFP4 (B), non-denatured EM- β galFP4 ($5 \mu\text{g ml}^{-1}$) (C), non-denatured GST (D) and non-denatured EM-GSTFP4 (E) as antigens tested with 12 individual anti-*E. multilocularis* sera of Alaskan origin (•) and normal human serum (—). Antigens were used at $2 \mu\text{g ml}^{-1}$ unless otherwise specified.



values greater than that produced by NHS (Fig. 3.5 B). The same number of sera reacted with the non-denatured antigen to give absorbance readings greater than that produced by NHS (Fig. 3.5 C). Sera reactivity with native β -gal was highly variable (Fig. 3.5 A), reflecting the large differences in anti- β -gal reactivity of individual patient sera. The generally higher absorbance readings obtained with this antigen, relative to EM- β galFP4, could have been produced by the increased amount of β -gal bound to the ELISA plate on account of there being no additional protein fused to the molecule. The native state of the β -gal may also have increased its antigenicity. The infection sera which reacted with β -gal to produce absorbance readings significantly greater than NHS may have produced absorbance values greater than NHS with EM- β galFP4 on account of their high anti- β -gal reactivity.

No significant increase in sensitivity was, therefore, produced using non-denatured EM- β galFP4. Absorbance readings were dramatically lowered, however, using the non-denatured antigen (Fig. 3.5 C), relative to those produced with the denatured form (Fig. 3.5 B). Reasons for this could have included, 1) folding configurations of the fusion-peptide which resulted in epitopes being obscured by the β -gal carrier protein, 2) decreased binding of the non-denatured protein to the ELISA plate, and 3) the application of less fusion-peptide than anticipated to the ELISA plate due to inaccurate fusion-peptide concentration determination, arising from *E. coli* contamination. EM- β galFP4, purified in both the denatured and non-denatured forms, produced antibody binding greater than that of NHS with approximately 60% of the human anti-*E. multilocularis* sera tested. The presence of the large β -gal carrier protein, fused to the antigen, may have been a contributory factor leading to the poor antigenicity demonstrated by the native fusion-peptide. This, together with problems of β -gal cross-reactivity, *E. coli* contamination and poor yield of purified protein, led to the testing of another *E. coli* expression system for the production of the EM4 antigen.

3.3.3.2 The production of the EM4 antigen using pGEX-1

In an attempt to overcome some of the disadvantages associated with the EM- β galFP4 antigen, it was decided to express the EM4 peptide

using another vector system, namely pGEX-1. It was anticipated that, with the smaller GST protein (26 kDa), there would be less likelihood of the carrier molecule interfering with antibody binding, as may have been the case with the β -gal (116 kDa) fusion. The affinity of GST for its glutathione substrate would also allow efficient purification of the fusion-peptide from *E. coli* contaminants using non-denaturing conditions.

The EM4 cDNA insert was subcloned into the Eco RI site of pGEX-1 and recombinants expressing antigenic GST/EM4 fusion-peptide were selected. The recombinant TG2 *E. coli* was designated pGEX-1/EM4. This was used to synthesize preparative amounts of fusion-peptide. The soluble fraction of the pGEX-1/EM4 lysate was incubated with glutathione agarose beads and an aliquot of the eluate was analysed (Fig. 3.6) by SDS-PAGE (lane 5), in parallel with an aliquot of the soluble fraction (lane 4). Aliquots of soluble extract and purified GST, produced by a non-recombinant pGEX-1 transformant, were also analysed (lanes 1 and 2, respectively).

The recombinant fusion-peptide lane (lane 5) contained two major bands of approximately 75 kDa and 68 kDa. This was the predicted M_r region of the GST fusion, considering the addition of the 46 kDa EM4 antigen to the 26 kDa GST carrier molecule. The presence of two bands in this region may have been due to either the rapid breakdown of the fusion-peptide, or *E. coli* contamination. The latter possibility was unlikely as neither of these bands was present in the non-recombinant GST lane (lane 2). The non-recombinant GST (lane 2) appeared to migrate at a higher M_r than was expected. This may have been due to poor resolution of the molecular weight marker bands in this region of the gel (lane 3). The smear below the GST band (lane 2) may have been caused by proteolytic degradation or poor resolution of the protein band. The GST fusion-peptide, designated EM-GSTFP4, was not observed in the soluble fraction of the non-recombinant pGEX-1 lysate (lane 1) and, conversely, a band corresponding to GST was not seen in the soluble fraction of the pGEX-1/EM4 lysate (lane 4).

The recombinant pGEX-1/EM4 was, therefore, capable of synthesizing the EM4 antigen as a fusion with GST. The fusion-peptide was soluble and could be readily obtained in a highly purified form using a simple affinity chromatography step. In addition, the fusion was expressed at a high level relative to λ gt11, facilitating the production of large quantities of protein from relatively small culture volumes. This system,

Figure 3.6 Analysis of affinity purified GST and EM-GSTFP4 by SDS-PAGE and Coomassie blue staining.

Lane 1, soluble fraction of non-recombinant pGEX-1 crude lysate.

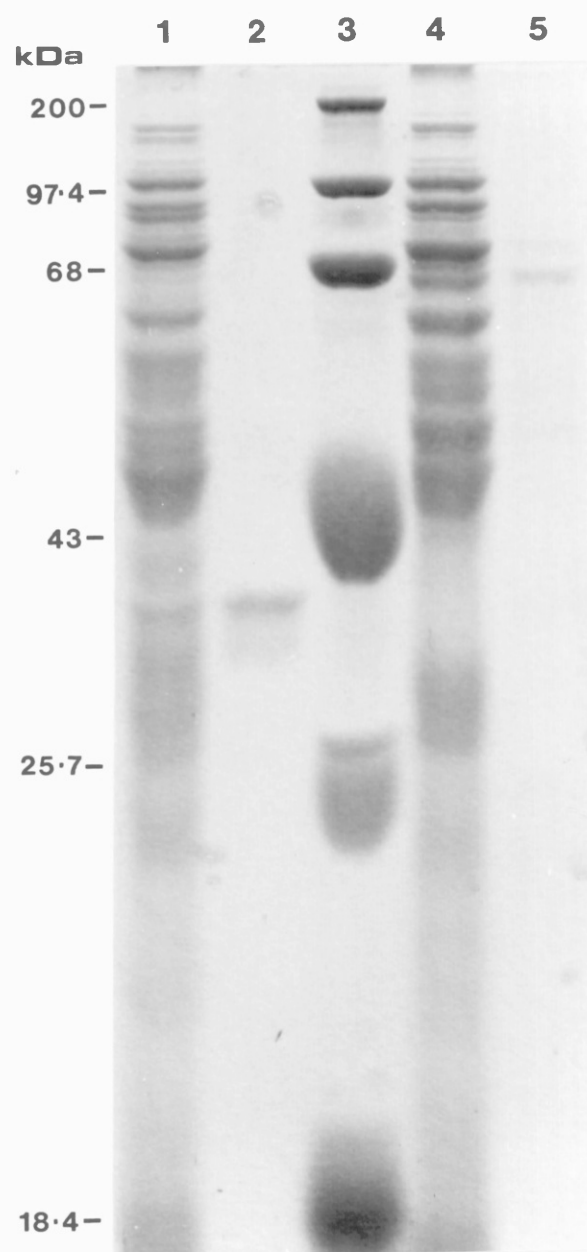
Lane 2, affinity purified GST.

Lane 3, molecular weight markers.

Lane 4, soluble fraction of pGEX-1/EM4 crude lysate.

Lane 5, affinity purified EM-GSTFP4.

Samples were reduced prior to electrophoresis.



appeared, therefore, to have overcome many of the disadvantages associated with expression using λ gt11.

Although a more practical antigen was now available, it was still unknown as to what effect this expression system had had upon the antigenicity of EM4 and, hence, upon its usefulness as a diagnostic reagent. In order to assess the properties of this recombinant antigen, therefore, it was tested using serum pools and individual human infection sera.

3.3.3.3 The immunological characterisation of EM-GSTFP4

3.3.3.3.1 Diagnostic sensitivity relative to EM- β galFP4

To determine whether any improvement in diagnostic sensitivity had been achieved by expressing the EM4 antigen as a GST fusion, EM-GSTFP4 was tested by ELISA with the 12 Alaskan anti-*E. multilocularis* sera used previously with EM- β galFP4 (Fig 3.5). 8 (67%) of the infection sera reacted with EM-GSTFP4 (Fig 3.5 E) producing absorbance readings greater than that of NHS. GST alone (Fig 3.5 D) produced a high background reactivity with all infection sera and NHS. This may be accounted for by there being no protein fused to the molecule, resulting in a greater amount of antigen bound to the ELISA plate. The masking of GST epitopes by the EM4 protein in the case of the fusion-peptide may account for the relatively low background reactivity of EM-GSTFP4 (Fig. 3.5 E). The positive reaction of EM-GSTFP4 with 67% of infection sera, relative to the value of 60% obtained with EM- β galFP4 (Fig. 3.5 B), suggested that a slight improvement in the diagnostic sensitivity of EM4 had been achieved using the pGEX-1 expression vector.

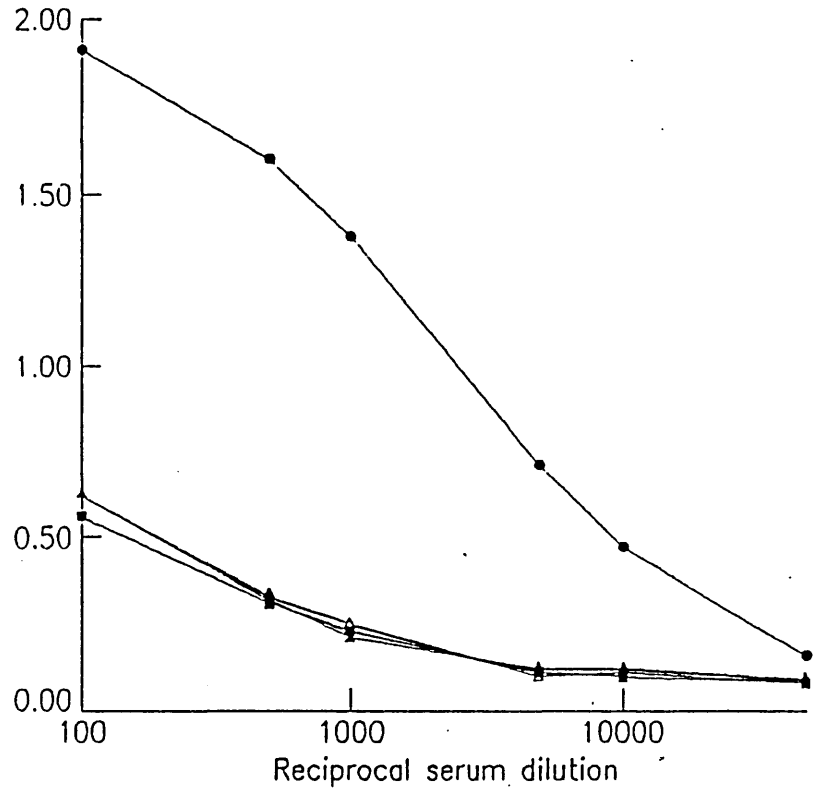
3.3.3.3.2 Diagnostic specificity tested with pools of human sera

To establish whether the GST carrier molecule had any deleterious affects upon the diagnostic specificity of the EM4 peptide, testing with a diverse range of pools of human infection sera was performed as described previously for EM- β galFP4 (Fig. 2.13 A). The result of testing with serial dilutions of anti-*E. multilocularis*, anti-*E. granulosus*, anti-*T. solium* and normal human serum pools (Fig. 3.7 A), showed significant antibody binding only with the anti-*E. multilocularis* serum pool. Anti-*E. granulosus* and anti-*T. solium* serum pools produced levels of binding

Figure 3.7 ELISA using EM-GSTFP4 (A) and GST (B) as antigens ($2 \mu\text{g ml}^{-1}$) tested with various human infection serum pools. Pool of human anti-*E. multilocularis* sera (●); pool of human anti-*E. granulosus* sera (■); pool of human anti-*T. solium* sera (▲); pool of normal human sera (Δ).

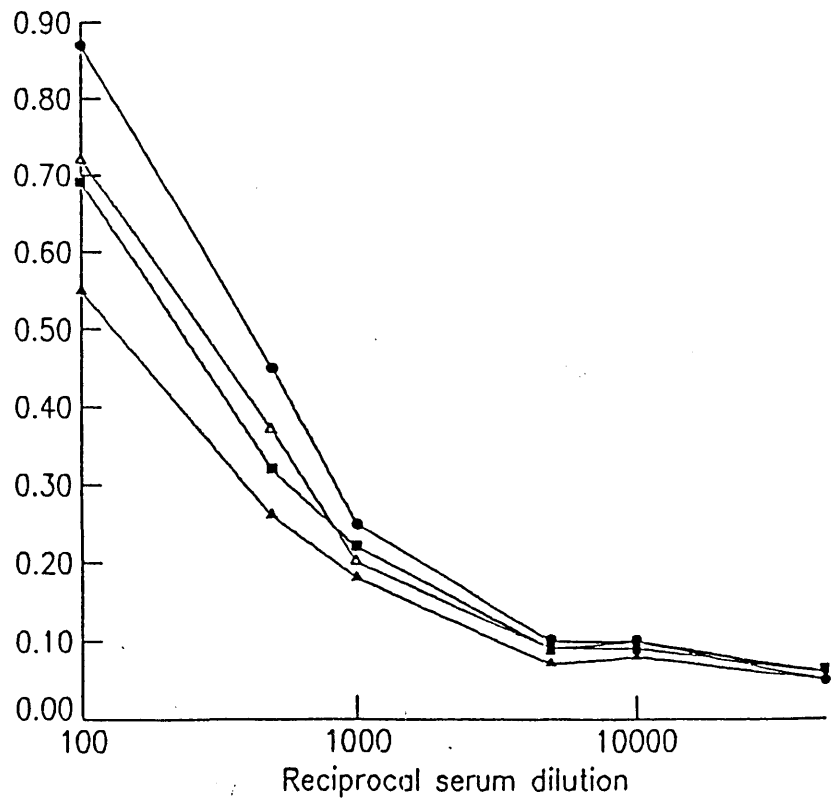
A

Absorbance OD 492 nm



B

Absorbance OD 492 nm



similar to that of NHS. The background level of binding produced by these serum pools appeared to be significantly less than that found with EM- β galFP4 (Fig. 2.13 A), particularly at low dilutions. GST alone produced a background level of binding with all serum pools similar to that of NHS (Fig. 3.7 B). This correlated with the result obtained with β -gal (Fig. 2.13 B), although background levels of cross-reactivity were reduced. The higher absorbance values produced by GST with all serum pools, relative to those produced by EM-GSTFP4 with anti-*E. granulosus*, anti-*T. solium* and NHS serum pools could be accounted for as mentioned previously (section 3.3.3.3.1).

Testing of EM-GSTFP4 and GST with other diverse human infection serum pools was also performed (Table 3.2). No significant level of anti-EM-GSTFP4 antibody binding was observed with any of the serum pools, apart from the anti-*E. multilocularis* serum pool. Some variation in background binding levels with GST was apparent, however, with different serum pools. For example, the anti-*Onchocerca volvulus* serum pool produced a significantly greater background response than the anti-*T. solium* or anti-*S. mansoni* serum pools. This was presumably due to some anti-GST response produced by this parasitic infection.

3.3.3.3.3 Diagnostic specificity tested with individual human sera

Individual human anti-*E. granulosus* and anti-*T. solium* sera were tested in ELISA with EM-GSTFP4 (Table 3.3). For interpretation of seropositivity a threshold value was calculated, as before, using the mean OD_{432nm} absorbance value plus 2 S.D. obtained from testing of the NHS pool, diluted 1 in 500, a total of 8 times against EM-GSTFP4 ($2 \mu g ml^{-1}$). A threshold value of 0.647 (OD_{432nm}) was calculated. Values equal to or greater than the threshold value were considered as positive. The anti-*E. granulosus* and anti-*T. solium* sera were each diluted 1 in 500 and tested in duplicate against EM-GSTFP4 ($2 \mu g ml^{-1}$) (Table 3.3). None of the sera tested produced a mean absorbance value equal to or exceeding the threshold value. Therefore, the EM-GSTFP4 antigen demonstrated 100% diagnostic species-specificity, under these conditions. The sera were also tested in parallel with GST alone ($2 \mu g ml^{-1}$) under identical conditions (data not shown). None of the test sera produced a mean absorbance value equal to or exceeding the threshold calculated for EM-GSTFP4, with this antigen. Anti-

TABLE 3.2

The diagnostic specificity of EM-GSTFP4, tested using diverse human infection serum pools in ELISA (compare with Table 2.1)

1 in 500 pooled human antiserum to:	Antigen	
	EM-GSTFP4	GST
<i>E. multilocularis</i>	1.56 (1.55, 1.57)	0.45 (0.45, 0.44)
<i>E. granulosus</i>	0.32 (0.30, 0.33)	0.32 (0.31, 0.33)
<i>T. solium</i>	0.32 (0.32, 0.31)	0.26 (0.26, 0.25)
<i>Ascaris lumbricoides</i>	0.34 (0.33, 0.35)	0.36 (0.35, 0.37)
<i>Brugia malayi</i>	0.42 (0.41, 0.42)	0.50 (0.50, 0.49)
<i>Onchocerca volvulus</i>	0.48 (0.50, 0.45)	0.58 (0.56, 0.60)
<i>Schistosoma mansoni</i>	0.22 (0.21, 0.22)	0.26 (0.26, 0.26)
Normal Human Serum	0.34 (0.32, 0.35)	0.37 (0.36, 0.38)

Figures represent OD_{492nm} absorbance readings in ELISA and are the means of the 2 determinations given in brackets

Antigens were used at 2 µg ml⁻¹

TABLE 3.3

The diagnostic specificity of EM- β galFP2 and EM-GSTFP4 assessed using individual human anti-*E. granulosus* and anti-*T. solium* infection sera

Serum No:	Origin	Case history	2	4
<i>E. granulosus</i> *				
infection sera				
1	Kenya	Liver cyst & DC. No surgery	+	-
2	"	Liver & abd. cysts. DC. 1 operation	+	-
3	"	No cyst detected by ultrasound	+	-
4	"	Liver & abd. cysts. DC. 3 operations	+	-
5	"	No cyst detected by U/S. Lung cyst ?	+	-
6	"	Liver cyst. DC. 1 operation	+	-
7	"	Liver cysts. DC. 1 operation	-	-
8	"	Liver & abd. cysts. DC? 1 operation	+	-
9	U.K.	Albendazole. No further data.	-	-
10	"	Liver cysts. 1 operation	+	-
11	"	No data available	-	-
<i>T. solium</i>				
infection sera				
1	Zimbabwe	No data available	-	-
2	"	"	-	-
3	"	"	-	-
4	"	"	+	-
5	"	"	-	-
6	"	"	+	-
7	"	"	+	-
8	"	"	-	-
9	Mexico	"	-	-
10	"	"	-	-
11	"	"	+	-
Total negatives:			10-	22-
% specificities:			45%	100%

+ Positive diagnosis

- Negative diagnosis

2 EM- β galFP2 (tested by immunoblotting)

4 EM-GSTFP4 (tested by ELISA)

* Sera used for differential screening of the cDNA library

DC Daughter cysts

GST reactivity did not, therefore, lead to the false interpretation of positive reactions with any of the sera tested.

3.3.3.3.4 Diagnostic sensitivity tested with individual human sera

Diagnostic specificity and to some extent sensitivity of EM-GSTFP4 had, therefore, been assessed, but in order to fully explore its potential as a diagnostic reagent, more extensive evaluation of its sensitivity was required. Testing of the antigen by ELISA using 49 individual human anti-*E. multilocularis* infection sera was carried out (Table 3.4). Using the same criterion as before (section 3.3.3.3), 18 of the 49 sera were diagnosed positive. Only 4 of the 8 Alaskan sera which had previously been shown to produce absorbance readings greater than NHS (Fig. 3.5 E), were positively diagnosed using the threshold value. This cut-off point was thought to be necessary, however, to prevent false-positive diagnosis resulting from technical variability and differing levels of anti-GST reactivity in individual sera. The EM-GSTFP4 antigen was, therefore, found to have a diagnostic sensitivity value of 37% under these conditions. This implied that, using this antigen, the majority of human *E. multilocularis* infections would not be successfully immunodiagnosed. From Table 3.4, it can be seen that antigen recognition was independent of serum origin and where data was available, did not have any obvious correlation with drug treatment. The EM-GSTFP4 antigen only positively diagnosed 6 out of 11 of the sera included in the anti-*E. multilocularis* serum pool used for library screening. This indicated, as with EM- β galFP2, that screening with pools of sera, as opposed to panels of individual sera, may not have been an appropriate strategy for the preliminary isolation of diagnostic clones.

3.3.4 THE RECOGNITION OF EM- β galFP2 AND EM-GSTFP4 BY INDIVIDUAL ANTI-*E. MULTILOCULARIS* SERA

Table 3.4 summarises the responses of individual human anti-*E. multilocularis* sera to the two recombinant antigens. The majority of sera which were diagnosed positive with EM-GSTFP4 also produced a positive reaction with EM- β galFP2. This may reflect the titre of these sera i.e. high titre sera may recognise both antigens, in contrast to low titre sera which may possess low levels of specific antibody to one or other, or both

TABLE 3.4

The diagnostic sensitivity of EM- β galFP2 and EM-GSTFP4 assessed using individual human anti-*E. multilocularis* infection sera

Ser.No:	Case history	2	4	Ser.No:	Case history	2	4
1*	ND	-	-	26	No chemotherapy	+	-
2*	"	-	-	27	Mebendazole	-	-
3*	"	-	+	28	No chemotherapy	+	-
4*	"	-	-	29	Mebendazole	-	-
5*	"	+	+	30	"	+	-
6*	"	+	+	31	"	-	-
7*	"	+	+	32	"	-	-
8*	"	-	-	33	"	-	-
9*	"	-	+	34	"	+	+
10*	"	+	+	35	No chemotherapy	+	-
11*	"	+	-	36	"	+	-
12	"	+	+	37	"	+	+
13	"	-	-	38	ND	+	+
14	"	+	+	39	"	+	-
15	"	-	-	40	"	-	+
16	"	-	-	41	"	+	+
17	"	-	-	42	"	-	-
18	No chemotherapy	+	+	43	"	+	-
19	"	+	+	44	"	+	-
20	Mebendazole	-	-	45	"	-	-
21	"	+	-	46	"	+	-
22	"	-	+	47	"	-	+
23	"	-	+	48	"	-	-
24	"	+	-	49	"	+	-
25	"	+	-				
Positive totals:		12+	12+			14+	6+
EM- β galFP2 total positives		= 26		% sensitivity		= 53%	
EM-GSTFP4 total positives		= 18		% sensitivity		= 37%	

Sera origins: 1-37 = Europe

38-49 = Alaska

+ Positive diagnosis

- Negative diagnosis

2 EM- β galFP2 (tested by immunoblotting)

4 EM-GSTFP4 (tested by ELISA)

* Sera used for differential screening of the cDNA library

ND No data available

antigens. The characteristics of individual *E. multilocularis* infections may also influence this co-recognition. Insufficient case history data was available to substantiate this, but EM- β galFP2 was recognised by more patient sera than EM-GSTFP4, although this may have been the result of the different diagnostic criteria used. The native antigen corresponding to EM2 may, therefore, be presented to the host immune system more frequently in AE cases than that corresponding to EM4.

3.3.5 THE ONSET OF HOST RECOGNITION OF CLONED ANTIGENS

A mouse model was used to establish whether the recombinant antigens EM- β galFP2 and EM- β galFP4 were recognised by infection sera shortly after infection. Following intraperitoneal inoculation of protoscoleces, 7 mice were bled sequentially every 14 days for a total of 168 days. 3 control mice were also bled at the same time intervals. The sera obtained were used to investigate changing antigen recognition profiles and the onset of recognition of the cloned antigens. Equal volumes of control mice sera, taken from over the full time course, were pooled and used as the normal mouse serum (NMS).

Sera were initially tested against EmAgPP (Fig. 3.8). This antigen extract was used instead of crude EmAgA as previous testing of immunoblots of EmAgPP and EmAgA with rabbit anti-rat serum (Sigma), showed the latter contained many cotton rat host antigens, whereas the former extract contained no detectable host contamination (data not shown). The use of EmAgPP thus prevented the detection of host proteins by anti-cotton rat antibodies generated by contaminating cotton rat proteins introduced with the purified protoscoleces inoculum. Sera from all mice, except number 6 (panel F), produced consistent immune recognition profiles over the time course of infection, major antigens not recognised by NMS (lane 1) of approximate M_r s 130, 86, 80, 67, 61, 55, 50 and 47 kDa, were detected. Several additional antigens of 44, 42, 22, 20, 18, 16, 14 and 12 kDa were also detected by sera from mice numbers 4, 5 and 7 (panels D, E and G, respectively). A gradual increase in the intensity of immune response, from the time of onset, was observed with most major antigens, the response being sustained over the time period. Some variation was apparent, however, in the time of onset and maintenance of response to some antigens. Sera

Figure 3.8 SDS-PAGE and immunoblotting of EmAgPP with mouse infection sera.

(A) mouse number 1 sera, (B) mouse number 2 sera, (C) mouse number 3 sera, (D) mouse number 4 sera, (E) mouse number 5 sera, (F) mouse number 6 sera and (G) mouse number 7 sera.

Lane 1, pool of normal mouse sera.

Lane 2, sera taken 14 days postinfection.

Lane 3, sera taken 28 days postinfection.

Lane 4, sera taken 42 days postinfection.

Lane 5, sera taken 56 days postinfection.

Lane 6, sera taken 70 days postinfection.

Lane 7, sera taken 84 days postinfection.

Lane 8, sera taken 98 days postinfection.

Lane 9, sera taken 112 days postinfection.

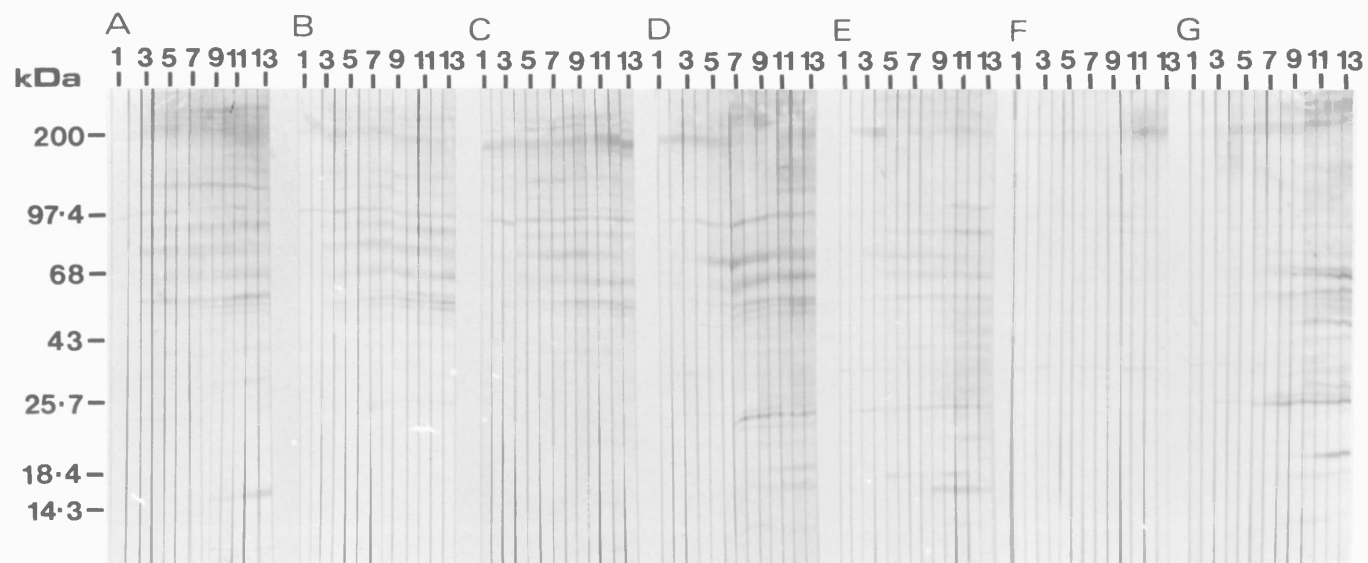
Lane 10, sera taken 126 days postinfection.

Lane 11, sera taken 140 days postinfection.

Lane 12, sera taken 154 days postinfection.

Lane 13, sera taken 168 days postinfection.

50 µg of protein was used per strip. Proteins were reduced prior to electrophoresis.



from mouse number 6 (panel F) only produced a weak response to the 86 kDa and 20 kDa antigens, which were not detected by NMS (lane 1).

Crude EM2 lysogen lysate was electrophoresed, immunoblotted and tested with the mouse infection sera (Fig. 3.9). Mouse anti-EM- β galFP2 serum was used to locate the fusion-peptide band (lane 1). No significant binding at the M_r of EM- β galFP2 was observed over the entire time course with any of the mouse infection sera. The native antigen corresponding to EM2 did not, therefore, stimulate a specific host immune response within the 168 day infection period in this type of animal model system.

Crude EM4 lysogen lysate was electrophoresed, immunoblotted and tested with the mouse infection sera (Fig. 3.10). Mouse anti-EM- β galFP4 serum was used to locate the fusion-peptide band (lane 1). With all mice sera, except number 6 (panel F), a gradual increase in antibody binding to EM- β galFP4 was observed. No decrease in reactivity was observed over the time period. The onset of detectable antibody binding to this antigen, as determined by a signal intensity greater than that produced by NMS (lane 2), was found with the sera from mice numbers 1 (panel A), 2 (panel B), 4 (panel D), 5 (panel E) and 7 (panel G), 42 days postinfection (lane 5). Mouse number 3 sera (panel C), produced a positive reaction after 70 days (lane 7). Mouse number 6 sera (panel F) produced no detectable antibody binding to this antigen throughout the time period. The relatively broad fusion-peptide band and the occasional presence of additional lower M_r bands in these immunoblots were produced by proteolytic degradation of the fusion-peptide and anti-*E. coli* cross-reactivity. To ensure that the increase in antibody binding was not due to anti- β -gal cross-reactivity, crude lysate of a non-recombinant λ gt11 lysogen was electrophoresed, immunoblotted and tested with pools of all mice infection sera (panel H). No significant increase in intensity of the β -gal band (lane 1) was observed over the time course, proving the recognition of EM- β galFP4 to be solely due to reaction with parasite-specific antibody.

Following completion of the 168 day infection period, the mice were killed by cervical dislocation and dissected (Fig. 3.11 A and B). No obvious abdominal cavity cyst mass could be seen in mouse number 6 (Fig. 3.11 A, number 6). The characteristics of each infection were noted, and a summary of these findings is presented in Table 3.5. The mice which produced a full immune response (numbers 1-5 and 7) contained large

Figure 3.9 SDS-PAGE and immunoblotting of crude EM2 lysogen lysate with mouse infection sera.

(A) mouse number 1 sera, (B) mouse number 2 sera, (C) mouse number 3 sera, (D) mouse number 4 sera, (E) mouse number 5 sera, (F) mouse number 6 sera and (G) mouse number 7 sera.

Lane 1, mouse anti-EM- β galFP2 serum.

Lane 2, pool of normal mouse sera.

Lane 3, sera taken 14 days postinfection.

Lane 4, sera taken 28 days postinfection.

Lane 5, sera taken 42 days postinfection.

Lane 6, sera taken 56 days postinfection.

Lane 7, sera taken 70 days postinfection.

Lane 8, sera taken 84 days postinfection.

Lane 9, sera taken 98 days postinfection.

Lane 10, sera taken 112 days postinfection.

Lane 11, sera taken 126 days postinfection.

Lane 12, sera taken 140 days postinfection.

Lane 13, sera taken 154 days postinfection.

Lane 14, sera taken 168 days postinfection.

50 μ g of protein was used per strip. Proteins were reduced prior to electrophoresis. The undegraded EM- β galFP2 band is arrowed.

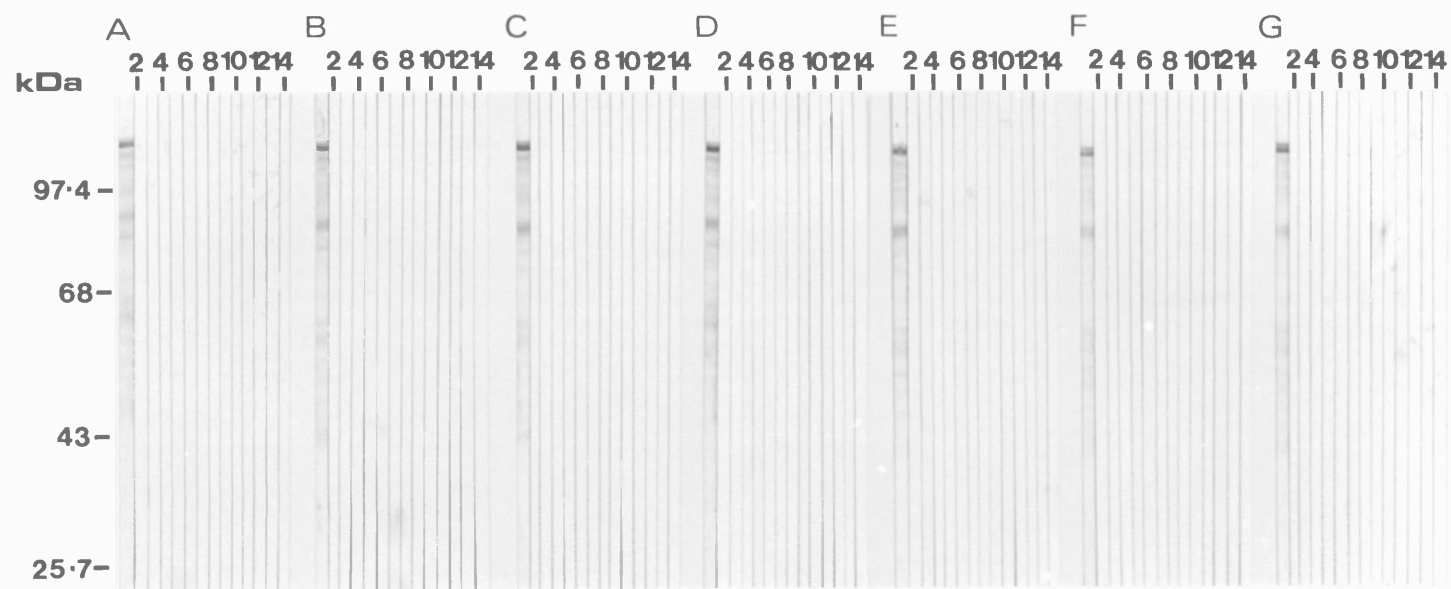


Figure 3.10 SDS-PAGE and immunoblotting of crude EM4 and λ gt11 lysogen lysates with mouse infection sera.

(A) mouse number 1 sera, (B) mouse number 2 sera, (C) mouse number 3 sera, (D) mouse number 4 sera, (E) mouse number 5 sera, (F) mouse number 6 sera and (G) mouse number 7 sera tested with crude EM4 lysogen lysate.

(H) pools of all mice sera tested with crude λ gt11 lysogen lysate.

Lane 1, mouse anti-EM- β galFP4 serum (A-G), mouse anti- β -gal serum (H).

Lane 2, pool of normal mouse sera.

Lane 3, sera taken 14 days postinfection (A-G), pool (H).

Lane 4, sera taken 28 days postinfection (A-G), pool (H).

Lane 5, sera taken 42 days postinfection (A-G), pool (H).

Lane 6, sera taken 56 days postinfection (A-G), pool (H).

Lane 7, sera taken 70 days postinfection (A-G), pool (H).

Lane 8, sera taken 84 days postinfection (A-G), pool (H).

Lane 9, sera taken 98 days postinfection (A-G), pool (H).

Lane 10, sera taken 112 days postinfection (A-G), pool (H).

Lane 11, sera taken 126 days postinfection (A-G), pool (H).

Lane 12, sera taken 140 days postinfection (A-G), pool (H).

Lane 13, sera taken 154 days postinfection (A-G), pool (H).

Lane 14, sera taken 168 days postinfection (A-G), pool (H).

50 μ g of protein was used per strip. Proteins were reduced prior to electrophoresis. The undegraded EM- β galFP4 (A-G) ($\blacktriangleleft$) and β -gal (H) ($\triangleleft$) bands are arrowed.

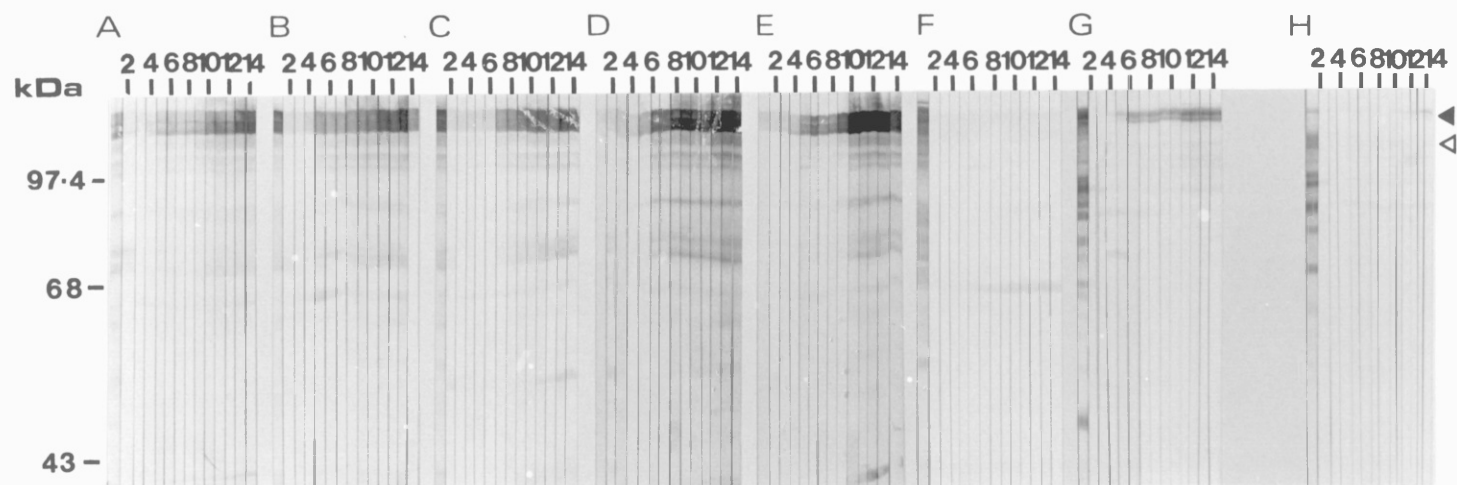


Figure 3.11 Dissection of experimentally infected mice to show characteristics of *E. multilocularis* within the abdominal cavity.

(A) 1-7, mice numbers 1-7.

(B) Detail of mouse number 1 infection. One large *E. multilocularis* cyst mass (pale white dappled colouration) in the posterior region of the abdominal cavity (foreground), a smaller cyst immediately adjacent to this and another small cyst attached to the liver in the anterior region of the abdominal cavity are visible.

A



B



TABLE 3.5

The characteristics of *E. multilocularis* from experimentally infected mice

Mouse	<u>Metacestode cyst characteristics</u>			
No:	Location	Morphology	No:	Mass
1	Liver/low.abd./TBC/1 large cyst	V. NP. No CC	12	2.18g
2	Liver/TBC	"	10	1.94g
3	Large liver cyst/TBC	"	12	3.23g
4	Liver/TBC	"	6	2.39g
5	"	"	8	2.76g
6	No liver cyst/Small S.C.? cyst	V. MP/FD. Large CC	1	0.10g
7	Liver/low.abd./TBC/1 v.large cyst	V. NP. No CC	4	2.84g

TBC	Throughout body cavity
S.C.	Subcutaneous
V.	Vesicles present
NP.	No protoscoleces present
MP/FD	Many protoscoleces, fully developed
CC	Calcereous corpuscles

cyst masses within the abdominal cavity (Fig. 3.11 A and B). All had at least one cyst mass attached to the liver, with the remainder spread throughout the body cavity. The cysts contained vesicles, typical of normal *E. multilocularis* metacystodes but all were sterile. Approximately the same mass of cyst material was present in each of these mice and all contained multiple cysts (Table 3.5). The remaining mouse (number 6) harboured only one very small cyst, found attached subcutaneously to the body cavity wall, and this contained many fully developed, viable protoscoleces. The atypical infection found in this case, explained the lack of immune response generated by this mouse. The difference in infection characteristics could have been due to inherent resistance of this mouse to infection or, as was more likely to be the case, due to technical error i.e. the protoscoleces could have been introduced into the subcutaneous region of the mouse abdominal cavity at the time of inoculation.

The immune response produced by the remaining mice proved to be the result of the steady growth of *E. multilocularis* metacystode cysts within the body cavity. The onset of recognition of EM- β galFP4 within 42 to 70 days postinfection was, therefore, solely due to the native antigen corresponding to this cloned antigen, being produced by the developing metacystodes. This suggests, although the human condition may differ in many respects from the animal system, that the native antigen corresponding to EM4 may well stimulate an immune response in the human host within a relatively short time period following infection. This might, therefore, facilitate early diagnosis in human cases where this antigen is recognised.

3.4 DISCUSSION

3.4.1 THE DIAGNOSTIC VALUE OF β -GAL FUSION-PEPTIDE ANTIGENS

The only *E. multilocularis*-specific immunodiagnostic antigen to have been isolated, to date, has been Em 2a (Gottstein *et al.*, 1983; Gottstein, 1985). This antigen exhibits high specificity (95%) and a high diagnostic sensitivity (94%). Recombinant antigens useful for immunodiagnosis, should, therefore, achieve comparable (>90%) levels of species-specificity and diagnostic sensitivity.

On testing with panels of individual human anti-*E. granulosus* and anti-*T. solium* infection sera, the EM- β galFP2 recombinant antigen was found to cross-react with a number of these heterologous sera. The antigen demonstrated a specificity value of 45%. Testing with individual human anti-*E. multilocularis* sera showed this antigen had a diagnostic sensitivity of 53%. Both of these values indicated that this antigen did not have the desired characteristics necessary for a diagnostic reagent. The antigen was initially shown to be species-specific, at the immunological level, using pools of human infection sera (Chapter 2). The effect of diluting out the anti-EM- β galFP2 antibody within high specific antibody titre individual sera when the sera were pooled was, therefore, the reason for the ambiguous results produced using the serum pools. The recent work of Vogel *et al.* (1988), reported similar findings. These workers differentially screened immunoblots of *E. multilocularis* fusion-peptide antigens, produced by lysogens of λ gt11 recombinant clones isolated from a metacestode cDNA library, using pools of human anti-*E. multilocularis* and anti-*E. granulosus* infection sera. Of the 11 clones tested, 6 were found to react strongly with the anti-*E. multilocularis* serum pool, and 2 of these were found to cross-react with the anti-*E. granulosus* serum pool. 4 clones were, therefore, putatively *E. multilocularis*-specific according to these data. On further testing of these by immunoblotting, using individual anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* infection sera, none of these recombinant antigens were found to possess the desired levels of species-specificity and diagnostic sensitivity necessary for use as diagnostic reagents. The subsequent testing, by these workers, of a recombinant clone whose lysogen did not produce a stable β -gal fusion-peptide, using

individual infection sera, did, however, reveal a clone with suitable immunodiagnostic operating characteristics (i.e. specificity and sensitivity >90%). The fusion produced by this clone was found to degrade to give two proteins of 31 kDa and 33 kDa. Immunoblotting of crude lysogen lysate of this clone was used to test the diagnostic potential of these peptides. The presence of many *E. coli* proteins within the size range of these recombinant peptides would, however, be a potential source of incorrect diagnosis should they be used routinely with immunoblotting as the diagnostic technique. The size of these proteins would also make their purification from *E. coli* components difficult by electroelution from SDS-PAGE gels or gel filtration chromatography for subsequent use in techniques such as ELISA.

All further work regarding diagnosis concentrated upon the EM4 antigen due to the inappropriate characteristics exhibited by the EM2 recombinant antigen. The testing of electroeluted EM- β galFP4 by ELISA, using a limited number of individual human anti-*E. multilocularis* sera, produced a diagnostic sensitivity value of 60%. This value was too low for diagnostic purposes and methods of improving the diagnostic sensitivity of the EM4 antigen had to be employed. The EM- β galFP4 antigen was denatured when purified by electroelution, effectively destroying any conformational epitopes that may have been formed. This could have been a contributory factor leading to the low levels of sensitivity demonstrated by this antigen. EM- β galFP4, was, therefore, purified using the non-denaturing technique of gel filtration. This method allows the recovery of soluble, native protein (Carroll and Laughon, 1987). The non-denatured EM- β galFP4 was tested with the same number of individual human anti-*E. multilocularis* sera but sensitivity was not significantly improved. The presence of the large β -gal carrier protein was thought to account for this. Although non-denaturation leaves conformational epitopes intact, it also allows the β -gal carrier protein to assume its native form. With such a large protein adjacent to the foreign peptide, the folding of the β -gal protein may have obscured many primary and secondary structure epitopes on the antigen molecule. The large overall decrease in OD_{492nm} absorbance readings produced using this non-denatured recombinant antigen, may have been a consequence of this reduction in the number of available epitopes.

In an attempt to improve the antigenicity of the EM4 peptide,

it was purified under non-denaturing conditions with the β -gal carrier protein having been replaced by a GST molecule. This was achieved by using one of the pGEX series of vectors which bestow a number of desirable characteristics upon recombinant peptides (Smith and Johnson, 1988), designed for use as cloned immunodiagnostic reagents.

3.4.2 THE IMMUNODIAGNOSTIC VALUE OF THE EM4 ANTIGEN SYNTHESIZED AS A GST FUSION-PEPTIDE

The pGEX vectors have been used to successfully express *P. falciparum* antigens, human auto-antigens, a fragment of the α -chain of the murine T-cell receptor and murine interleukin 4 (Smith and Johnson, 1988). The size of these fusion-peptides ranged from 32 kDa to 84 kDa and the majority were soluble and could, therefore, be purified by affinity chromatography on immobilised glutathione. It was anticipated, therefore, that this vector system would be capable of synthesizing the 46 kDa EM4 antigen as a 72 kDa fusion with the 26 kDa GST carrier protein. The recombinant antigen produced could then be purified by affinity chromatography using non-denaturing conditions. The small GST carrier protein, relative to β -gal, would, it was anticipated, not interfere to the same extent with antibody binding to the EM4 antigen when in the non-denatured state.

The EM4 cDNA was subcloned into the pGEX-1 vector and fusion-peptide synthesis was induced by the addition of IPTG. A recombinant peptide of approximately 70 kDa was produced, as predicted. It was purified from *E. coli* lysates by immobilised glutathione affinity chromatography, a process made possible by the solubility of the EM4/GST fusion. Detection of recombinant colonies by immunoassay proved the antigenicity of EM4 had been maintained, indicating that the recombinant molecule had been correctly synthesized. The ease of purification of this fusion made it particularly suitable for ELISA.

The GST fusion demonstrated a slight increase in immunological sensitivity relative to its β -gal counterpart on testing with a limited number of individual anti-*E. multilocularis* sera, and testing with pools of infection sera indicated that diagnostic specificity had been maintained. The absorbance readings produced with the anti-*E. multilocularis* serum pool

had increased, and those obtained with the anti-*E. granulosus*, anti-*T. solium* and NHS serum pools had decreased, relative to the values obtained with the EM4 antigen fused to β -gal. The antigenicity of the EM4 peptide, had, therefore, improved as a GST fusion and its background reactivity had been lowered. It was anticipated that certain parasitic infections, particularly schistosomiasis, may produce an anti-GST immune response within patient sera (Taylor *et al.*, 1988), which may cross-react with the GST carrier protein in immunoassays. This did not appear to be the case when the anti-*S. mansoni* serum pool was tested against the EM4/GST fusion and GST alone. Possible reasons for the lack of recognition may have been due to dilution of high anti-GST antibody sera when the sera were pooled, or discrepancies in the structure of the GST molecules (Smith *et al.*, 1986; Taylor *et al.*, 1988). The anti-*B. malayi* and anti-*O. volvulus* serum pools produced the highest background OD_{492nm} absorbance values of 0.42 and 0.48, respectively (Table 3.2). High background was not produced by the β -gal fusion with these serum pools (Table 2.1). Therefore, these parasites stimulate a host immune response which cross-reacts with the *S. japonicum* GST carrier molecule. The GST produced by these organisms must, therefore, show significant homology to that of *S. japonicum*. The background OD_{492nm} absorbance readings produced by these two serum pools, although significantly higher than those produced by the other serum pools, did not approach the threshold OD_{492nm} absorbance value of 0.647, used as the basis for determining seropositivity. The EM4 antigen expressed as a fusion with GST proved, therefore, to be a better ELISA reagent than the same antigen fused to β -gal.

In order to assess the immunodiagnostic value of this recombinant antigen further, it was tested by ELISA using panels of human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* infection sera. None of the anti-*E. granulosus* and anti-*T. solium* sera were diagnosed positive with this antigen, resulting in a diagnostic specificity of 100%. Only 18 of the 49 anti-*E. multilocularis* sera were diagnosed positive with this antigen using a diagnostic threshold value, resulting in a diagnostic sensitivity of 37%. In terms of specificity, therefore, the EM-GSTFP4 antigen had characteristics suitable for immunodiagnosis i.e. >90%. Sensitivity was not, however, in the region required (>90%), rendering the

antigen, if used alone, inappropriate for diagnosis based on the detection of circulating free antibody.

3.4.3 THE EARLY HOST RECOGNITION OF EM2 AND EM4 CLONED ANTIGENS

The diagnostic polypeptide Em 2a, isolated by conventional methods, has been assumed, on account of anti-Em 2a antibodies being present in almost all *E. multilocularis* infected patients, to stimulate an early humoral response following infection (Vogel *et al.*, 1988). In the current study a mouse infection model was used to determine the time taken before onset of a humoral immune response which could be detected by the two cloned antigens, EM2 and EM4.

Sera taken at regular intervals from infected mice were tested against immunoblots of protoscolex material and β -gal fusion-peptide antigens. All but one of the mice recognised most of the *E. multilocularis* antigens detected previously by human anti-*E. multilocularis* infection sera (Fig. 2.6 A). Lingelbach and Hinz (1984) reported similar findings using sera obtained 90 days postinfection from intraperitoneally infected NMRI-mice. None of the mice sera detected the recombinant EM2 antigen for the entire time course of infection. This may have resulted from discrepancies between the experimental animal and human infections, which have been discussed earlier (section 3.1.5). The recombinant EM4 antigen was detected, however, by most mice sera after 42 days, with the sera of one mouse producing a reaction after 70 days. Of these infected mice, none harboured fertile cysts which is the usual condition found in the case of human infection (Rausch and Wilson, 1973). In this respect, therefore, the mouse infections appeared to closely mimic the human condition. The native parasite antigen corresponding to the EM4 clone, was, therefore, shown to be capable of initiating an early immune response in *E. multilocularis* mouse infections. Should this be the case in man, the rapid response generated might allow early diagnosis of AE using the recombinant EM4 antigen.

3.4.4 THE DIAGNOSTIC APPLICATION OF THE CLONED ANTIGENS EM2 AND EM4

The recombinant antigens expressed by EM2 and EM4 did not possess all of the characteristics required for practical immunodiagnosis.

EM- β galFP2 lacked specificity and EM-GSTFP4 was only capable of positively diagnosing relatively few AE cases. Despite these problems, a suitable *E. coli* expression system for the production and purification of immunodiagnostic antigens has been identified, namely, the pGEX-1 - 3 vector family. The use of the pGEX-2 and 3 vectors may have improved the diagnostic sensitivity of the EM4 antigen as the GST carrier molecule can be cleaved from the foreign peptide, thereby eliminating all problems associated with masking of epitopes by the carrier molecule and cross-reactivity with anti-GST antibody.

The use of the recombinant EM4 antigen alone, in ELISA assays, for the detection of circulating free anti-EM4 antibodies may be improved by expression of the antigen without a carrier protein. If this does not improve sensitivity, alternative diagnostic recombinant antigens would have to be identified. This could be achieved by testing the fusion-peptides produced by the remaining moderately reactive, putatively specific clones 1, 3 and 6 (Chapter 2, Fig. 2.8) following immunoblotting, with panels of individual human infection sera. This type of large scale screening was used by Vogel *et al.* (1988) to characterise potentially diagnostic recombinant *E. multilocularis* antigens. Antigens which appear to demonstrate high levels of diagnostic specificity and sensitivity could then be expressed using the pGEX vectors. The subsequent use of these antigens in conjunction with EM4 as an 'antigen cocktail', could then potentially overcome the problem of low diagnostic sensitivity demonstrated by the recombinant EM4 antigen used alone for the detection of circulating unbound antibody.

The use of alternative immunoassay techniques may also enhance the diagnostic usefulness of the EM4 cloned antigen. Anti-EM4 antibody, raised using EM-GSTFP4, could be used to detect circulating native EM4 antigen within human sera (Gottstein, 1984; Craig and Nelson, 1984; Craig, 1986). The treatment of human sera to dissociate immune-complexes (Craig and Nelson, 1984; Craig, 1986), prior to testing for circulating antibody and/or antigen, may also improve the sensitivity of these assays. These improvements may help to overcome the lack of diagnostic sensitivity of the recombinant EM4 antigen, making it more applicable for the immuno-diagnosis of AE.

CHAPTER 4

4 THE MOLECULAR CHARACTERISATION OF THE EM2 AND EM4 CLONED ANTIGENS

4.1 INTRODUCTION

4.1.1 GENERAL

Many cloned parasite antigens have now been characterised at the molecular level, *S. mansoni* myosin (Newport *et al.*, 1987), a high molecular weight antigen of *Babesia bovis* (Gill *et al.*, 1987), a potentially protective antigen of *Brugia malayi* (Perrine *et al.*, 1988), a *Plasmodium knowlesi* antigen (Perler *et al.*, 1987) and a *P. falciparum* protective antigen (Horii *et al.*, 1988), to name but a few. Cloned immunodiagnostic antigens of *Leishmania donovani* (Blaxter *et al.*, 1988) and *S. mansoni* (Klinkert *et al.*, 1989) have also been subject to molecular characterisation.

Very limited studies have been reported with regard to the characterisation of cloned cestode antigens. In both the work on *Taenia taeniaeformis* (Bowtell *et al.*, 1984; 1986) and *T. ovis* (Howell and Hargreaves, 1988) antigens, characterisation consisted of the preliminary identification of native antigens. Analyses at the molecular level, to date, have only been performed with regard to *E. granulosus* antigen B (Shepherd, 1988). This work included the elucidation of the nucleotide sequence of a cloned gene fragment which corresponded to the 3' portion of the entire gene, leading to the putative identification of the native antigen's function as a protease inhibitor.

No such studies have been performed relating to *E. multilocularis* proteins. The only *E. multilocularis* antigen to have been characterised to any extent, to date, has been Em 2a (Gottstein, 1985). The protein has a M_r of 54 kDa and a pI value of 4.8, but no data at the molecular level is available. The antigen gene clones EM2 and EM4 were thus analysed in an effort to shed some light on this previously unexplored area.

4.1.2 THE CHARACTERISATION OF THE EM2 AND EM4 NATIVE ANTIGENS

Preliminary identification of the native proteins corresponding to cloned *T. taeniaeformis* and *T. ovis* antigens has been reported (Bowtell *et al.*, 1984; 1986 and Howell and Hargreaves, 1988, respectively). A similar approach was taken in the current study to allow identification of the native antigens which correspond to EM2 and EM4. This facilitated preliminary localisation of these antigens within the *E. multilocularis* metacestode. The related parasites *E. granulosus* and *T. solium* were also analysed to ascertain the reason for diagnostic species-specificity of EM4 and to establish whether any variation in the EM2 antigen exists between different species. Similar studies have been reported for an immunodiagnostic *L. donovani* antigen (Blaxter *et al.*, 1988). Identification of the EM2 and EM4 native antigens also provided preliminary data regarding the glycosylation of these proteins.

To obtain information relating to the expression of these antigens by the parasite, at the levels of transcription and translation, the mRNA transcripts were identified and translated *in vitro*. This allowed potential avenues of processing of the antigens to be identified. Studies of this type have been reported with regard to *S. mansoni* myosin (Newport *et al.*, 1987), a protective *B. malayi* antigen (Nilsen *et al.*, 1988), *Eimeria tenella* antigens (Profous-Juchelka *et al.*, 1988), a diagnostic antigen of *L. donovani* (Blaxter *et al.* 1988) and two diagnostic *S. mansoni* antigens (Klinkert *et al.*, 1989).

The genes coding for the native EM2 and EM4 antigens were identified and analysed to establish the nature of their genomic organisation, similar investigations having been reported previously relating to the gene(s) for *S. mansoni* myosin (Newport *et al.*, 1987), a protective *B. malayi* antigen (Perrine *et al.*, 1988), *P. falciparum* antigens (Hori *et al.*, 1988), *E. tenella* antigens (Profous-Juchelka *et al.*, 1988), a diagnostic *L. donovani* antigen (Blaxter *et al.*, 1988) and, most recently, two diagnostic *S. mansoni* antigens (Klinkert *et al.*, 1989). Where EM2 and EM4 related genes occurred in other parasites, conservation of their genomic organisation between species was investigated, work of this type having been reported previously with regard to the genes coding for protective *P. falciparum*

antigens (Horii *et al.*, 1988) and a diagnostic *L. donovani* antigen (Blaxter *et al.*, 1988).

Many parasite antigen genes have now been sequenced at the nucleotide level. The inferred amino acid sequences of cloned *S. mansoni* peptides have revealed homologies with the protein myosin (Newport *et al.*, 1987) and the proteases cathepsin B and haemoglobinase (Klinkert *et al.*, 1989). *P. falciparum* antigen genes have been sequenced and analysed for nucleotide and amino acid homologies with other genes and proteins, respectively (Horii *et al.*, 1988). One of these genes was found to have a degree of similarity to exon 23 and 24 of chicken vitellogenin and the *engrailed* locus of *Drosophila*. An *E. granulosus* antigen B cDNA clone has also been shown to code for a recombinant peptide which bears significant amino acid sequence homology to a protease inhibitor (Shepherd, 1988). Nucleotide sequencing of the EM4 cDNA was, therefore, used in an attempt to determine a possible function for this *E. multilocularis* antigen and to aid in its further characterisation.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

4.2.1.1 Antigens

EmAgA and EgSCF antigens (section 2.2.1.1) and EmAgPP antigen (section 3.2.1.1) were prepared as described earlier.

Crude *T. solium* whole antigen extract (TsAg) (cysticerci from Mexican patients) was prepared from whole cyst material in the same manner as EmAgA.

Pure *E. granulosus* protoscolex antigen extracts from sheep and horse strain material (EgAgPP-S and EgAgPP-H, respectively) were prepared from protoscoleces purified by pepsin treatment in the same manner as EmAgPP.

E. multilocularis cyst fluid antigen (EmAgCF) (aspirated from cotton rat alveolar cysts) was prepared as described for EgSCF.

E. multilocularis protoscolex excreted-secreted (EmAgE/S) antigen products were obtained by *in vitro* culture of purified protoscoleces under sterile conditions in RPMI-1640 medium (Gibco) for 5 days with slow rotation at 37 °C. The medium was changed daily and collection commenced after the first two days of culture. The medium was passed through an Acrodisc filter (0.45 µm) and concentrated by ultrafiltration as described for cyst fluid antigens (section 2.2.1.1).

4.2.1.2 Antisera

Mouse anti-β-gal and anti-fusion-peptide sera were prepared as described previously (section 2.2.1.2).

Affinity purified rabbit anti-EM-βgalFP4 antibody was prepared as described previously (section 2.2.2.5).

Human anti-*E. multilocularis* and anti-*E. granulosus* infection serum pools and the NHS pool were constructed as described previously (section 2.2.1.2).

4.2.1.3 *E. multilocularis* protoscolex total RNA extraction

Total RNA was extracted from protoscolexes, purified as previously described (section 2.2.2.1.1), using reagents and protocols provided by Amersham International (RNA extraction kit, code no; RPN.1254). This procedure utilised selective purification of RNA with lithium chloride. The yield of total RNA, estimated from OD_{260/280nm} absorbance readings, was approximately 100 µg from 1 ml of packed protoscolexes.

4.2.1.4 Genomic DNA extraction

Total nucleic acids were extracted from purified *E. multilocularis* and *E. granulosus* protoscolexes and from *T. solium* and *T. saginata* cysticerci by the method described by McManus *et al.* (1985). This involved disruption of parasite material by proteinase K digestion in the presence of SDS, deproteinisation with phenol, RNase digestion, phenol, phenol/chloroform and chloroform extractions, followed by ethanol precipitation of DNA. Estimations of DNA concentration and purity were made from OD_{260/280nm} absorbance readings.

4.2.2 METHODS

4.2.2.1 SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as described previously (section 2.2.2.3).

4.2.2.2 Lectin blotting

Biotinylated Lectin kits I (code no: BK-1000) and II (code no: BK-2000) were obtained from Vector Laboratories. A total of 14 lectins were used with a range of specificities summarised in Table 4.1.

Proteins were separated by SDS-PAGE and immunoblotted as above. All incubations were carried out at ambient temperature. Nitrocellulose strips were blocked with 1% (w/v) BSA in LBB [150 mM NaCl/0.1 mM CaCl₂/0.1 mM MnSO₄/20 mM Tris-HCl pH 7.5] for 60 minutes. Following

TABLE 4.1

Summary of biotinylated lectin binding specificities

No:	Biotinylated lectin	Specificity
1	Concanavalin A (Con A)	Mannose
2	Soybean agglutinin (SBA)	N-acetylgalactosamine, galactose
3	Wheat germ agglutinin (WGA)	N-acetylglucosamine (sialylated and non-sialylated residues)
4	Dolichos biflorus agglutinin (DBA)	N-acetylgalactosamine
5	Ulex europaeus agglutinin I (UEA I)	Fucose
6	Ricinus communis agglutinin I (RCA I)	Galactose, N-acetylgalactosamine
7	Peanut agglutinin (PNA)	Galactosyl β -1.3 N-acetyl-galactosamine ('T-antigen')
8	Bandeiraea simplicifolia lectin I (BSL I)	N-acetylgalactosamine, galactose
9	Pisum sativum agglutinin (PSA)	Mannose
10	Lens culinaris agglutinin (LCA)	Mannose
11	Phaseolus vulgaris agglutinin-E (PHA-E)	-
12	Phaseolus vulgaris agglutinin-L (PHA-L)	-
13	Sophora japonicum agglutinin (SJA)	N-acetylgalactosamine, galactose
14	Succinylated wheat germ agglutinin	Non-sialylated N-acetylglucosamine residues

a 1 × 10 minute wash in LBB, strips were incubated with solutions of each biotinylated lectin consisting of 1 µg ml⁻¹ lectin/1% (w/v) BSA/LBB for 30 minutes. Unbound lectin was removed by 3 × 10 minute washes in LBB. Avidin-HRP conjugate, diluted in LBB according to the manufacturer's recommendations, was incubated with strips for 30 minutes followed by removal of unbound reagent by washing as before. Lectin binding was revealed by incubation with DAB substrate (section 2.2.2.3) for 30 minutes. The colour reaction was terminated by washing with water and the strips were air dried.

4.2.2.3 Northern blotting

20 µg of *E. multilocularis* total RNA was fractionated under denaturing conditions using a 1.5% agarose gel containing formaldehyde (Maniatis *et al.*, 1982). The RNA was transferred to a Hybond-N nylon membrane (Amersham Int.) according to the manufacturer's recommended procedure. Hybridisation with radiolabelled probes was carried out using standard conditions as described by Maniatis *et al.* (1982). In addition, the hybridisation solution was supplemented with 50 µg ml⁻¹ Poly-A (BCL) to reduce background signal levels. The filter was washed to high stringency using 2 × 15 minute washes at 45 °C with 0.1% (w/v) SDS/0.3 M NaCl/0.03 M Na₃ citrate, 1 × 30 minute wash at 45 °C with 0.1% (w/v) SDS/0.15 M NaCl/0.015 M Na₃ citrate and 2 × 15 minute washes at ambient temperature with 0.1% (w/v) SDS/0.015 M NaCl/0.0015 M Na₃ citrate. The filter was wrapped in plastic film and autoradiographed at -70 °C with an intensifying screen.

Probes were radiolabelled with [α -³²P] dCTP using a multi-prime kit (Amersham Int., code no; RPN.1601) according to the manufacturer's recommended protocol. The specific activity of probes was estimated, by TCA precipitation (Maniatis *et al.*, 1982), to be in the region of 10⁸ cpm µg⁻¹.

Removal of probes from the blot was achieved by incubating the filter for 2 h at 65 °C in 0.005 M Tris-HCl pH 8.0/0.002 M EDTA/0.002% (w/v) BSA/0.002% (w/v) ficoll/0.002% (w/v) polyvinyl pyrrolidone. Complete removal of probes was ensured by re-autoradiography of the filter.

4.2.2.4 *In vitro* translation and immunoprecipitation

10 µg of *E. multilocularis* total RNA was *in vitro* translated using rabbit reticulocyte lysate (Amersham Int., code no; N.90), in the presence of [³⁵S] methionine (Amersham Int.), according to the manufacturer's recommended conditions.

Each immunoprecipitation utilised 5 µl of translation products and an equal volume of undiluted antiserum or affinity purified antibody in a total volume of 50 µl. The procedure used was identical to that described by McManus *et al.* (1985).

4.2.2.5 Southern blotting

10 µg of each genomic DNA was restricted with the appropriate restriction enzyme (Amersham Int.; BRL) using the manufacturer's recommended conditions, and was fractionated through a 0.8% agarose gel (Maniatis *et al.*, 1982). The DNA was transferred onto Hybond-N nylon membranes (Amersham Int.) according to the manufacturer's recommended procedure. Hybridisation with radiolabelled probes was carried out using standard conditions as described by Maniatis *et al.* (1982). Filters were washed to high stringency using 2 × 15 minute washes at 65 °C with 0.3 M NaCl/0.03 M Na₃ citrate, 1 × 30 minute wash at 65 °C with 0.1% (w/v) SDS/0.3 M NaCl/0.03 M Na₃ citrate and 1 × 10 minute wash at 65 °C with 0.015 M NaCl/0.0015 M Na₃ citrate. The filters were autoradiographed as described above.

Probes were radiolabelled as described above and were removed from blots by incubating the filters for 30 minutes at 45 °C in 0.4 M NaOH followed by a further 30 minute wash at 45 °C in 0.1% (w/v) SDS/0.015 M NaCl/0.0015 M Na₃ citrate/0.2 M Tris-HCl pH 7.5. Complete removal of probes was ensured by re-autoradiography of the filters.

4.2.2.6 Nucleotide sequencing

M13mp18 and M13mp19 (Messing, 1983) subclones were constructed using reagents and protocols provided by BRL, as previously described (section 3.2.2.3.2). Recombinants were grown and single strand DNA

templates for sequencing were prepared as described by the manufacturer. Preparation of double stranded RF DNA, as a source of insert DNA for further fragment subcloning, was performed as previously described (section 3.2.2.3.3).

The determination of subclone insert orientation was achieved using the complementarity test (C-test) (Messing, 1983) as described by Miller (1987).

Sequencing was performed using the dideoxynucleotide chain termination method (Sanger *et al*, 1977), for which protocols and reagents were supplied by United States Biochemical Corporation (USB). In addition, a 15 mer primer (BRL, code no; 8228SA), which anneals 14 bases upstream of the M13mp19 Eco R1 site, was used to prime reactions involving Eco R1 inserts. This facilitated sequencing of the 5' cloning site thus enabling the extreme ends of the insert to be sequenced. This allowed the β -gal reading frame to be determined. A modified bacteriophage T7 DNA polymerase (Sequenase) (USB) was used for strand synthesis. Reactions were performed using both dGTP and its analogue dITP (USB), in order to reduce ambiguities caused by secondary structure 'compressions'. Strands were radiolabelled with [α - ^{35} S] dATP (Amersham Int.).

Reaction products were electrophoresed under denaturing conditions on 'field gradient' gels, using a BRL model S2 apparatus and an LKB power source, according to the manufacturer's recommended procedures. Acrylamide monomer (National Diagnostics, sequencing grade reagent) percentage of gels and length of run were varied according to the desired size range to be read. Gels were fixed, dried and autoradiographed according to BRL procedures.

The derived amino acid sequence of EM4 was compared with previously reported amino acid sequences in the PSEQIP Protein Sequence Database of the Biocomputing Research Group at Edinburgh University.

4.3 RESULTS

4.3.1 THE IMMUNOBLOTTING OF ANTI-FUSION-PEPTIDE ANTIBODY WITH PARASITE ANTIGEN EXTRACTS

4.3.1.1 The identification of EM2 and EM4 native antigens

Preliminary characterisation of clones EM2 and EM4 involved identification of the native parasite antigens which they encoded. Antibody specific to the fusion-peptides was isolated either, in the case of EM- β galFP4, by affinity purification, or by hyperimmunisation of mice in the case of both EM- β galFP2 and EM- β galFP4. Various *E. multilocularis* antigen extracts were subjected to SDS-PAGE, immunoblotted and tested with mouse anti-EM- β galFP2 serum (Fig. 4.1). A single major band of 70 kDa was observed with EmAgA and EmAgPP (lanes 3-5). No change in mobility was observed under reducing or non-reducing conditions (lanes 3 and 4) indicating a lack of disulphide bound subunits within the protein. No apparent reaction with EmAgCF (lane 6) suggested that it was not an E/S product. A weak signal was detected, however, when *in vitro*-derived EmAgE/S extract was tested (lane 7). This may have been the result of dead, dying or disrupted protoscoleces which had released their somatic antigens into the medium during culture.

Figure 4.2 shows an immunoblot of both affinity purified rabbit anti-EM- β galFP4 antibody (lanes 4, 6, 8, 10 and 12) and mouse anti-EM- β galFP4 serum (lanes 3, 5, 7, 9 and 11) against various *E. multilocularis* antigen extracts, following their fractionation by SDS-PAGE. All lanes, except the controls and lane 10, showed three bands of 62 kDa, 49 kDa and 44 kDa. No change in mobility was observed under reducing or non-reducing conditions (lanes 3-6) indicating the absence of disulphide linked subunits in the three proteins. The three bands were present in the cyst fluid lane tested with mouse antiserum (lane 9) suggesting that all three are E/S products. However, the cyst fluid lane tested with affinity purified rabbit antibody (lane 10) failed to display the 62 kDa band. The reason for this discrepancy could be low abundance of this particular antigen within cyst fluid and/or a low level of affinity-purified rabbit antibody specific for it. The presence of the three bands in the *in vitro*-derived E/S extract

Figure 4.1 SDS-PAGE and immunoblotting of various *E. multilocularis* antigen extracts with mouse anti-EM- β galFP2 serum.

EmAgA was probed with: lane 1, normal mouse serum; lane 2, mouse anti- β -gal serum; lane 3, mouse anti-EM- β galFP2 serum.

Mouse anti-EM- β galFP2 serum was used to probe: lane 4, non-reduced EmAgA; lane 5, EmAgPP; lane 6, EmAgCF; lane 7, EmAgE/S.

50 μ g of protein was used per strip apart from EmAgE/S for which 6 μ g/strip was used. All antigens were reduced prior to electrophoresis unless otherwise specified.

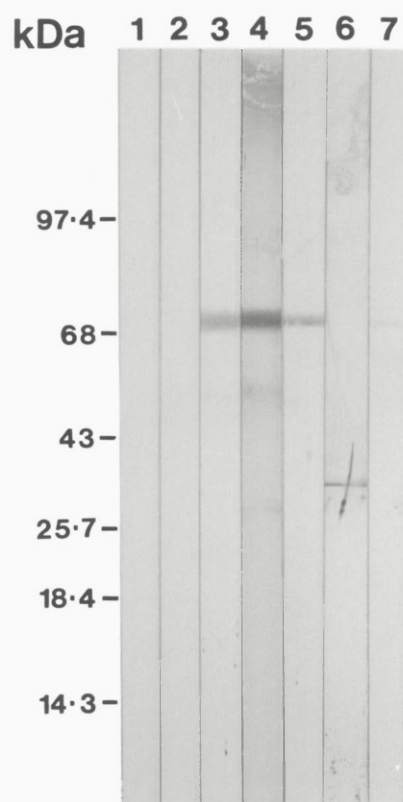


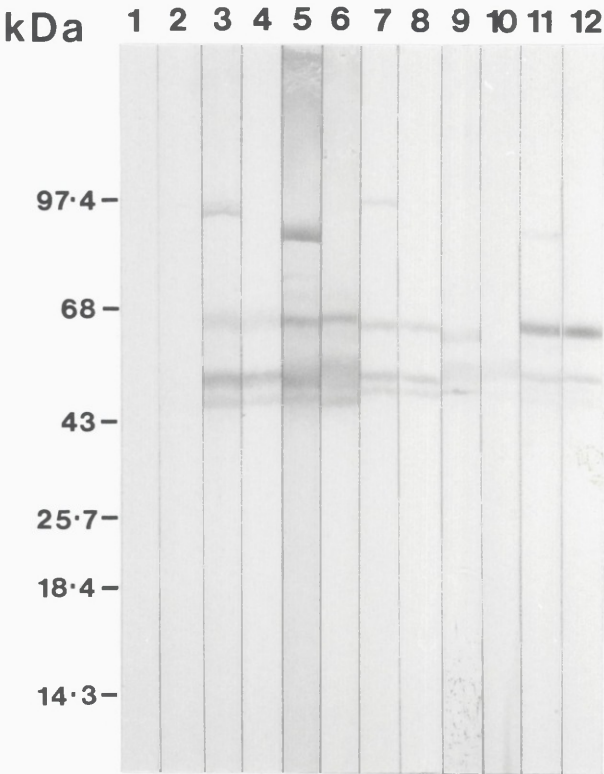
Figure 4.2 SDS-PAGE and immunoblotting of various *E. multilocularis* antigen extracts with mouse anti-EM- β galFP4 serum and affinity purified anti-EM- β galFP4 antibody.

EmAgA was probed with: lane 1, normal mouse serum; lane 2, mouse anti- β -gal serum; lane 3, mouse anti-EM- β galFP4 serum; lane 4, affinity purified rabbit anti-EM- β galFP4 antibody.

Mouse anti-EM- β galFP4 serum was used to probe: lane 5, non-reduced EmAgA; lane 7, EmAgPP; lane 9, EmAgCF; lane 11, EmAgE/S.

Affinity purified rabbit anti-EM- β galFP4 antibody was used to probe: lane 6, non-reduced EmAgA; lane 8, EmAgPP; lane 10, EmAgCF; lane 12, EmAgE/S.

50 μ g of protein was used per strip apart from EmAgE/S for which 6 μ g/strip was used. All antigens were reduced prior to electrophoresis unless otherwise specified.



(lanes 11 and 12) suggests that these proteins are excreted/secreted by protoscoleces of *E. multilocularis*. The strong signal produced by the 62 kDa band in the E/S lanes relative to the other two protein bands indicated that, of the three, this protein was the most abundant *in vitro* E/S product. A band of approximately 97 kDa appeared in several mouse antiserum lanes (lanes 3, 5, 7 and 11). This band was not apparent in the control lanes or the cyst fluid lane probed with mouse antiserum (lane 9). Affinity purified rabbit antibody, isolated from preabsorbed antiserum did not bind antigen at this M_r . The presence of cross-reactive, non-specific antibody within the mouse antiserum is, therefore, the most probable explanation for the appearance of this band.

4.3.1.2 The species-specificity of EM2 and EM4 native antigens

In order to determine whether these antigens were *E. multilocularis*-specific, extracts of *E. granulosus* protoscoleces, sheep and horse strains (EgAgPP-S and EgAgPP-H, respectively) and *T. solium* cysticerci (TsAg) were tested with fusion-peptide-specific antibody (Fig. 4.3). Native proteins corresponding to the M_r 's established previously for both clones, were present within EgAgPP-S and EgAgPP-H (sheep strain, lanes 6-8; horse strain, lanes 10-12). A 70 kDa antigen related to that of EM2, was also present in TsAg (lane 14), as was a 62 kDa antigen related to that of EM4 (lane 16). In addition, protein bands of the expected M_r for the EM4 antigens were apparent in TsAg when tested with mouse antiserum (lane 15). These bands were also produced, however, by mouse anti-EM- β galFP2 serum (lane 14). Cross-reactive antibody in this mouse antiserum appeared, therefore, to be binding at the same M_r as the EM4 antigens. The presence of native antigens related to those of EM2 and EM4 within these parasite extracts, suggests that they are conserved between the various taeniid species. However, the antigens were not detected in EgSCF (lanes 2-4) suggesting that they are not excreted/secreted into the lumen of the unilocular cyst, although they are present in protoscoleces.

Figure 4.3 SDS-PAGE and immunoblotting of *E. granulosus* and *T. solium* antigen extracts with anti-fusion-peptide antibody.

EgSCF was probed with: lane 1, mouse anti- β -gal serum; lane 2, mouse anti-EM- β galFP2 serum; lane 3, mouse anti-EM- β galFP4 serum; lane 4, affinity purified rabbit anti-EM- β galFP4 antibody.

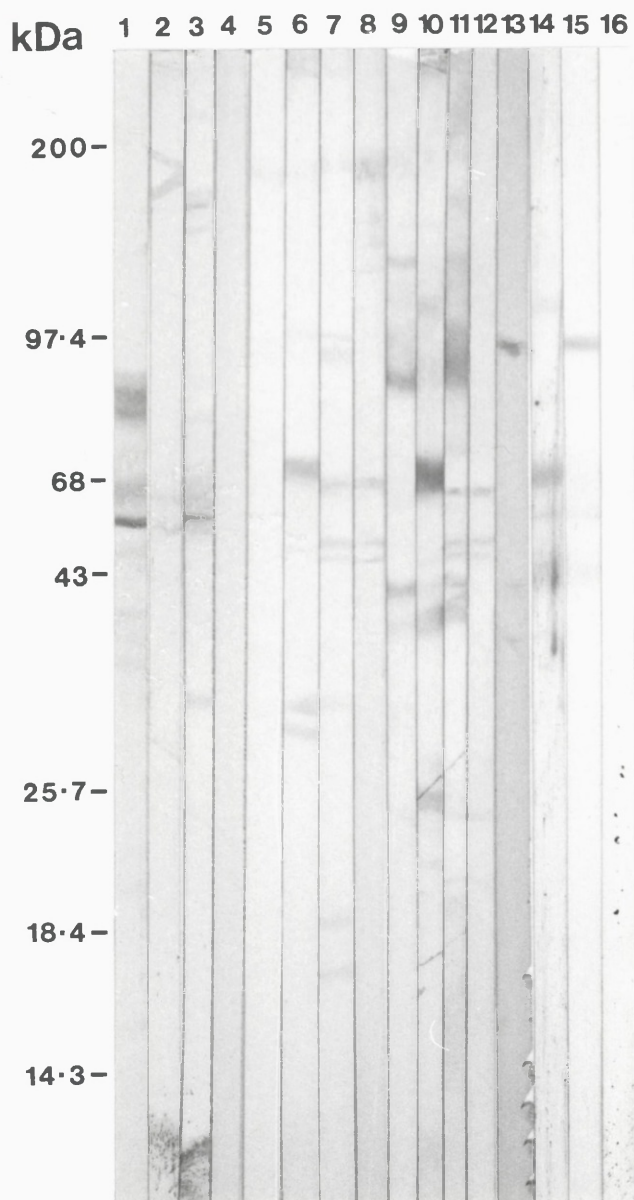
EgAgPP-S was probed with: lane 5, mouse anti- β -gal serum; lane 6, mouse anti-EM- β galFP2 serum; lane 7, mouse anti-EM- β galFP4 serum; lane 8, affinity purified rabbit anti-EM- β galFP4 antibody.

EgAgPP-H was probed with: lane 9, mouse anti- β -gal serum; lane 10, mouse anti-EM- β galFP2 serum; lane 11, mouse anti-EM- β galFP4 serum; lane 12, affinity purified rabbit anti-EM- β galFP4 antibody.

TsAg was probed with: lane 13, mouse anti- β -gal serum; lane 14, mouse anti-EM- β galFP2 serum; lane 15, mouse anti-EM- β galFP4 serum; lane 16, affinity purified rabbit anti-EM- β galFP4 antibody.

50 μ g of protein was used per strip. Proteins were reduced prior to electrophoresis.

Molecular weights of native antigens are arrowed: EM2 ($\triangleleft$); EM4 ($\blacktriangleleft$).



4.3.2 GLYCOSYLATION OF THE EM2 AND EM4 NATIVE ANTIGENS

To further characterise the native proteins corresponding to clones EM2 and EM4, EmAgPP antigen was immunoblotted and probed with a range of carbohydrate-binding lectin proteins (Fig. 4.4). Heavy background binding was observed in the high and low M_r regions but distinct glycoprotein bands within the 30 kDa to 80 kDa M_r range could be identified. Bands corresponding to the M_r 's of the EM2 and EM4 antigens were visible but could have been due to other glycoproteins which co-migrate at the same M_r as these antigens. The lectins SJA (lane 13) and succinylated WGA (lane 14) do not appear to have bound to the same extent as the other lectins (lanes 1 to 12) or the pool (lane 15). This was probably due to a technical aspect rather than the absence of the sugar structures specific for these lectins on the blotted proteins, particularly as the specificities of these two lectins were duplicated by others (SBA and WGA). The same glycoprotein bands bound all lectins, except SJA and succinylated WGA, indicating significant levels of varied glycosylation.

4.3.3 THE IDENTIFICATION OF EM2 AND EM4 mRNA TRANSCRIPTS

A blot of total *E. multilocularis* RNA was probed with λ gt11/EM2 (lane 2) and EM4 insert (lane 3) DNA (Fig. 4.5). The EM2 cDNA could not be excised from λ gt11 using Eco R1 (data not shown) possibly owing to damage to one or both of the Eco R1 cloning sites during library construction. Therefore, vector DNA containing insert was used as probe. The blot was autoradiographed for the same time period with each probe. The non-recombinant λ gt11 control probe (lane 1) showed no cross-hybridisation with the parasite RNA.

The EM2 probe produced two very weak bands at approximately 1 660 bp and 1 288 bp and a large smear at 900 bp. This latter effect was probably due to RNA degradation. Only one native antigen of 70 kDa was detected with anti-EM2 antibody. Consequently only one mRNA species was expected to give rise to this protein. Although the protein is probably glycosylated, a transcript in the region of 1 660 bp is more likely to code for a molecule of this size than the smaller transcript identified. The band of 1 660 bp may, therefore, correspond to the correct EM2 transcript, the

Figure 4.4 SDS-PAGE and lectin blotting of EmAgPP with a range of biotinylated lectins.

Lane 1, Con A.

Lane 2, SBA.

Lane 3, WGA.

Lane 4, DBA.

Lane 5, UEA I.

Lane 6, RCA I.

Lane 7, PNA.

Lane 8, BSL I.

Lane 9, PSA.

Lane 10, LCA.

Lane 11, PHA-E.

Lane 12, PHA-L.

Lane 13, SJA.

Lane 14, succinylated WGA.

Lane 15, pool.

50 µg of protein was used per strip. Proteins were reduced prior to electrophoresis.

Molecular weights of native antigens are arrowed: EM2 (◁); EM4 (◀).

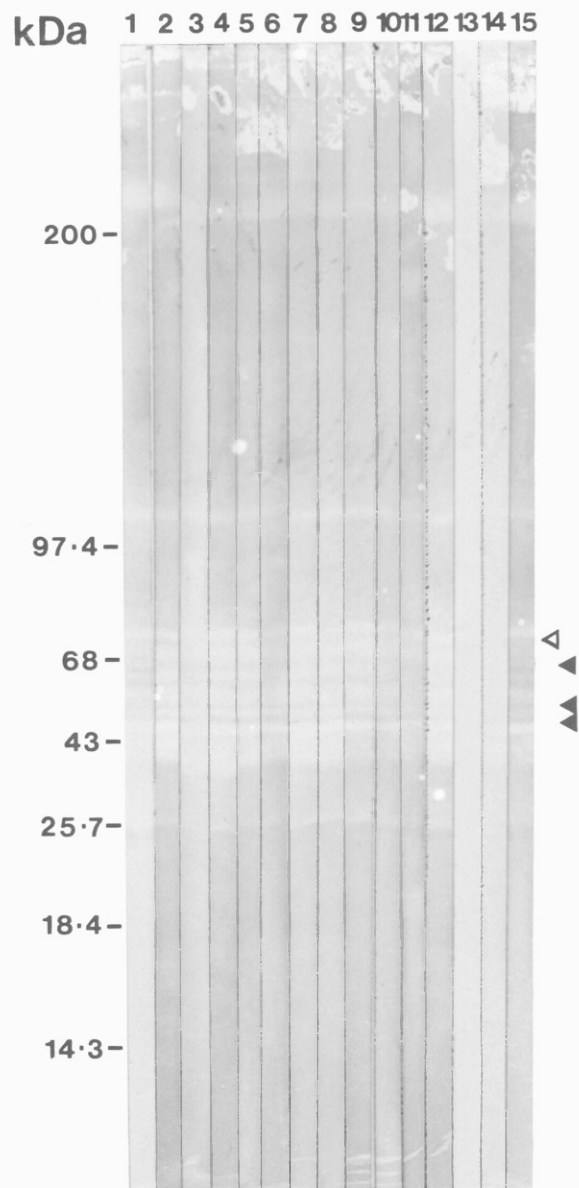


Figure 4.5 Northern blot analysis of EM2 and EM4 transcripts. Total *E. multilocularis* protoscolex RNA was fractionated, blotted and probed with:

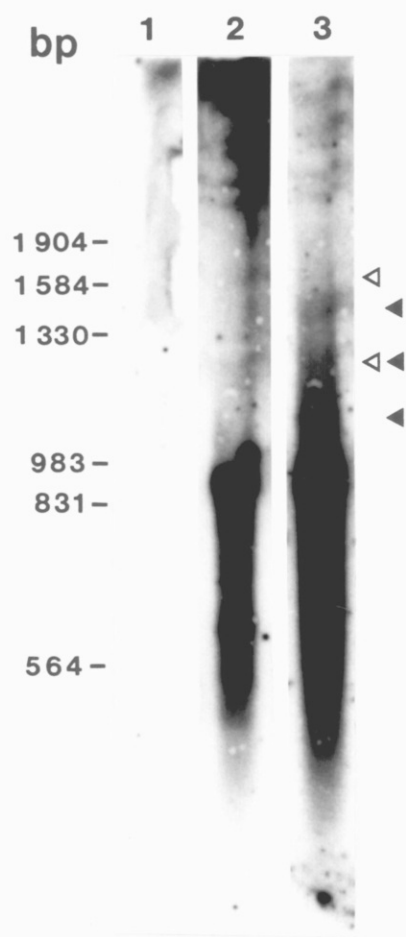
Lane 1, non-recombinant λ gt11 DNA.

Lane 2, λ gt11/EM2 DNA.

Lane 3, EM4 cDNA insert.

20 μ g of RNA was used per lane.

Putative EM2 ($\blacktriangleleft$) and EM4 ($\blacktriangleleft$) transcripts are arrowed.



lower M_r band resulting from degradation of this message. A similar phenomenon has been reported recently with regard to *S. mansoni* transcripts (Klinkert *et al.*, 1989). The EM2 fusion-peptide contained 39 kDa of the EM2 antigen (section 2.3.4.1), which corresponds to a cDNA of approximately 1 050 bp. If the 1 660 bp band does correspond to the correct mRNA then the cloned cDNA molecule was not of full length.

The EM4 probe (lane 3) hybridised to three distinct bands of 1 514 bp, 1 288 bp and 1 096 bp and a smear, similar to that produced by EM2, at 900 bp. The latter effect was again probably due to degradation of message. The signals produced were significantly greater than those produced with EM2, perhaps reflecting the greater abundance of these transcripts. Three native antigens of 62 kDa, 49 kDa and 44 kDa were detected with anti-EM4 antibody. If each of these proteins is coded for by a distinct mRNA species, then the EM4 cDNA may only correspond to one of these antigens, the other two merely possessing common epitopes. The transcripts identified would have the coding capacity for proteins of 55 kDa, 45 kDa and 40 kDa, respectively. The addition of carbohydrate residues would account for the discrepancy between these M_r 's and those of the native antigens identified. The size of the EM4 cDNA has been found to be 1 114 bp following nucleotide sequencing (Fig. 4.9 B), therefore, as the sizes of the transcripts identified are approximate, this molecule could have originated from any of the three putative mRNA species identified. An alternative explanation for the origin of the three bands could be the artefactual production of two smaller bands by degradation of the large 1 514 bp transcript, as suggested above with the EM2 transcript. This would suggest that only one mRNA species codes for the native protein(s).

In order to explore these possibilities further, total RNA was translated in a cell-free system and the products precipitated with specific antisera.

4.3.4 THE IMMUNOPRECIPITATION OF *IN VITRO* TRANSLATION PRODUCTS

In order to establish the relationship between the EM2 and EM4 transcripts and their native proteins, total RNA was translated and the products immunoprecipitated with antisera raised to EM2 and EM4 recombinant antigens (Fig. 4.6).

Figure 4.6 SDS-PAGE analysis of *E. multilocularis* protoscolex total RNA *in vitro* translation products immunoprecipitated by anti-fusion-peptide antibody.

Lane 1, no added RNA control.

Lane 2, total translation products.

In vitro translation products immunoprecipitated by:

Lane 3, normal human serum.

Lane 4, pool of human anti-*E. multilocularis* sera.

Lane 5, pool of human anti-*E. granulosus* sera.

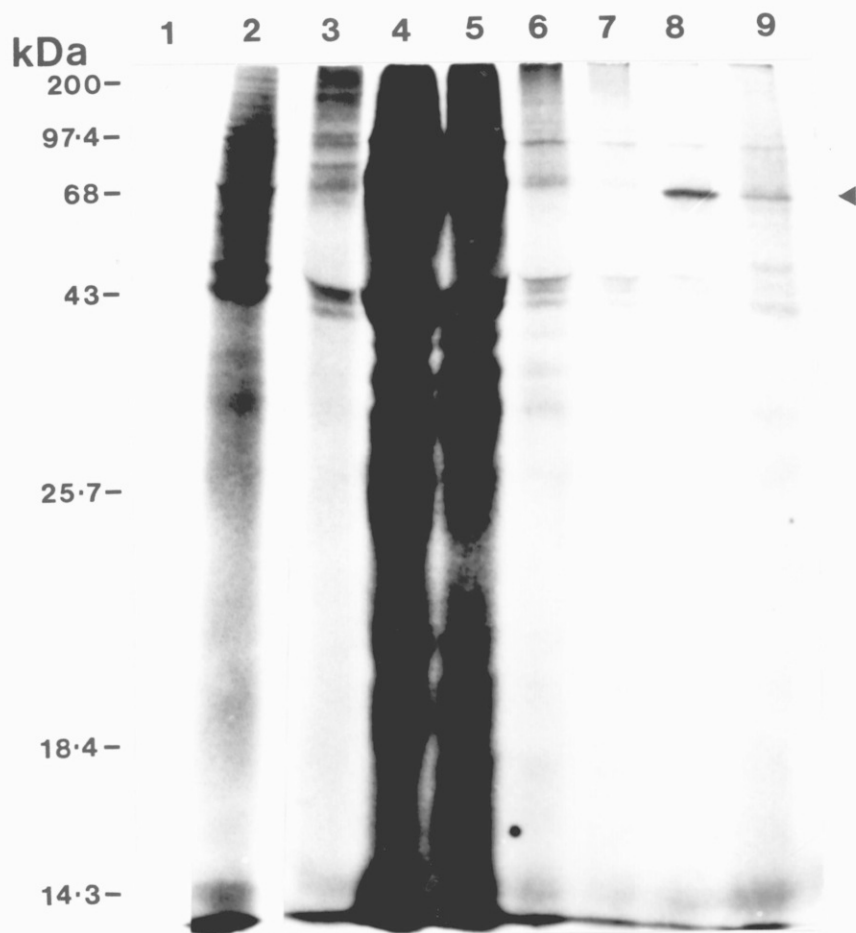
Lane 6, mouse anti- β -gal serum.

Lane 7, mouse anti-EM- β galFP2 serum.

Lane 8, mouse anti-EM- β galFP4 serum.

Lane 9, affinity purified rabbit anti-EM- β galFP4 antibody.

Samples were reduced prior to electrophoresis. The 66 kDa protein precipitated by anti-EM- β galFP4 antibody is arrowed.



No additional proteins other than those precipitated by the mouse anti- β -gal control serum (lane 6), were precipitated by mouse anti-EM- β galFP2 serum (lane 7). Significant amounts of the EM2 native antigen had not, therefore, been synthesized *in vitro*. This may have been due to low abundance of the EM2 transcript, as suggested by Northern blotting, or degradation of the available message. The possibility of the 1 660 bp band corresponding to the EM2 transcript was not, therefore, substantiated by this experiment.

With the mouse anti-EM- β galFP4 serum (lane 8) and the affinity purified rabbit anti-EM- β galFP4 antibody (lane 9), a major band of approximately 66 kDa was observed. This band was not seen with the mouse anti- β -gal control serum (lane 6). A minor band of approximately 50 kDa was apparent with the rabbit anti-EM- β galFP4 antibody (lane 9). This band was not produced, however, with mouse anti-EM- β galFP4 serum (lane 8) and was probably artefactual. No discernible variation in the proteins precipitated by the human anti-*E. multilocularis* (lane 4) and anti-*E. granulosus* (lane 5) serum pools was apparent in the 66 kDa region.

The precipitation by anti-EM- β galFP4 antibody of one protein instead of three, suggested that one transcript may code for all three native antigens. Within the limits of experimental error, the 1 514 bp transcript has sufficient coding capacity for the 66 kDa immunoprecipitated protein. This message may, therefore, correspond to the genuine EM4 transcript. The two additional transcripts detected by Northern blot analysis could have resulted from degradation of the single mRNA species, as discussed earlier. If the 1 114 bp EM4 cDNA did originate from a 1 514 bp message then the cloned cDNA molecule is incomplete.

If all three native antigens that react with anti-EM4 antibody do originate from the same transcript, then some form of post-translational modification of a single 'precursor' protein would be necessary. The larger size of the *in vitro* translate product (66 kDa), relative to the native antigens, may in part be due to the retention of a signal peptide by the former molecule. The EM4 native antigens have been shown to be excreted/secreted from the parasite, therefore, a signal peptide is probably present within the 'precursor' protein. The removal of this signal sequence upon excretion/secretion of the native antigens would account for some of the reduction in M_r . Such post-translational modification would not take

place within a cell-free system, allowing retention of the signal peptide by the 'precursor' molecule. Additional post-translational modifications to the pre-protein would also be required, however, to account for the size of the two smaller E/S antigens.

4.3.5 THE GENOMIC ORGANISATION OF THE EM2 AND EM4 GENES

E. multilocularis genomic DNA was digested with a number of restriction enzymes, Southern blotted and probed with non-recombinant λ gt11 (panel A), λ gt11/EM2 (panel B) and EM4 cDNA insert (panel C) (Fig. 4.7). No cross-hybridisation of the non-recombinant λ gt11 DNA with the parasite DNA was apparent (panel A).

The EM2 probe (panel B) produced a complex hybridisation pattern. Multiple bands were present in all lanes, the lowest M_r band of 2 300 bp being produced by Bam HI (lane 1). Because no restriction map of the EM2 cDNA was available and the fact that the cDNA was probably not of full length, the origins of these restriction sites could not be predicted. The hybridisation pattern obtained, however, suggested the EM2 native antigen may be coded for by either a complex single-copy gene containing many introns, or a multi-copy gene family. Additional work is required to resolve this further.

The EM4 probe (panel C) produced a much simpler hybridisation pattern compared to that of EM2. This confirmed that these two antigens are unrelated. From nucleotide sequencing data (Fig. 4.9) it was known that of the restriction enzymes used, only Sst I (Sac I) (lane 5) had restriction sites within the EM4 cDNA. Single, high M_r bands were produced in all lanes except Hind III (lane 3) and Sst I (lane 5). The band of approximately 600 bp (lane 5) may correspond to the 646 bp Sst I fragment within the cDNA sequence (Fig. 4.9 A). Because two bands were produced with Hind III (lane 3) it is probable that there is an intronic sequence within the gene which contains a Hind III site. This restriction site may also be present, however, in the uncloned region of the transcribed gene, assuming that an incomplete cDNA had been cloned. The relatively simple hybridisation pattern suggested that the EM4 native antigens may be coded for by a single-copy gene. The lack of cross-hybridisation of the EM4 cDNA to other genes confirmed that one type of gene codes for all three native antigens

Figure 4.7 Southern blot analysis of restriction enzyme cut *E. multilocularis* DNA probed with non-recombinant λ gt11 DNA (A), λ gt11/EM2 DNA (B) and EM4 cDNA insert (C).

Restriction enzymes used were:

Lane 1, Bam HI.

Lane 2, Eco RI.

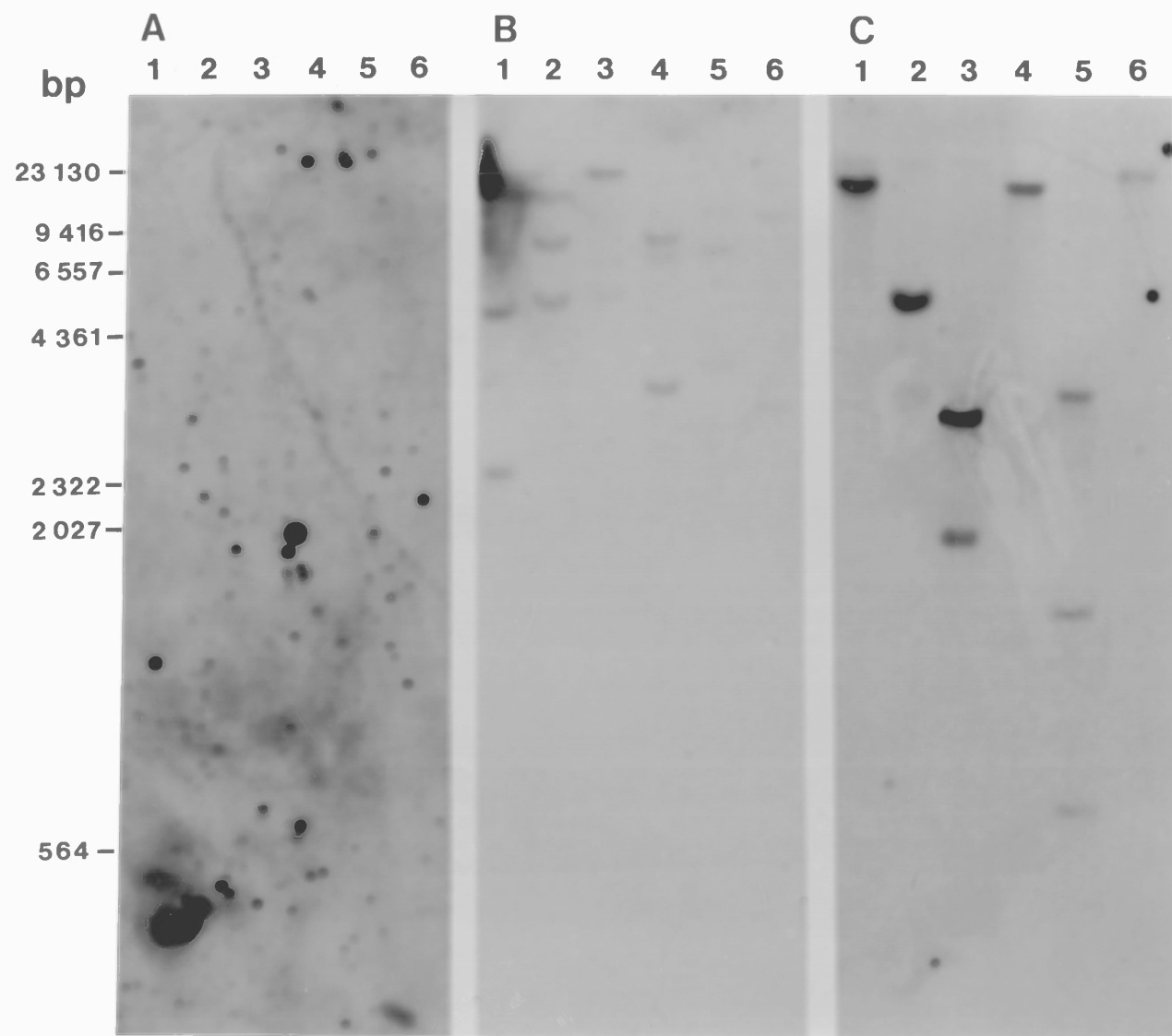
Lane 3, Hind III.

Lane 4, Pst I.

Lane 5, Sst I.

Lane 6, Xba I.

10 μ g of DNA was used per lane.



identified. Consequently, it follows that these antigens originate from the post-translational modification of a pre-protein which is coded for by a single mRNA species.

4.3.6 THE ORGANISATION OF EM2 AND EM4 GENES IN RELATED PARASITE GENOMES

It has been established that native antigens related to those of EM2 and EM4 are produced by *E. granulosus* and *T. solium* (Fig. 4.3). To investigate the genomic organisation of their corresponding genes in these parasite species, blots of restricted genomic DNA were probed with the EM2 and EM4 cloned sequences (Fig. 4.8).

The EM2 probe (panel B) produced distinctly different hybridisation patterns with restricted genomic DNA from the four species. No two restriction patterns produced using the same restriction enzyme were the same, indicating significant differences in the genomic organisation of the gene(s), even with *E. granulosus*, the most closely related to *E. multilocularis* of those tested. Relatively weak signals were produced by *T. solium* and *T. saginata* DNAs (lanes 9 and 10, respectively), presumably due to decreased sequence homology with the gene(s) of *E. multilocularis*.

The EM4 probe (panel C) hybridisation pattern differed with various parasite DNAs as found with the EM2 probe. Differences in the *E. multilocularis* (lanes 1 to 4) and *E. granulosus* (lanes 5 to 8) hybridisation patterns indicated specific differences in the structure of the gene of these two species. It was apparent that the *E. granulosus* gene possesses an internal Eco RI site (lane 5) and lacks an internal Hind III site (lane 6), in contrast to the *E. multilocularis* gene (lanes 1 and 2). The same number of bands were produced by Sst I and Eco RI/Sst I digestion of *E. granulosus* (lanes 7 and 8) and *E. multilocularis* (lanes 3 and 4) DNAs, although the fragment lengths appeared to vary considerably, indicating further differences in the organisation of the gene in *E. granulosus* and *E. multilocularis*. *T. solium* and *T. saginata* DNAs hybridised weakly (lanes 9 and 10, respectively) presumably due to decreased sequence homology with the *E. multilocularis* gene, as with EM2 above.

Figure 4.8 Southern blot analysis of restriction enzyme cut taeniid DNAs probed with non-recombinant λ gt11 DNA (A), λ gt11/EM2 DNA (B) and EM4 cDNA insert (C).

Lanes 1-4, *E. multilocularis* DNA.

Lanes 5-8, *E. granulosus* DNA.

Lane 9, *T. solium* DNA.

Lane 10, *T. saginata* DNA.

Restriction enzymes used were:

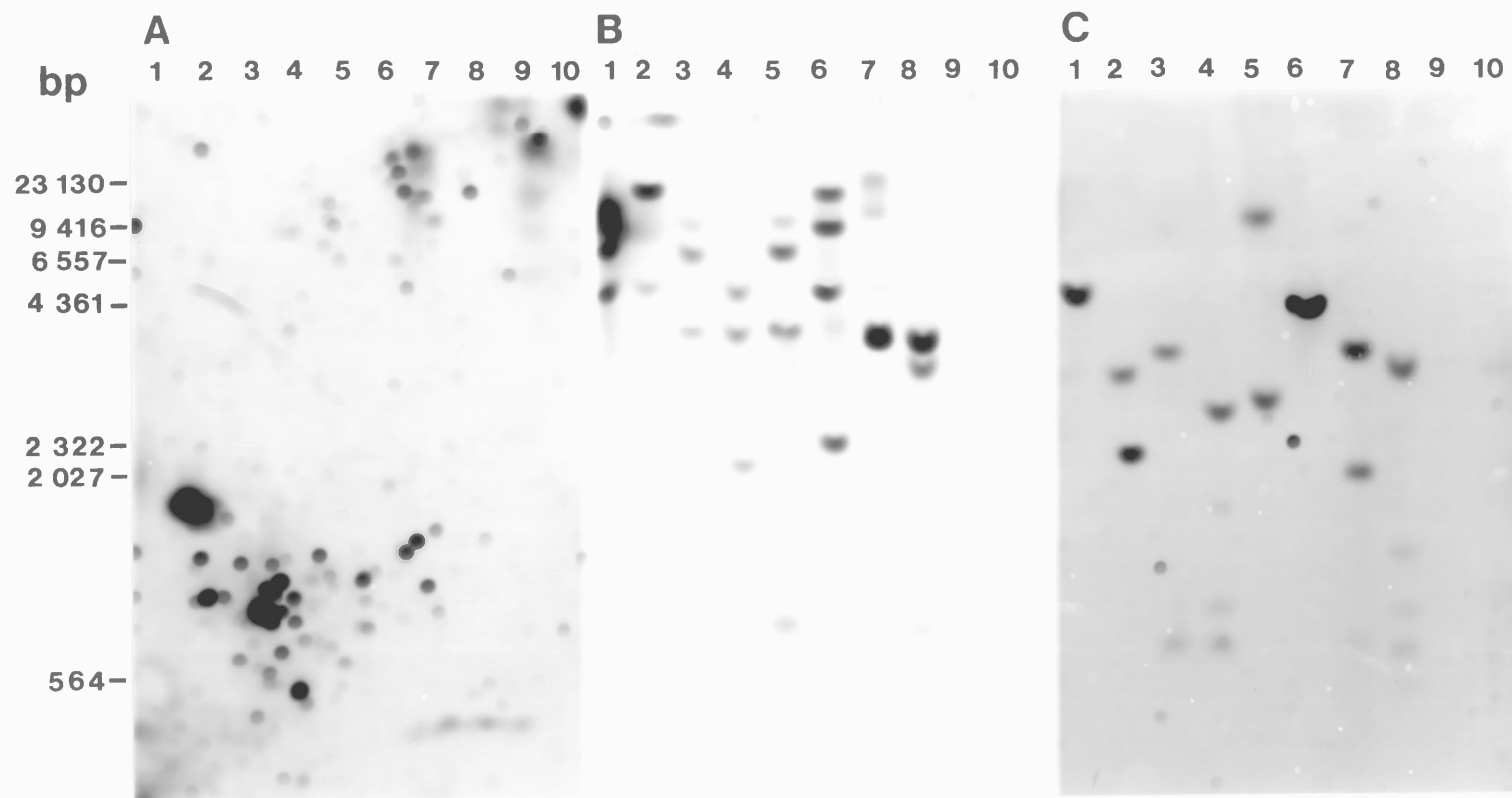
Lanes 1 and 5, Eco RI.

Lanes 2 and 6, Hind III.

Lanes 3 and 7, Sst I.

Lanes 4, 8, 9 and 10, Eco RI/Sst I.

10 μ g of DNA was used per lane.



4.3.7 NUCLEOTIDE SEQUENCING OF THE EM4 cDNA

In order to further characterise the protein coded for by this molecule, the nucleotide sequence of the cDNA was obtained (Fig. 4.9 B). The entire cDNA insert was excised from λ gt11 and subcloned into the M13mp19 Eco RI site in both orientations, as confirmed by the C-test (Miller, 1987) (data not shown). Preliminary sequencing of these subclones revealed the location of two convenient Sst I sites within the molecule. These were used to subclone the 646 bp Sst I fragment into M13mp19 and the 350 bp Eco RI/Sst I fragment into M13mp18. These clones facilitated complete sequencing of the cDNA. The overall strategy is outlined in Figure 4.9 A. The coding strand was identified by the presence of a polyadenylated tail which also enabled the identification of the carboxyl terminal of the expressed protein. The putative polyadenylation signal in the 3' non-coding sequence is underlined. This is an unusual but not unknown variant on the more common 'AATAAA' signal sequence (McLauchlan *et al.*, 1985). The complete inserted cDNA sequence is 1 114 bp in length.

The three possible reading frames of this sequence were analysed and of these, only one appeared to be appropriate and is shown in Figure 4.9 B. The other reading frames were not thought to be correct as they resulted in premature termination of the protein 72 bp and 158 bp from the 5' end. The longest reading frame found was also in frame with the *lacZ* gene of λ gt11 (Young and Davis, 1983). In addition, the 354 amino acids coded for by the open reading frame corresponded to a protein of 39 kDa which was the approximate size of the foreign peptide expressed by EM4. Therefore, the derived amino acid sequence corresponded to that expressed by the EM4 clone. The first ATG (Met) codon was found 120 bp downstream of the 5' end, this may represent an initiation codon or an internal methionine residue. If it is an initiation codon then 313 amino acids would be coded for, resulting in a 34 kDa protein. This did not correspond to the 66 kDa protein immunoprecipitated by anti-EM4 antibody (Fig. 4.6). Further evidence that this was not the genuine initiation codon was provided by the fact that the 5 bases immediately upstream of this ATG did not conform to the eukaryotic initiation site consensus sequence 'CC(A/G)CCATG' (Kozak, 1984). This codon is, therefore, unlikely to represent the true initiation signal. The genuine signal is probably situated, going by the transcript size,

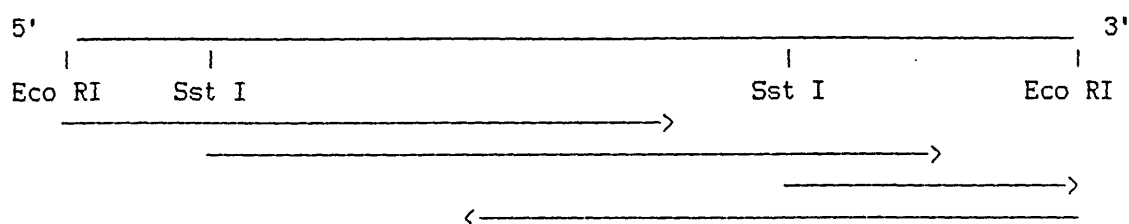
Figure 4.9 Nucleotide sequencing of EM4 cDNA.

(A) Partial restriction map and sequencing strategy.

(B) Nucleotide and derived amino acid sequences.

An asterisk indicates the translation stop codon. The putative polyadenylation signal upstream of the poly(A) tract is underlined as is an N-linked carbohydrate acceptor site situated at amino acids 240-242. Eco RI linker sequences used for cloning are not shown.

A



B

CAG	AAG	TTC	ATC	ATC	AAA	CCG	GCA	GAC	AAG	TCC	GCA	AAG	GAG	TTT	TTC	48
Gln	Lys	Phe	Ile	Ile	Lys	Pro	Ala	Asp	Lys	Ser	Ala	Lys	Glu	Phe	Phe	16
TTC	TTG	GTG	GAA	AAA	TCC	AAG	ATT	AAC	AAG	CGC	ATT	TTG	GCA	TTG	TGT	96
Phe	Leu	Val	Glu	Lys	Ser	Lys	Ile	Asn	Lys	Arg	Ile	Leu	Ala	Leu	Cys	32
ACT	GGC	AAC	CAT	GAG	CTC	TAC	ATG	CGT	AGA	AGA	AAG	TCA	GAC	TCT	ATT	144
Thr	Gly	Asn	His	Glu	Leu	Tyr	Met	Arg	Arg	Arg	Lys	Ser	Asp	Ser	Ile	48
GAG	GTG	CAA	CAG	ATG	AAG	ATT	CAG	GCC	AAG	GAG	GAA	CGT	GAA	TTG	AAG	192
Glu	Val	Gln	Gln	Met	Lys	Ile	Gln	Ala	Lys	Glu	Glu	Arg	Glu	Leu	Lys	64
GAG	GCT	GAG	AGA	CAA	CGC	CTG	AAG	GAG	GAA	CGA	TTG	CAA	CGT	ATG	GAA	240
Glu	Ala	Glu	Arg	Gln	Arg	Leu	Lys	Glu	Glu	Arg	Leu	Gln	Arg	Met	Glu	80
AAT	GAA	CAG	AAA	CTG	CGG	GAG	CTT	CGT	GCT	CAA	ATG	GTC	GAA	AAG	GAG	288
Asn	Glu	Gln	Lys	Leu	Arg	Glu	Leu	Arg	Ala	Gln	Met	Val	Glu	Lys	Glu	96
TCT	GAC	TTA	GCG	GAT	ATG	AAG	AAT	AAG	GCA	TCT	GCC	TAT	GAG	AGT	AAG	336
Ser	Asp	Leu	Ala	Asp	Met	Lys	Asn	Lys	Ala	Ser	Ala	Tyr	Glu	Ser	Lys	112
ATT	GCG	GAG	CTG	GAG	ATG	CTG	CTA	CAG	CAG	GAG	CGA	CAT	GCG	CGT	GAG	384
Ile	Ala	Glu	Leu	Glu	Met	Leu	Leu	Gln	Gln	Glu	Arg	His	Ala	Arg	Glu	128
AGT	CTT	CAG	AAG	AGC	CAA	GAC	AAA	CTG	GCG	GAG	ATG	AAC	AGA	AAG	CTG	432
Ser	Leu	Gln	Lys	Ser	Gln	Asp	Lys	Leu	Ala	Glu	Met	Asn	Arg	Lys	Leu	144
AAG	GAG	GAG	ACT	GCG	GCA	TCA	GCC	GAA	GAG	CGC	GAC	CGT	CTG	ATG	GCC	480
Lys	Glu	Glu	Thr	Ala	Ala	Ser	Ala	Glu	Glu	Arg	Asp	Arg	Leu	Met	Ala	160
CAG	CGT	GAC	GAA	GTG	CAA	CGC	GAA	GTT	GAG	GCT	CAG	AAG	GTC	GCC	ATG	528
Gln	Arg	Asp	Glu	Val	Gln	Arg	Glu	Val	Glu	Ala	Gln	Lys	Val	Ala	Met	176
GCC	AAG	AAG	GAA	GCT	GAA	AAG	GCT	CAG	GCT	GAA	GCT	GAG	CTT	CGC	AGA	576
Ala	Lys	Lys	Glu	Ala	Glu	Lys	Ala	Gln	Ala	Glu	Ala	Glu	Leu	Arg	Arg	192

Continued....

EM4 cDNA sequence continued.....

ATG CTG AGA ACA CGA TGC AAG CAC AAG TCC AGG TCA ATG GCA GTG GTG	624
Met Leu Arg Thr Arg Cys Lys His Lys Ser Arg Ser Met Ala Val Val	208
ACG CTC GCA GAT GAT GAA AGT GAA GCC AAG GAA CTT GAG GTG ATA CCA	672
Thr Leu Ala Asp Asp Glu Ser Glu Ala Lys Glu Leu Glu Val Ile Pro	224
AAT GTG AGG CGG ACG GAG GAA TCG AGG GTG ACG GCC GTC TCT AAG AAT	720
Asn Val Arg Arg Thr Glu Glu Ser Arg Val Thr Ala Val Ser Lys <u>Asn</u>	240
GAG ACA CTC AGA CGA AGC TGG CCA ACT AAA ATG GAG TTG AGC TCG ACA	768
<u>Glu Thr</u> Leu Arg Arg Ser Trp Pro Thr Lys Met Glu Leu Ser Ser Thr	256
CGC GAT CAG TCG AAA ATG CGC GAC ATT GAT CGT CGT CAT GAG TAC AAT	816
Arg Asp Gln Ser Lys Met Arg Asp Ile Asp Arg Arg His Glu Tyr Asn	272
GTG CGG GAG GGT AAT GAC AAG TAC AAG ACA CTG CGC AAC ATT CGC AAG	864
Val Arg Glu Gly Asn Asp Lys Tyr Lys Thr Leu Arg Asn Ile Arg Lys	288
GGC AAC ACC ATG TGT CGT GTT GAA CAG TTT GAG TCG ATG TAC AAA TGT	912
Gly Asn Thr Met Cys Arg Val Glu Gln Phe Glu Ser Met Tyr Lys Cys	304
TAC AGT TGT CTT CAT TCC CCT CAT CTT TCT GCA ATT TTG GAC CCT CTA	960
Tyr Ser Cys Leu His Ser Pro His Leu Ser Ala Ile Leu Asp Pro Leu	320
TCA CTA CGG TTA CTT CCT CAA TCA TGC TGC TAC AGT GCT ATC CAA CAT	1 008
Ser Leu Arg Leu Leu Pro Gln Ser Cys Cys Tyr Ser Ala Ile Gln His	336
TCC ATT TTT ATT CTC GCG ATG CAC ACC TGC CTT TTC ATT CTG CCC CTT	1 056
Ser Ile Phe Ile Leu Ala Met His Thr Cys Leu Phe Ile Leu Pro Leu	352
CTT ATC TAACGTCCACTTT <u>ATTAA</u> CTGCTATCGTACGCTAAAAAAAAAAAAAAAAAAAA	1 114
Leu Ile *	354

approximately 500 bp upstream of this codon. This region of 5' sequence was absent from the incomplete EM4 cDNA analysed.

One N-linked oligosaccharide acceptor site (Asn-Xxx-Ser/Thr) was situated at amino acids 240-242 (underlined). This was predicted as secreted proteins are usually glycosylated. Data regarding O-linked carbohydrate could not be obtained from the amino acid sequence on account of the recognition sequence for this modification being poorly defined (Bitter, 1987).

The lack of any recognisable hydrophobic domains suggestive of membrane spanning or anchoring was in accordance with the finding that the EM4 native antigens are excreted/secreted and not membrane bound, however, no hydrophobic signal peptide was found despite the fact that the native proteins are excreted/secreted. Such a peptide would occur at the amino-terminal end of the protein, corresponding to the 5' end of the cDNA. The lack of data relating to the 5' end of the sequence, resulting from the analysis of a partial cDNA, probably accounts for the missing signal peptide.

No obvious major repeat sequences were detected within the sequence, as have been found in some cloned parasite antigens (Newport *et al.*, 1987; Perler *et al.*, 1987; Horii *et al.*, 1988).

In order to ascribe some function to the cloned peptide, and hence to the native antigens, the inferred amino acid sequence was compared with previously reported sequences in the PSEQIP Protein Sequence Database. A wide variety of proteins contained sequences with limited similarity but none showed extensive homology, examples of those which demonstrated the greatest homology are given in Figure 4.10. *E. coli* IF-2, Fruit fly myosin and *S. mansoni* paramyosin are unlikely to correspond to the EM4 native antigens. No definitive information regarding the function of the recombinant EM4 peptide was, therefore, obtained by comparison of amino acid sequence data. The sequence of a complete EM4 cDNA molecule may enable a more comprehensive analysis.

Figure 4.10 Comparison of the EM4 recombinant peptide amino acid sequence, derived from the nucleotide sequence of the cDNA, with those of previously characterised proteins using the PSEQIP Protein Sequence Database.

Numbers 1 (*E. coli* IF-2) and 2 (Fruit fly myosin-heavy chain) showed the greatest homology with number 15 (*S. mansoni* paramyosin) being the most homologous protein of parasite origin.

(*), matching amino acids.

(.), conservative substitutions.

>FIEC2 894 - Initiation factor IF-2 - Escherichia coli

12.792 0.345 -0.150 0.006 1.618 72 3168
 No. 1 Score= 129 Quality 21.192 Loc. Exp. 0.1646E-02 SD 19.596
 IDs= 25 CONS= 18 MisMatch= 30 Gaps= 2 Exp. No. 0.1248E-02 SD 36.3
 Ratio(Found/Expected) Identities 0.774 Positives 0.827 Negatives

2

.* **.* **.* **.* **.* **.* **.* **.*
 121 EESAKREAGQKAEERAAEDAKREAAEDAKREAAEKDKVSNQDDMTKNADAERAEDEA
 122 QERHARESLOKSQDKLAEM NRKLKEETAASAEERDLMAQRDEVQREVEADQVAMAKKE

**.* **.* **.*
 191 AELKREKAEERARRKL 195
 191 AE KADAEELRRML 194

>A19942 165 - Myosin heavy chain - Fruit fly (fragment)

13.920 0.366 -0.165 0.006 3.626 78 4183
 No. 2 Score= 117 Quality 15.354 Loc. Exp. 0.4272E-02 SD 14.987
 IDs= 24 CONS= 20 MisMatch= 47 Gaps= 0 Exp. No. 0.8182E-02 SD 26.1
 Ratio(Found/Expected) Identities 0.612 Positives 0.697 Negatives

5

..* **.* **.*.*.* **.* **.* **.*
 19 LDEAEANALKGKKAIQKLEQVRLELENELDGEQRRHADQKNLAKSERRVKELSFQSEE
 63 LKEAEFGRLKEERLQRMENEQKLELRAQNVKESDLADMKNKASAYESKIAELEMLLQD

.*.*.* **.*.*.*
 79 DFKNHERMODLVQKLQKIKTYKRQIEEAE 109
 123 ERHARESLOKSQDKLAEMNRKLKEETAASAE 153

>A25993 437 - Paramyosin - Fluke (Schistosoma mansoni) (fragment)

12.521 0.799 -0.165 0.015 1.381 61 1124
 No. 15 Score= 99 Quality 31.935 Loc. Exp. 0.2240E-01 SD 4.752
 IDs= 13 CONS= 9 MisMatch= 14 Gaps= 0 Exp. No. 0.1775E+00 SD 9.4
 Ratio(Found/Expected) Identities 0.838 Positives 0.882 Negatives

9

***.*.*.*.* **.*.*.*
 233 YESNIADLEIQDITANKANANLMKENKNLSQRVKDL 268
 109 YESKIAELEMLLQGERHARESLOKSQDKLAEMNRKL 144

4.4 DISCUSSION

The M_r of the native proteins corresponding to EM2 and EM4 cloned antigens has been established. The discrepancy in M_r between the recombinant antigens and their corresponding native proteins may be due to the cloning of incomplete gene sequences and/or the existence of carbohydrate residues on the native proteins. Although these proteins are expressed by protoscoleces, their ability to produce an antibody response within the human host despite the usual absence of protoscoleces within the human alveolar cyst (Rausch and Wilson, 1973), suggests that they are also present in or on the germinal layer, or, in the case of EM4, excreted/secreted from this layer. A 70 kDa antigen was identified by Lingelbach and Hinz (1984) as being characteristic of hydatid infection. This antigen was recognised by both human anti-*E. multilocularis* and anti-*E. granulosus* infection sera and may, therefore, be the same antigen as that encoded by EM2. EM4 codes for a recombinant peptide that has epitopes shared by 62 kDa, 49 kDa and 44 kDa native antigens. It is probable that these antigens arise from the post-translational modification of a single precursor protein. All three proteins appear to be E/S products which concurs with the finding of high antibody levels specific to these proteins in human sera, as excreted/secreted antigens released into the host system are generally good immunogens (Yong and Heath, 1984).

Antigens related to EM2 and EM4 native antigens are present in protoscoleces of both U.K. strains of *E. granulosus* and in *T. solium* cysticerci, although they are not present in *E. granulosus* sheep cyst fluid. This accounts for the cross-reactivity of the recombinant EM2 antigen with human anti-*E. granulosus* and anti-*T. solium* infection sera. The conservation of the EM4-related native proteins suggests that the diagnostic *E. multilocularis*-specificity demonstrated by the recombinant EM4 antigen is the result of immunological factors. The fact that these proteins are not excreted/secreted into the unilocular *E. granulosus* cyst lumen may account for this species-specific immunological recognition. The presence of related proteins in taeniids other than *E. multilocularis* may create diagnostic complications when using the cloned EM4 antigen as a diagnostic reagent, particularly in the case of drug treated patients. Drug treatment has been found to produce disruption of cystic material (Eckert, 1986) which leads to

the release of parasite antigens into the host system that would not normally be presented during the course of an infection. Antibody to this potentially immunodiagnostic recombinant antigen may, therefore, be found in drug-treated *E. granulosus* and *T. solium* infected patients and could result in incorrect diagnosis, although this problem was not apparent with the drug treated patient sera tested in the current study.

Lectin binding studies indicate that the native antigens may be glycosylated, as would be expected of membrane bound (EM2) and excreted/secreted (EM4) proteins. Glycosylation of the EM4 proteins has been confirmed by the presence of an N-linked carbohydrate binding site within the recombinant peptide amino acid sequence.

The mRNA transcripts corresponding to both clones have been putatively identified. The EM2 message is in the region of 1 660 bp and the EM4 message is approximately 1 514 bp in length. A potentially protective *B. malayi* antigen has been shown to originate from a 1 800 bp transcript (Nilsen *et al.*, 1988) and an *E. tenella* antigen from a 2 150 bp mRNA (Profous-Juchelka *et al.*, 1988). A diagnostic *L. donovani* antigen is coded for by a 20 000 bp transcript (Blaxter *et al.*, 1988) and two diagnostic proteins of *S. mansoni* have been found to originate from 1 400 bp and 1 500 bp mRNAs (Klinkert *et al.*, 1989). From the size of the foreign peptide synthesized by EM2 and nucleotide sequencing of EM4, the length of the cloned cDNAs were 1 050 bp and 1 114 bp, respectively. It was apparent, therefore, that both clones contained only partial sequences. It was shown by Northern analysis that greater levels of EM4, relative to EM2, message were present within the protoscolex developmental stage used as the RNA source. The artificial activation of the protoscoleces by pepsin digestion may, however, have influenced expression levels of certain genes.

Only one protein was precipitated by anti-EM4 antibody following *in vitro* translation of protoscolex total RNA. No protein was precipitated by anti-EM2 antibody, perhaps confirming the low expression levels of the EM2 gene(s). The detection of only one protein with anti-EM4 serum indicated either low abundance of transcripts coding for the two smaller native proteins or, alternatively, that the three native antigens originated from one mRNA species. The confirmation by Southern blotting that the native antigens arise from one type of gene led to the suggestion that the 66 kDa protein synthesized *in vitro* undergoes post-translational

modification to produce the three smaller excreted/secreted native antigens. Post-translational processing has been shown to be involved in the generation of the immunodiagnostic *S. mansoni* proteases, cathepsin B and haemoglobinase, in their active forms (Klinkert *et al.*, 1989). Removal of a signal peptide could account for the reduction in M_r to produce the 62 kDa EM4 native antigen. The close size correlation of the 62 kDa native antigen with the 66 kDa cell-free translate product indicates that the native antigen is not highly glycosylated. This may account for the high level of antibody present in some human anti-*E. multilocularis* infection sera specific for the unglycosylated recombinant antigen. The two smaller (49 kDa and 44 kDa) EM4 native proteins must arise from modifications to the 66 kDa pre-protein in addition to the removal of a signal peptide, to account for their decreased M_r .

Southern blot analysis indicated that the EM2 native antigen may be coded for by a complex single-copy gene or by a multi-copy gene family. A potentially protective *B. malayi* antigen has been shown to be coded for by a multi-copy gene from similar studies (Perrine *et al.*, 1988). Data relating to EM4 indicates that these native antigens are probably coded for by a single-copy gene. *S. mansoni* myosin (Newport *et al.*, 1987), a diagnostic *L. donovani* antigen (Blaxter *et al.*, 1988) and diagnostic *S. mansoni* proteins (Klinkert *et al.*, 1989) may be coded for by single-copy genes as shown by similar studies. The genes coding for antigens related to EM2 and EM4 native antigens in *E. granulosus*, *T. solium* and *T. saginata*, do not have the same genomic organisation as the *E. multilocularis* genes. Similar findings have been reported with regard to a diagnostic *L. donovani* antigen gene also found in *L. tropica* (Blaxter *et al.*, 1988), however, Horii *et al.* (1988) have found that the genomic organisation of the genes coding for two potentially protective *P. falciparum* antigens is conserved between two different strains of this parasite. The *E. granulosus* genes vary in organisation but retain significant homology at the nucleotide level to the *E. multilocularis* genes, whereas the *T. solium* and *T. saginata* genes, because of reduced hybridisation levels, appear also to differ at the nucleotide level from the *E. multilocularis* genes. This may account, in part, for the lack of cross-reactivity of the EM4 recombinant antigen with human anti-*T. solium* infection sera.

Nucleotide sequencing of the EM4 cDNA revealed that the

inferred amino acid sequence did not exhibit any strong homology with previously reported sequences. This has also been found with several other sequenced recombinant antigens, for example, an *S. mansoni* protective antigen (Balloul *et al.*, 1987), a *B. bovis* antigen (Gill *et al.*, 1987) and a potentially protective *B. malayi* antigen (Nilsen *et al.*, 1988). No homology was also apparent with the recently reported *S. mansoni* protease protein sequences of Klinkert *et al.* (1989). The lack of homology may have been attributable to the cloning of a novel or highly divergent gene and the fact that the full gene sequence was not analysed. The sequence contained no repeated elements at the nucleotide or amino acid levels which has also been found to be the case in a *B. bovis* antigen (Gill *et al.*, 1987) and a protective *S. mansoni* antigen (Balloul *et al.*, 1987). Repetitive elements have, however, been found in *S. mansoni* myosin (Newport *et al.*, 1987), an abundant *P. knowlesi* antigen (Perler *et al.*, 1987) and a potentially protective *P. falciparum* antigen (Horii *et al.*, 1988). A hydrophobic signal peptide was not found in the EM4 sequence despite the fact that the native antigens are excreted/secreted. Sequencing of the 5' end of a complete cDNA molecule may resolve this and may also allow homology with an existing protein sequence to be found, thereby allowing a possible function for this cloned peptide to be determined. Only one other *Echinococcus* sp. antigen has been characterised at the molecular level (Shepherd, 1988). The amino acid sequence of the recombinant *E. granulosus* peptide when compared to existing data, indicated a possible protease inhibition function. This was subsequently confirmed by biochemical analysis of the excreted/secreted native antigen (antigen B). Although the EM4 native antigens are excreted/secreted from the parasite, the recombinant peptide did not demonstrate sequence homology with the cloned *E. granulosus* peptide and furthermore, no difficulty was experienced in expressing the EM4 recombinant antigen in *E. coli*, in contrast to the cloned *E. granulosus* peptide which proved to be poorly expressed by the λ gt11 system, possibly on account of its protease inhibition properties (Shepherd, 1988). Therefore, if the EM4 recombinant antigen is biologically active, it is unlikely to perform a protease inhibition function. This remains to be confirmed, however, by further studies.

CHAPTER 5

5 SUMMARY AND FUTURE INVESTIGATIONS

5.1 SUMMARY

Little work has been performed on the characterisation of antigens from *E. multilocularis* (Schantz and Gottstein, 1986), although a preliminary study (Lingelbach and Hinz, 1984) has identified those antigens characteristically immunogenic in human *E. multilocularis* infection. Only recently has the requirement for large quantities of well characterised antigen for the immunodiagnosis of AE been recognised (Gottstein *et al.*, 1983). The most successful attempt, to date, to rectify this, has been the work of Gottstein *et al.* (1983) with the resultant isolation of an *E. multilocularis*-specific antigen, designated Em 2a (Gottstein, 1985). Provision of this antigen, however, relies heavily on a readily available source of parasite material. In this thesis, molecular cloning technology has been employed to overcome this problem and to facilitate antigen characterisation.

An *E. multilocularis* λ gt11 expression library has been constructed and differentially screened with pools of human anti-*E. multilocularis* and anti-*E. granulosus* infection sera. Two putatively *E. multilocularis*-specific clones were identified and tested using pools of various heterologous human infection sera and panels of individual human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* infection sera. The β -gal fusion-peptide synthesized by one clone (EM2) was shown to cross-react with individual heterologous sera and, therefore, has limited diagnostic value. The β -gal fusion-peptide produced by the other clone (EM4), purified in both the denatured and non-denatured states, was shown to have poor diagnostic sensitivity. Infected mice produced antibody specific for this antigen shortly after infection, however, indicating it's potential for application in early diagnosis. Expression of the EM4 antigen fused to GST using a plasmid vector, allowed confirmation of it's 100% immunological species-specificity and produced a slight improvement in diagnostic sensitivity, this proved insufficient, however, to enable its practical use as a reagent for the detection of circulating antibody. The

alternative vector did, however, produce major improvements with regard to the production and purification of the recombinant antigen. Background antibody binding with human sera was also reduced with the GST carrier protein relative to that produced by β -gal.

The molecular cloning strategy, in addition to facilitating the identification and production of a potentially immunodiagnostic reagent, has allowed detailed characterisation of the native *E. multilocularis* antigens. The native antigen corresponding to EM2 has a M_r of 70 kDa and those recognised by anti-EM4 antibody have M_r 's of 62 kDa, 49 kDa and 44 kDa. In addition to being produced by protoscoleces, the EM2 antigen may be localised in or on the germinal layer and the EM4 antigens may be excreted/secreted from this layer. Antigens related to EM2 and EM4 native antigens have been found in *E. granulosus* (sheep and horse strain) and *T. solium* extracts. This accounted for the cross-reactivity of the recombinant EM2 antigen and showed that the diagnostic specificity of EM4 was due to differential immunological recognition. This may be attributable to the lack of excretion/secretion of the EM4 related native antigens by the metacestodes of some taeniids, coupled with low levels of immunological cross-reactivity in the case of antigens produced by distantly related parasite species. The native antigens corresponding to both clones may be glycosylated. This has been substantiated in the case of EM4 by the finding of an N-linked glycosylation site within the cloned cDNA sequence.

The mRNA transcripts corresponding to these clones have been putatively identified. The size of these indicates that the cDNA inserts of EM2 and EM4 do not represent entire coding sequences. The EM2 transcript may originate from either a single or multi-copy gene. The EM4 transcript, on the other hand, probably arises from a single-copy gene. This transcript appears to code for a precursor peptide which undergoes post-translational processing to give rise to three native antigens. The genomic organisation of EM2 and EM4 related genes is not conserved across species boundaries, the degree of heterogeneity increasing the more distantly related the parasite, as was anticipated.

Comparison of the recombinant EM4 antigen amino acid sequence with existing data has revealed no significant homologies and possible reasons for this have been discussed. A potential function for the EM4 native antigens has not, therefore, been determined.

5.2 FUTURE INVESTIGATIONS

The diagnostic sensitivity of the EM4 recombinant antigen when used to detect specific antibody may be improved by, 1) expression of a full-length cDNA clone, isolated from the original library using the existing EM4 cDNA insert as a probe, and 2) removal of the GST carrier protein from the antigen, following expression using either pGEX-2 or 3. Should these approaches fail to produce the desired result, specific anti-EM4 antibodies, produced by the hyperimmunisation of animals with EM-GSTFP4, could be used in an attempt to detect circulating native EM4 antigens in human sera (Gottstein *et al.*, 1984; Craig and Nelson, 1984; Craig, 1986). The detection of both circulating anti-EM4 antibody and native EM4 antigens may also be enhanced by the prior treatment of test sera to dissociate immune complexes (Craig and Nelson, 1984; Craig, 1986).

If the EM4 recombinant antigen alone proves to be inadequate for effective diagnosis, characterisation of the remaining λ gt11 putatively specific clones, particularly 1, 3 and 6, will be necessary. This may reveal a clone (or clones) with suitable diagnostic properties when used alone, or which could be used in conjunction with EM4 in an antigen 'cocktail'.

Precise localisation of the EM2 and EM4 native antigens within the protoscolex should be determined by probing tissue sections with anti-fusion-peptide antibody, followed by labelling with fluorescent conjugated second antibody. Regions of antibody binding will then be revealed upon microscopical examination.

Detailed molecular characterisation of EM2 may necessitate the prior isolation of a clone from which the cDNA insert can be excised. This will require rescreening of the library with mouse anti-EM- β galFP2 serum. An alternative approach (Gyllenstein and Erlich, 1988) would be to use the polymerase chain reaction in conjunction with λ gt11 primers that flank the insertion site, to synthesize single strand DNA from the existing EM2 clone which could be sequenced directly.

The putative function of the EM4 native antigens may be elucidated following the determination of the nucleotide sequence of a full-length cDNA. Should no homology with previously characterised proteins be found, testing of the recombinant peptide with a range of biochemical assays may determine the function of the corresponding native antigens.

A further potential use of the cloned antigens may be with regard to inducing protective immunity within the intermediate or definitive hosts. This could be tested relatively easily in the case of the intermediate host, as the metacystode infection can be produced readily in laboratory mice. It has already been shown in this study that the EM4 native antigens are capable of stimulating a strong humoral response in these animals, which suggests that the recombinant peptide may prove to be of value as a vaccine candidate.

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The isolation, by differential antibody screening, of *Echinococcus multilocularis* antigen gene clones with potential for immunodiagnosis

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A λ gt11 cDNA expression library consisting of a million recombinant clones has been constructed from *Echinococcus multilocularis* protoscolex mRNA. Differential screening of the library with pools of *E. multilocularis* and *Echinococcus granulosus* human infection sera has revealed 13 potentially immunodiagnostic clones. On the basis of plaque immunoassays and lysogen characteristics, two of these clones, designated EM2 and EM4, have been further characterised. The recombinant fusion-peptides have been purified and their potential as immunodiagnostic reagents has been assessed by immunoblotting and, in the case of one fusion-peptide (EM4), by enzyme-linked immunosorbent assay (ELISA). Furthermore, the native parasite antigens coded for by these clones have been identified. EM2 corresponds to a 70 kDa protein and epitopes coded for by EM4 have been found on three antigens of 62, 49 and 44 kDa. The native antigens of both clones are present in the protoscolex and those corresponding to EM4 appear to be excreted/secreted products. They are not recognised in ELISA by a variety of human parasitic infection sera other than sera taken from patients infected with *E. multilocularis*. Nevertheless, the native antigens for both clones are present in *E. granulosus* protoscolexes and *Taenia solium* cysticerci. These antigens are not detectable in *E. granulosus* cyst fluid, and this may, in part, explain the lack of immune response to them in human *E. granulosus* and *T. solium* infections.

Key words: *Echinococcus multilocularis*; λ gt11 cDNA library; Differential antibody screening; Immunodiagnosis

Introduction

One of the most lethal zoonotic diseases known to man, 'alveolar echinococcosis' (AE), is caused by the metacestode stage of *Echinococcus multilocularis*. The symptoms of the disease are often confused with those of hepatic carcinoma and

correct diagnosis is usually only made when the condition is well advanced. Consequently, only 20–40% of cases are surgically resectable and, of the remaining non-resectable cases, 90% die within 10 years of diagnosis [1]. The closely related species *E. granulosus*, the causative agent of 'cystic echinococcosis' (CE), is often found in the same geographic locality [2] which further complicates precise diagnosis.

Methods for the diagnosis of AE and CE have been reviewed by Schantz and Gottstein [1]. Non-immunological methods, e.g. computerised axial tomography, ultrasound scanning and angiography are relatively complex, require highly trained operatives, produce data which is often difficult to interpret, are expensive and offer poor prospects for early diagnosis. Immunological techniques can overcome many of these problems but, with the use of crude antigen extracts, they too are limited by cross-reactivity which often results in false positive reactions. The availability of well characterised parasite material for use as antigen

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Abbreviations: SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; PMSF, phenylmethylsulphonyl fluoride; IPTG, isopropyl β -D-thiogalactopyranoside; HRP, horseradish peroxidase; β -gal, β -galactosidase; 2-me, 2-mercaptoethanol; DAB, 3,3'-diaminobenzidine; AE, alveolar echinococcosis; CE, cystic echinococcosis; PBS, phosphate buffered saline; TBS, tris-buffered saline; TNT, Tween-20/Nonidet-P40/TBS; NHS, normal human serum.

in these tests is also a further drawback. In an attempt to reduce cross-reactivity, an *E. multilocularis* species-specific antigen has been purified by classical affinity chromatography techniques [3]. This has subsequently been shown to be effective in enzyme-linked immunosorbent assay (ELISA) producing a 95% discrimination rate between AE and CE [4]. Unfortunately, this work has not overcome the problems of antigen availability.

The recent advances in molecular biology can potentially provide unlimited amounts of pure, well-characterised parasite protein. Work of this type has been performed on many types of infectious organism [5], although the only recorded studies with cestodes have been the construction of a *Taenia taeniaeformis* λ gt11-amp 3 cDNA expression library [6] and the cloning of *Taenia ovis* antigen genes into the pEX1/2/3 series of expression plasmids [7] in order to facilitate vaccine development. Most studies, to date, concerned with cloning of parasite antigen genes have concentrated on the production of putative protective molecules, whereas very little work has been performed with regard to diagnostic antigens. One example of the latter is the work performed by Klinkert et al. [8] concerning the immunodiagnosis of *Schistosoma mansoni*.

In this paper, we describe the isolation of two *E. multilocularis* antigen gene clones directly from a λ gt11 cDNA expression library using a differential antibody probing technique. The diagnostic efficacy of the recombinant peptides synthesized by these clones is demonstrated by immunoblotting and ELISA and preliminary characterisation of the corresponding native parasite antigens is presented.

Materials and Methods

Preparation of parasite material. *E. multilocularis* metacestode material was obtained from laboratory reared cotton rats infected by interperitoneal passage.

Crude *E. multilocularis* antigen extract (AgA) and *Taenia solium* whole antigen extract (cysticerci from Mexican patients) were prepared from whole cyst material by one freeze-thaw cycle, sonication for 2×30 s at maximum amplitude (MSE Soniprep 150) and 5 strokes in a dounce

homogeniser.

Pools of *E. granulosus* sheep cyst fluid (aspirated from abattoir-collected unilocular hydatid cysts) and *E. multilocularis* cyst fluid (aspirated from cotton rat alveolar cysts) were clarified by centrifugation at $4000 \times g$ for 15 min at 4°C and concentrated in an Amicon ultrafiltration cell (Amicon Corp., U.S.A.) over a YM2 (1 kDa cut-off) membrane.

'Purified' protoscoleces of *E. multilocularis* and *E. granulosus* were prepared by the pepsin digestion technique described earlier [9,10]. *E. multilocularis* protoscoleces excreted-secreted (E/S) products were obtained by in vitro culture of purified protoscoleces under sterile conditions in RPMI-1640 medium (Gibco) for 5 days with slow rotation at 37°C. The medium was changed daily and collection commenced after the first two days of culture. The medium was then passed through an Acrodisc filter (0.45 μ m) and concentrated by ultrafiltration as described above.

Antisera. Human *E. multilocularis* infection sera were obtained from 13 confirmed French AE patients. The *E. granulosus* human infection sera were obtained from Kenyan (9 individual sera and a pool), U.K. (3 individual sera) and Spanish (a pool of 15 sera) confirmed CE patients. Other human parasitic infection sera were obtained from confirmed cysticercosis patients (a pool of 19 Zimbabwean and 4 Mexican sera) and the *Ascaris suum*, *Brugia malayi*, *Onchocerca volvulus*, *Schistosoma haematobium* and *Schistosoma mansoni* serum pools were from various endemic regions. Normal human serum (NHS) was a pool of 4 sera from U.K. laboratory workers.

Rabbit antiserum was raised by subcutaneous injection of 250 μ g *E. multilocularis* AgA protein in Freund's complete adjuvant as primary inoculation, followed, after 14 days, by a secondary boost in Freund's incomplete adjuvant. Subsequent booster inoculations of 5 mg parasite protein in 0.15 M NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 (pH 7.3) (PBS) were administered at 14 day intervals.

Mouse antiserum was raised as above using 50 μ g for each inoculation at 7 day intervals.

Protein concentration was estimated by the Bradford method [11].

Immunoblotting and ELISA. Proteins were separated by SDS-PAGE according to Laemmli [12] using thin-slab 5–20% gradient gels in an LKB electrophoresis apparatus. Samples were loaded in the buffer described by Studier [13] with 5 min boiling prior to loading with or without 2-mercaptoethanol (2-me). Transfer of proteins to nitrocellulose [14] was performed in a BioRad transfer cell using 80 mA overnight. Non-specific binding was blocked by incubation of nitrocellulose strips with 5% (w/v) skimmed milk (blotto) in PBS for 1 h at room temperature. Unbound primary and secondary antibody was removed by extensive washing with 0.1% (v/v) Triton X-100/0.05% (v/v) Tween-20 in PBS (wash solution). Antisera and horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, 1:1000 dilution) were diluted in wash solution/1% (w/v) blotto. Incubations were for 2 h at room temperature and bound antibody was detected using 3,3'-diaminobenzidine (DAB) substrate.

The ELISA procedure [15] used HRP-conjugated second antibody (Sigma, 1:1000 dilution) and *o*-phenylene diamine as chromogenic substrate.

Construction of E. multilocularis lgt11 expression library. Total RNA was isolated from purified protoscoleces using a variation of the method of Chirgwin et al. [16]. Protoscoleces were disrupted by dounce homogenisation in the presence of guanidinium thiocyanate and RNA was isolated by caesium chloride gradient centrifugation.

Partial purification of poly(A)⁺ mRNA was achieved with one cycle of enrichment on 'Hybond' messenger affinity paper (Amersham) according to the manufacturer's recommended procedure.

Double stranded cDNA was synthesized from 1 µg of mRNA according to the ribonuclease H method [17]. This was subsequently cloned [18] into *lgt11* arms (Stratagene). Reagents and protocols for cDNA synthesis and cloning were supplied by Amersham International. The library was amplified once prior to screening.

Differential antibody screening. Plaques were screened using a variation of the protocol of

Huynh et al. [18]. Replica nitrocellulose filters (Schleicher and Schuell), taken after incubating a fresh isopropyl β-D-thiogalactopyranoside (IPTG) saturated filter on the plate for a further 2 h at 37°C, following removal of the first filter, were blocked with 5% (w/v) blotto in 50 mM Tris-HCl pH 8.0, 150 mM NaCl (TBS) for 1 h at room temperature. They were then incubated for 2 h at room temperature with the respective antibody probe diluted in 1% (w/v) blotto in 0.05% (v/v) Tween-20/0.05% (v/v) Nonidet-P40/TBS (TNT). The optimum dilution of the probe antisera was determined previously by immunoblotting against AgA. Unbound antibody was removed by extensive washing with TNT. Bound antibody was detected with HRP-conjugated second antibody diluted 1:1000 (Sigma) together with DAB substrate as chromogen.

Human sera used for screening were subjected to several cycles of absorption against lysed, *lgt11* infected *E. coli* cells bound to nitrocellulose filters prior to use, in order to reduce background signal levels.

Spot-lysis arrays of purified clones were produced by spotting 1 µl dots of phage stock onto a lawn of Y1090 cells containing 10 mM IPTG. Following diffusion of the phage into the lawn, the incubation of plates and processing of filters was identical to that described above.

Lysogen construction and fusion-peptide purification. Lysogens of recombinant clones were produced [18] by infecting exponentially growing Y1089 cells with *lgt11* phage. Lysogenic colonies were selected according to their temperature sensitivity.

Fusion-peptides were produced [18] by inducing exponentially growing lysogens at 42°C for 20 min and, following the addition of IPTG to 10 mM, incubating for a further 2 h at 37°C. Cells were harvested at room temperature by centrifugation at 4000 × *g* for 5 min. The pellet was resuspended in 1/100 original volume of PBS/1 mM EDTA/1 mM phenylmethylsulphonyl fluoride (PMSF) and one freeze-thaw cycle plus sonication at 4°C for 5 × 10 s at maximum amplitude was used to lyse the cells and decrease chromosomal DNA viscosity.

To purify recombinant peptides, crude lysate,

equivalent to 100 ml of culture volume, was loaded under reducing conditions on a 7.5% preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Following electrophoresis, protein bands were visualised by precipitating unbound SDS with 4 M sodium acetate at room temperature. The fusion-peptide band was excised and the protein electroeluted at 100 V overnight using a modified LKB tube-gel apparatus which consisted of dialysis tubing fixed to the base of the tube containing the gel slice. Extensive dialysis against PBS was used to remove excess SDS and the sample was concentrated to a final volume of 1 ml in a Minicon-B (Amicon) ultrafiltration cell (15 kDa cut-off). The approximate yield and purity was estimated by SDS-PAGE of fusion-peptide ali-

quots alongside known concentrations of commercially available β -gal (Sigma). The fusion-peptides were then tested by immunoblotting and ELISA.

Affinity purification of specific anti-fusion-peptide antibody. 10^5 phage of the clone of interest were plated out on Y1090 cells in the presence of 10 mM isopropyl β -D-thiogalactopyranoside (IPTG). Following growth for 4 h at 42°C, a nitrocellulose disc was placed on the lawn and, after a further 2 h incubation at 37°C, the disc was removed and blocked with 5% (w/v) blotto/tris-buffered saline (TBS) as described previously. The filter was then incubated as before with preabsorbed rabbit anti-*E. multilocularis* AgA serum. Unbound antibody was washed off as before and the remaining bound

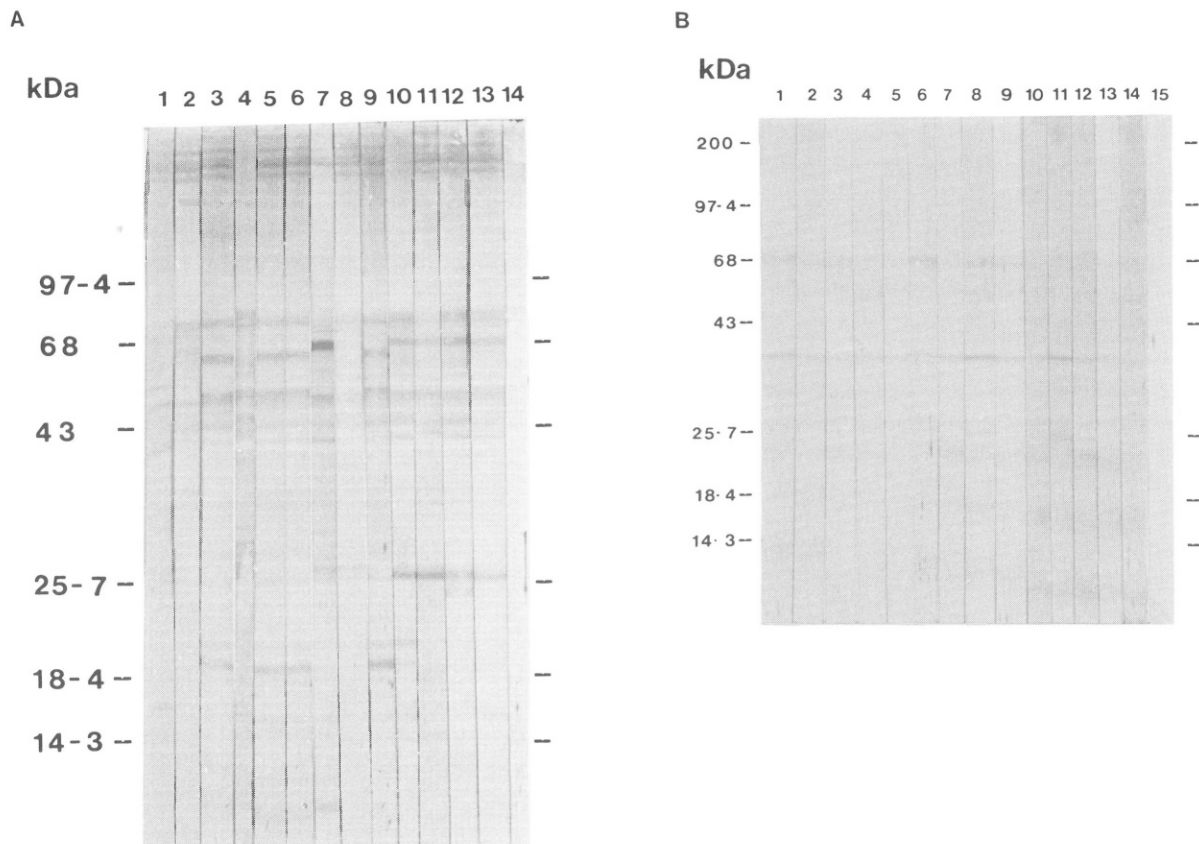


Fig. 1. SDS-PAGE and immunoblotting of human infection sera. (A) AgA tested with: lanes 1–13, individual human anti-*E. multilocularis* sera (French origin); lane 14, normal human serum. (B) Sheep cyst fluid tested with: lanes 1–14, human anti-*E. granulosus* sera; lanes 1–3, U.K. individual sera; lane 4, Spanish pool; lanes 5–13, Kenyan individual sera; lane 14, Kenyan pool; lane 15, NHS. 10 μ g of protein was used per strip for both antigens. Proteins were reduced prior to electrophoresis.

antibody was stripped from the filter by incubation with 0.1 M glycine pH 2.6, 0.15 M NaCl for 1 min at room temperature. The eluate was collected and immediately re-equilibrated with 1/25 volume 2 M Tris-HCl pH 8.0.

Results

Construction and differential screening of the λ gt11 library. The λ gt11 library, titred using the chromogen 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside in the presence of IPTG, was found to contain 10^6 recombinant clones. Prior to screening of clones, the individual *E. multilocularis* and *E. granulosus* human infection sera to be used were tested by immunoblotting against AgA (Fig. 1A) and sheep cyst fluid (Fig. 1B), respectively. This was to ensure that any serum which recognised anomalous antigens could be excluded from the serum pool used for screening. In the case of the anti-*E. multilocularis* sera, only one appeared to present a substantially different antigen recognition profile (Fig. 1A, lane 4). This serum was therefore omitted from the pool used for screening. The anti-*E. granulosus* sera all appeared to recognise the same major antigens, although some

heterogeneity was observed regarding the minor antigens. The consequences of this, however, would have been minimised by the pooling of the sera.

Following amplification, 10^6 recombinant phage were probed with the pooled human *E. multilocularis* infection sera. 364 clones produced positive signals and these were picked and pooled. These clones were then differentially screened with *E. multilocularis* and *E. granulosus* human infection sera pools and 13 putatively *E. multilocularis*-specific clones were isolated. In order to ascertain the degree of specificity and immunogenicity of these clones, spot-lysis arrays were made and probed with the two serum pools (Fig. 2). Colour intensity varies significantly depending on the degree of antibody binding and, using this criterion, high levels of antibody to clone 4 were present only in the human anti-*E. multilocularis* pool. Clones 1, 2, 3 and 6 also appeared to produce darker plaques with the *E. multilocularis* antibody probe. Clone 1 reacted strongly with the anti-*E. granulosus* pool, although this may have been an artifact produced during the screening procedure, possibly due to uneven protein transfer to the nitrocellulose filter.

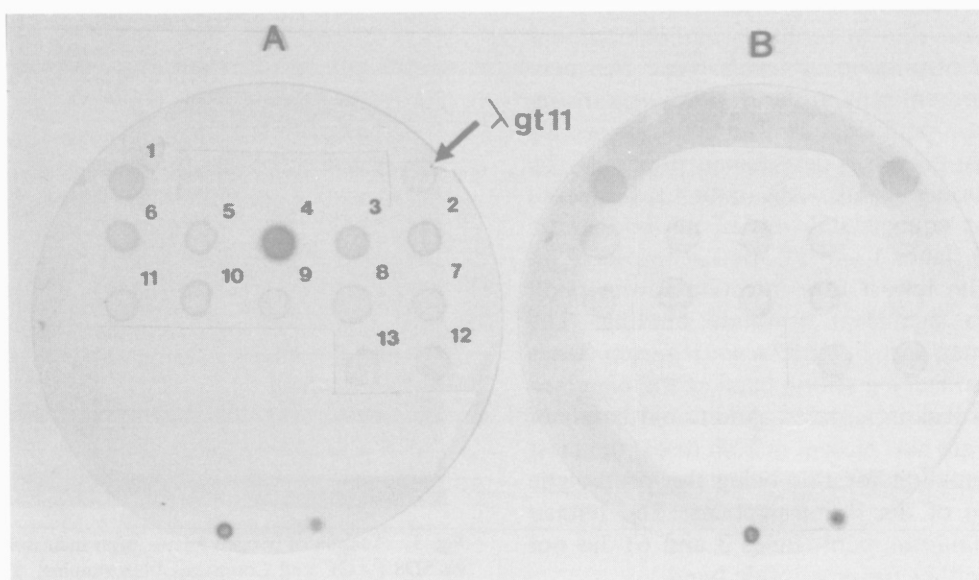


Fig. 2. Differential screening of antigen-expressing clone replica plaque arrays with human infection sera. Recombinant clones are numbered 1–13 and the λ gt11 negative control is arrowed. Filters were probed with: (A) preabsorbed pooled human anti-*E. multilocularis*; (B) preabsorbed pooled human anti-*E. granulosus*.

Analysis of recombinant fusion-peptides. Lysogens were constructed of all 13 putatively *E. multilocularis*-specific clones together with non-recombinant λ gt11 and an aliquot of crude lysate of each was electrophoresed on a 7.5% SDS-PAGE gel (data not shown). Clones 1, 3 and 6, although immunogenic by plaque immunoassay, did not produce lysogens which would yield sufficient fusion-peptide to justify their inclusion in further studies using this expression vector. Clones 2 and 4 (designated EM2 and EM4 respectively) produced lysogens which did yield significant amounts of fusion-peptide (Fig. 3), and further work was, therefore, concentrated on these clones. The fusion-peptide of EM2 (designated EM- β galFP2) (lane 2) had an apparent size of 155 kDa. This corresponds, when compared to the 116 kDa of β -gal (lane 1), to approximately 39 kDa of *E. multilocularis* peptide. The EM4 fusion (designated EM- β galFP4) (lane 3) had an apparent size of 162 kDa corresponding to approximately 46 kDa of parasite peptide.

Diagnostic potential of fusion-peptides as tested by immunoblotting and ELISA. Purified EM- β galFP2 and EM- β galFP4 were subjected to SDS-PAGE and immunoblotting. Each was tested with the pools of human anti-*E. multilocularis* and anti-*E. granulosus* sera to confirm whether relatively high levels of antibody towards these two peptides was present only in the *E. multilocularis* infection serum pool (Fig. 4). The dilution used for each human pool was determined previously by immunoblotting against AgA (data not shown) to ensure that equivalent levels of antibody were used. NHS (lanes 1 and 4), used at the same dilution as the lowest titre infection serum pool, showed no significant antibody binding. The pooled human anti-*E. multilocularis* serum (lanes 2 and 5) produced a strong band at the expected M_r of both fusion-peptides. Additional bands of lower M_r were also present in both cases, the most likely explanation for this being the proteolytic degradation of the fusion-peptides. The human anti-*E. granulosus* pool (lanes 3 and 6) did not react with either fusion-peptide band.

The incorporation of purified fusion-peptide into an ELISA assay proved possible only with EM- β galFP4. EM- β galFP2 did not bind antibody

when used with this technique, possibly because of its relatively weak antigenicity combined with its inefficient binding to the ELISA plate in the presence of SDS. Pools of human anti-*E. multilocularis*, anti-*E. granulosus* and anti-*T. solium* sera and NHS were tested by ELISA against EM- β galFP4 (Fig. 5A) and native β -gal (Fig. 5B). EM- β galFP4 bound high levels of antibody only with the human anti-*E. multilocularis* serum. Hu-

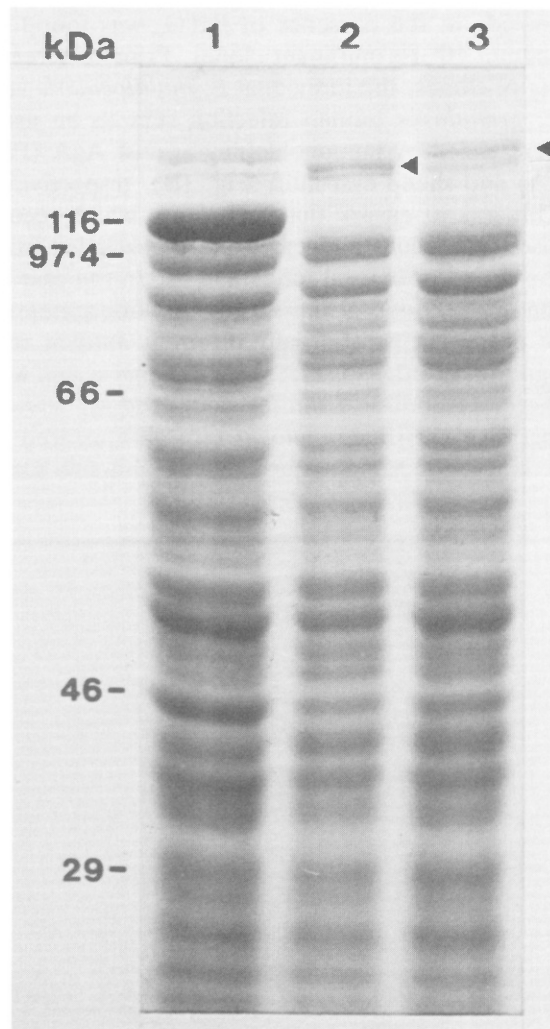


Fig. 3. Analysis of lysogen lysates, after induction with IPTG, by SDS-PAGE and Coomassie blue staining. Y1089 *E. coli* cells were lysogenised with: lane 1, nonrecombinant λ gt11; lane 2, recombinant clone 2 (EM2); lane 3, recombinant clone 4 (EM4). Extracts were reduced prior to electrophoresis. The fusion-peptide bands are arrowed.

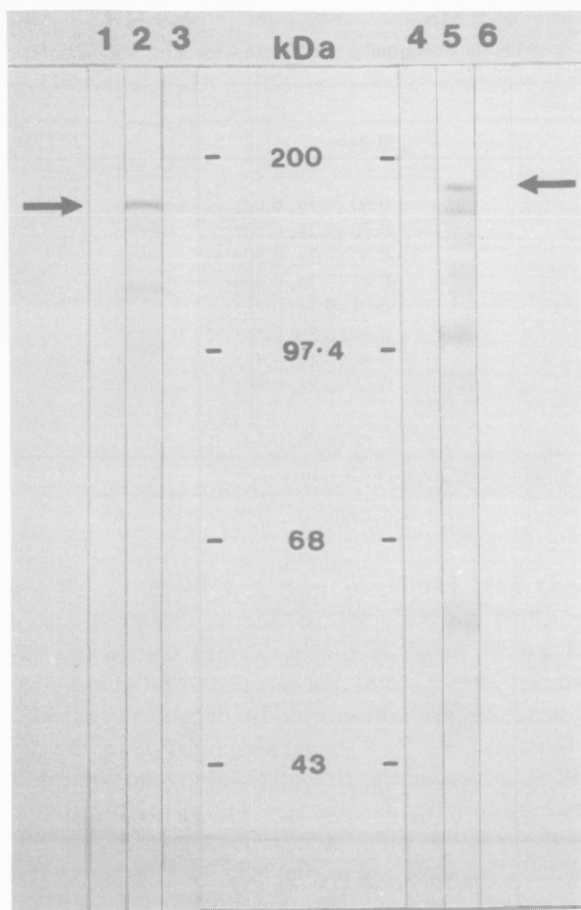


Fig. 4. SDS-PAGE and immunoblotting of purified fusion-peptides with human infection sera. Lanes 1-3, EM-βgalFP2 probed with: lane 1, normal human serum; lane 2, pooled human anti-*E. multilocularis*; lane 3, pooled human anti-*E. granulosus*. Lanes 4-6, EM-βgalFP4 probed with: lane 4, normal human serum; lane 5, pooled human anti-*E. multilocularis*; lane 6, pooled human anti-*E. granulosus*. 2 µg of purified fusion-peptide was used per strip. Proteins were reduced prior to electrophoresis. The undegraded fusion-peptide bands are arrowed.

man anti-*E. granulosus* and anti-*T. solium* sera produced levels of binding similar to that of NHS. β-gal produced a background level of binding with all sera similar to that of NHS. Table I shows the ELISA values obtained when various, diverse human infection sera were tested against EM-βgalFP4 and β-gal. No significant level of anti-EM-βgalFP4 antibody was bound with any of the sera apart from the human anti-*E. multilocularis* serum pool. All sera produced similar levels of

binding to β-gal. High levels of anti-β-gal antibody were present at low serum dilution due to the inherent human response to β-gal produced by, for example, gut microflora [8].

Immunoblotting of anti-fusion-peptide antibody with parasite antigen extracts. Preliminary characterisation of clones EM2 and EM4 involved identification of the native parasite antigens which they encoded. Antibody specific to the fusion-peptides was isolated either, in the case of EM-βgalFP4, by affinity purification, or by hyperimmunisation of mice in the case of both EM-βgalFP2 and EM-βgalFP4. Various *E. multilocularis* antigen extracts were subjected to SDS-PAGE, immunoblotted and tested with mouse

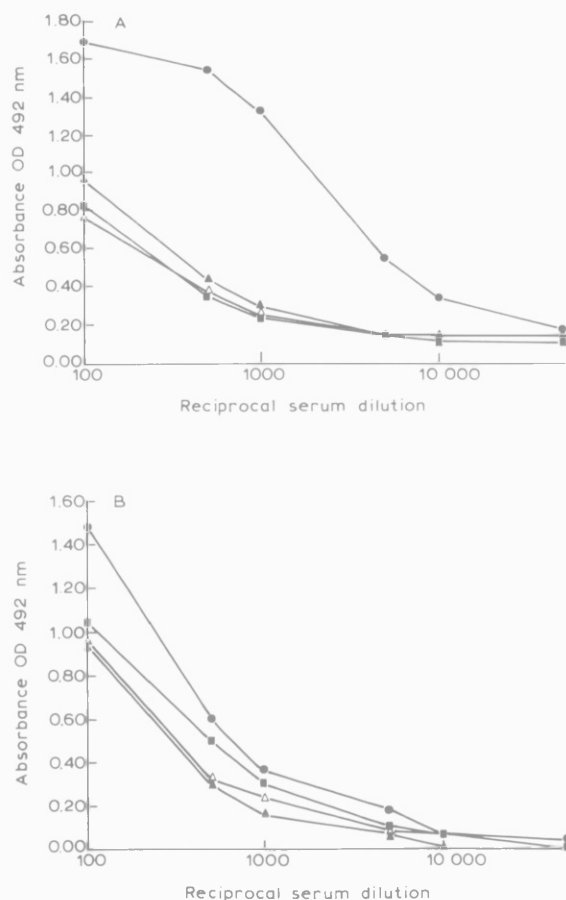


Fig. 5. ELISA using EM-βgalFP4 (A) and β-gal (B) as antigens (2 µg ml⁻¹) tested with various human infection sera. Pooled human anti-*E. multilocularis* (●); pooled human anti-*E. granulosus* (■); pooled human anti-*T. solium* (▲); NHS (△).

TABLE I

Results of ELISA showing that EM- β galFP4 binds significant levels of antibody with human anti-*E. multilocularis* serum only

	Antigen	
	EM- β galFP4	β -gal
1/500 pooled human antiserum to		
<i>E. multilocularis</i>	1.51 (1.52, 1.50)	0.60 (0.56, 0.64)
<i>E. granulosus</i>	0.35 (0.34, 0.35)	0.50 (0.28, 0.72)
<i>T. solium</i>	0.43 (0.42, 0.44)	0.30 (0.24, 0.36)
<i>Ascaris suum</i>	0.61 (0.58, 0.64)	0.42 (0.36, 0.48)
<i>Brugia malayi</i>	0.26 (0.26, 0.26)	0.34 (0.36, 0.32)
<i>Onchocerca volvulus</i>	0.34 (0.37, 0.30)	0.56 (0.56, 0.56)
<i>Schistosoma haematobium</i>	0.27 (0.27, 0.27)	0.46 (0.44, 0.48)
<i>Schistosoma mansoni</i>	0.30 (0.32, 0.28)	0.50 (0.52, 0.48)
NHS	0.25 (0.24, 0.26)	0.32 (0.36, 0.28)

Figures represent OD₄₉₀ in an ELISA and are the means of the 2 determinations given in brackets.

Antigens were used at 2 μ g ml⁻¹.

anti-EM- β galFP2 serum (Fig. 6). A single major band of 70 kDa was observed with AgA and protoscolex antigen (lanes 3–5). No change in mobility was observed under reducing or non-reducing conditions (lanes 3 and 4) indicating a lack of disulphide bound subunits within the protein. The lack of reaction with cyst fluid as antigen (lane 6) suggested that it was not an E/S product. A weak signal was detected, however, when in vitro-derived E/S antigen extract was tested (lane 7). This may have been the result of dead, dying or disrupted protoscoleces which had released their somatic antigens into the medium during culture.

Fig. 7 shows an immunoblot of both affinity purified anti-EM- β galFP4 antibody (lanes 4, 6, 8, 10 and 12) and mouse anti-EM- β galFP4 serum (lanes 3, 5, 7, 9 and 11) against various *E. multilocularis* antigen extracts following their fractionation by SDS-PAGE. All lanes, except the controls and lane 10, showed three bands of 62, 49 and 44 kDa. No change in mobility was observed under reducing or non-reducing conditions (lanes 3–6) indicating the absence of disulphide links between any subunits of the three proteins. The three bands were present in the cyst fluid lane tested with mouse antiserum (lane 9) suggesting that all three are E/S products. However, the cyst fluid lane tested with affinity purified antibody (lane 10) failed to display the 62 kDa band. The reason for this discrepancy could be low abundance of this particular antigen within

cyst fluid combined with low levels of affinity purified antibody specific for it. The presence of the three bands in the in vitro-produced E/S extract (lanes 11 and 12) suggests that these proteins are secreted/excreted by protoscoleces of *E. multilocularis*. The strong signal produced by the 62 kDa band in the E/S lanes relative to the other two protein bands indicates that of the three, this protein is the most abundant in vitro E/S product. A band of approximately 97 kDa appeared in several mouse antiserum lanes (3, 5, 7 and 11). This band was not apparent in the control lanes or the mouse anti-cyst fluid lane (lane 9). Affinity purified antibody, isolated from preabsorbed antiserum did not bind antigen at this M_r . The presence of cross-reactive non-specific antibody within the mouse antiserum is, therefore, the most probable explanation for the appearance of this band.

In order to determine whether these antigens were *E. multilocularis*-specific, extracts of *E. granulosus* protoscoleces (sheep and horse strains) and *T. solium* cysticerci were tested with fusion-peptide-specific antibody (Fig. 8). Native proteins corresponding to the M_r values, established previously for both clones, were present within *E. granulosus* (sheep strain, lanes 6–8; horse strain, lanes 10–12). The 70 kDa antigen corresponding to EM2 was also present in *T. solium* (lane 14), as was the 62 kDa antigen of EM4 (lane 16). In addition, protein bands of the expected M_r for the

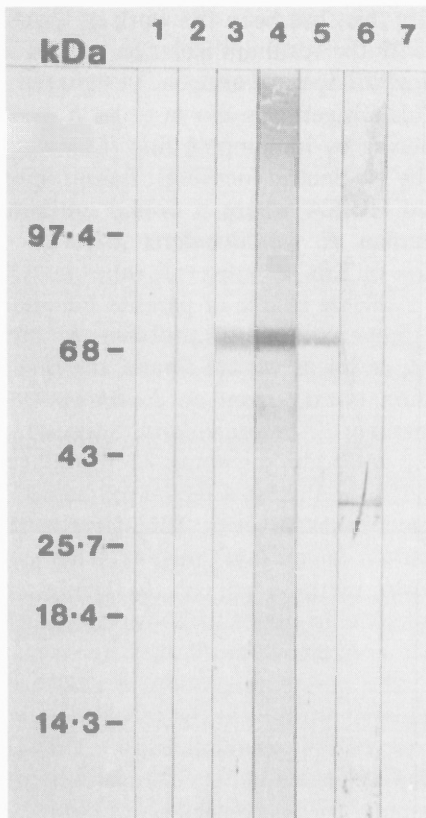


Fig. 6. SDS-PAGE and immunoblotting of various *E. multilocularis* antigen extracts with mouse anti-EM-βgalFP2 serum. AgA was probed with: lane 1, normal mouse serum; lane 2, mouse anti-β-gal serum; lane 3, mouse anti-EM-βgalFP2 serum. Mouse anti-EM-βgalFP2 serum was used to probe: lane 4, non-reduced AgA; lane 5, purified protoscolex antigen; lane 6, cyst fluid antigen; lane 7, in vitro excreted/secreted products. 50 µg of protein was used per strip apart from the E/S antigen for which 6 µg per strip was used. All antigens were reduced prior to electrophoresis unless otherwise specified.

EM4 antigens were apparent in the *T. solium* extract when tested with mouse antiserum (lane 15). These bands were also produced, however, by mouse anti-EM-βgalFP2 serum (lane 14). Cross-reactive antibody in the mouse antisera appeared, therefore, to be binding at the same M_r as the EM4 antigens. The presence of the cloned antigens within these parasite extracts suggests that they are conserved between the various taeniid species. However, the antigens were not detected in *E. granulosus* sheep cyst fluid (lanes 2–4), suggesting that they are not secreted/excreted into the lumen of the unilocular

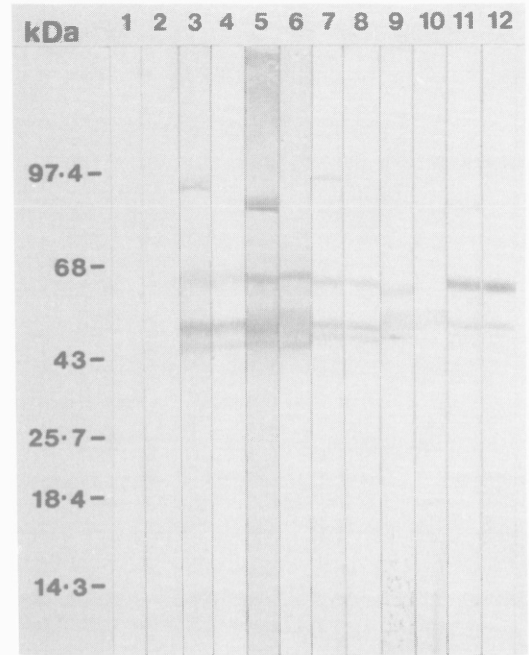


Fig. 7. SDS-PAGE and immunoblotting of various *E. multilocularis* antigen extracts with mouse anti-EM-βgalFP4 serum and affinity purified anti-EM-βgalFP4 antibody. AgA was probed with: lane 1, normal mouse serum; lane 2, mouse anti-β-gal serum; lane 3, mouse anti-EM-βgalFP4 serum; lane 4, affinity purified rabbit anti-EM-βgalFP4 antibody. Mouse anti-EM-βgalFP4 serum was used to probe: lane 5, non-reduced AgA; lane 7, purified protoscolex antigen; lane 9, cyst fluid antigen; lane 11, in vitro excreted/secreted products. Affinity purified rabbit anti-EM-βgalFP4 antibody was used to probe: lane 6, non-reduced AgA; lane 8, purified protoscolex antigen; lane 10, cyst fluid antigen; lane 12, in vitro excreted/secreted products. 50 µg of protein was used per strip apart from the E/S antigen for which 6 µg per strip was used. All antigens were reduced prior to electrophoresis unless otherwise specified.

cyst, although they are present in protoscoleces.

Discussion

Little work has been performed on the characterisation of antigens from *E. multilocularis* [1], although a preliminary study [19] has identified those antigens characteristically immunogenic in human *E. multilocularis* infection. Only recently has the requirement for large quantities of well characterised antigen, for use as immunodiagnostic reagents for alveolar echinococcosis, been recognised [3]. The most successful attempt, to date,

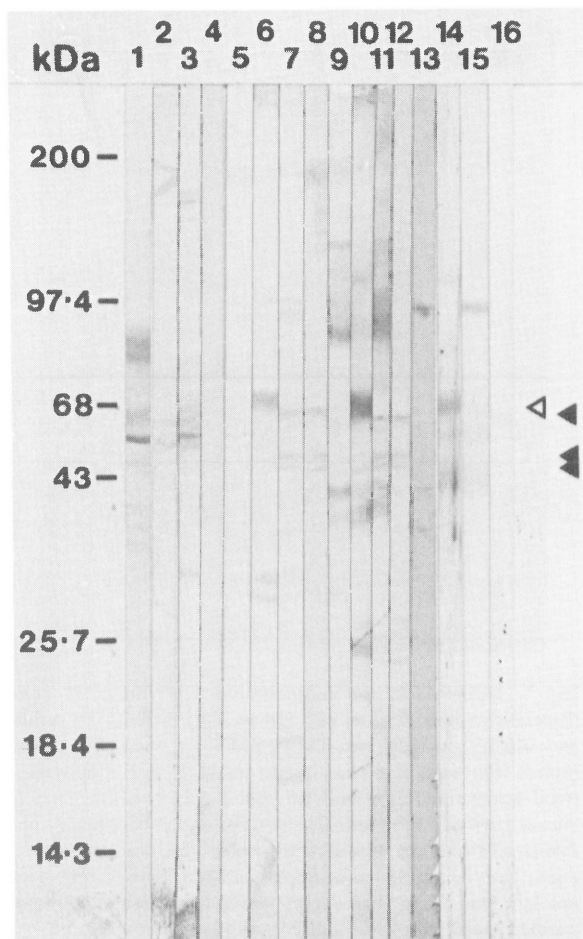


Fig. 8. SDS-PAGE and immunoblotting of *E. granulosus* and *T. solium* antigen extracts with anti-fusion-peptide antibody. *E. granulosus* sheep strain cyst fluid was probed with: lane 1, mouse anti- β -gal serum; lane 2, mouse anti-EM- β galFP2 serum; lane 3, mouse anti-EM- β galFP4 serum; lane 4, affinity purified rabbit anti-EM- β galFP4 antibody. *E. granulosus* sheep strain protoscolex antigen was probed with: lane 5, mouse anti- β -gal serum; lane 6, mouse anti-EM- β galFP2 serum; lane 7, mouse anti-EM- β galFP4 serum; lane 8, affinity purified rabbit anti-EM- β galFP4 antibody. *E. granulosus* horse strain protoscolex antigen was probed with: lane 9, mouse anti- β -gal serum; lane 10, mouse anti-EM- β galFP2 serum; lane 11, mouse anti-EM- β galFP4 serum; lane 12, affinity purified rabbit anti-EM- β galFP4 antibody. *T. solium* cysticercus antigen was probed with: lane 13, mouse anti- β -gal serum; lane 14, mouse anti-EM- β galFP2 serum; lane 15, mouse anti-EM- β galFP4 serum; lane 16, affinity purified rabbit anti-EM- β galFP4 antibody. Molecular weights of native antigens were arrowed: EM2 ($\triangleleft$); EM4 ($\blacktriangleleft$). 50 μ g of protein was used per strip. Proteins were reduced prior to electrophoresis.

to rectify this, has been the work of Gottstein et al. [3] with the resultant isolation of a 54 kDa *E. multilocularis*-specific antigen, designated Em 2a [20]. This antigen was shown to be *E. multilocularis*-specific by immunoblotting (following separation by isoelectric focusing), immunoprecipitation and ELISA where it bound antibody only with human *E. multilocularis* infection serum. Provision of Em 2a, however, relies heavily on a readily available source of parasite material. With a view to overcoming this problem, we have constructed, as far as we are aware, the first cDNA expression library from an *Echinococcus* organism, namely *E. multilocularis*. Subsequent, differential antibody screening of this library has been performed using a technique based on that developed by Stahl et al. [21] for the study of *Plasmodium falciparum* antigens. This technique was shown, by these workers, to facilitate the isolation of specific clones without prior purification of either antigens or antibodies by conventional means. The major adaptation of this technique, in the current study, was the use of plaque as opposed to colony immunoassay. This allowed screening of large numbers of recombinants without the need for lysogenisation of clones.

The differential screening approach was adopted in favour of probing with antiserum raised to a specific antigen such as Em 2a for several reasons. Primarily, if an antigen is cloned and subsequently expressed in a prokaryotic system as a fusion-peptide, this could alter the antigenicity to such an extent as to make it unsuitable as a diagnostic tool. These changes could be attributed to the inability of the system to recreate carbohydrate epitopes, incorrect folding and masking of certain epitopes by the fusion-peptide carrier protein or the cloning of cDNA sequences which are not of full length and, therefore, do not code for the complete antigenic peptide. An approach based on differential screening also allows several potentially specific clones to be isolated simultaneously. This is important as several antigens may be required in an immunodiagnostic test to overcome the effects of differing host response to individual parasite antigens. Furthermore, differential screening also allows very rapid identification of antigen clones recognised in human infections without the need for preliminary

purification or characterisation of these antigens.

Following initial isolation, testing of the two cloned antigens (EM2, EM4) by the probing of immunoblots of the purified fusion-peptides, confirmed human *E. multilocularis* infection sera-specific antibody binding (Fig. 4). Inclusion of the recombinant peptides in an ELISA assay proved feasible only with EM- β galFP4. The ELISA data produced with this antigen on testing with various human infection sera, using β -gal as control, confirmed the immunodiagnostic potential of this clone (Fig. 5 and Table I).

The M_r of the native parasite proteins corresponding to these cloned antigens was also established. The discrepancy in M_r between the recombinant antigens and their corresponding native proteins may have been due to the cloning of incomplete gene sequences and/or the existence of carbohydrate residues on the native proteins. Their ability to produce an antibody response within the human host, despite the usual absence of protoscoleces within the human alveolar cyst [22], suggests that these proteins are either present in or on the germinal layer, or, in the case of EM4, excreted/secreted from this layer. A 70 kDa antigen was identified by Lingelbach and Hinz [19] as being characteristic of hydatid infection. This antigen was recognised by both human *E. multilocularis* and *E. granulosus* infection sera. There is, therefore, little likelihood of this being the same antigen as that encoded by EM2. EM4 codes for an antigen that has epitopes shared by 62, 49 and 44 kDa native parasite antigens. It has yet to be established whether one protein is coded for by this clone which undergoes post-translational modification to produce three distinct antigens, or whether the one protein shares epitopes with other dissimilar antigens. All three proteins appear to be E/S products, which concurs with the finding of high antibody levels specific to these proteins in human sera as excreted/secreted an-

tigens released into the host system are generally good immunogens [23].

Both EM2 and EM4 native protein antigens are present in protoscoleces of both U.K. strains of *E. granulosus* and in *T. solium* cysticerci, although they do not appear to be present in *E. granulosus* sheep cyst fluid. This indicates that these antigens are not *E. multilocularis*-specific at the parasite level and that they are apparently not secreted/excreted into the unilocular *E. granulosus* cyst lumen. The latter characteristic may account for the lack of immune response directed towards these antigens by unilocular hydatid patients. The lack of *E. multilocularis* specificity of these clones may create diagnostic complications, particularly in the case of drug treated patients. Drug treatment has been found to produce disruption of cystic material [24] which leads to release of parasite antigens into the host system that would not normally be presented during the course of an infection. Antibody to these potentially immunodiagnostic cloned antigens may, therefore, be found in drug-treated cystic echinococcosis or *T. solium* infected patients and could result in an incorrect diagnosis. The extensive testing of recombinant antigens, encoded by EM2 and EM4, against large numbers of individual human infection sera, including those from drug treated patients, is now in progress in order to establish their suitability as diagnostic reagents.

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