SOME RECENT ADVANCES IN THE MOLECULAR CHARACTERIZATION OF *ECHINOCOCCUS* AND *TAENIA SOLIUM*

Donald P McManus, Josephine Bowles,
Graham Leggatt and Eduardo Garcia-Zepeda

Tropical Health Program, Queensland Institute of Medical Research,
Bramston Terrace, Herston, Brisbane, Queensland 4006, Australia.

Abstract. Some recently obtained data from our laboratory on the molecular characterization of *Echinococcus* and *Taenia solium* are described and are complimented by relevant new information obtained by other groups. Progress made in the development of satisfactory immunodiagnostic assays and in the production of recombinant molecules, suitable for application in serology of hydatid disease and cysticercosis, is highlighted. Results arising from the application of polymerase chain reaction and direct sequencing, using primers homologous to evolutionarily conserved sequences, in phylogenetic studies and for distinguishing individual taeniid species are also discussed.

INTRODUCTION

The Taeniidae is the most important cyclophyllidean cestode family from the medical or veterinary standpoint. Three of the most important species belonging to this group are *Echinococcus granulosus*, which causes unilocular (or cystic) hydatid disease in man and domestic livestock, the closely related *E. multilocularis*, the cause of human alveolar hydatid disease and *Taenia solium*, the pig tapeworm which causes taeniasis (infection due to the adult worm) and cysticercosis (infection due to the cysticercus - *Cysticercus cellulosae*) in man. The three species occur widely in Asia. *E. granulosus* is commonly found on the Indian sub-continent (India, Bangladesh, Pakistan), in Southeast Asia (Malaysia, Indonesia, Indo-China) and Eastern Asia (Japan, China, parts of the USSR); human cases of alveolar hydatid occur frequently in China, northern Japan and parts of the USSR; endemic areas for *T. solium* include northem Thailand, northeast and north central China and Indonesia.

The biology, physiology and biochemistry (including molecular biology) of these three zoonotic taeniid species has been recently reviewed (Smyth and McManus, 1989). The eventual control of these parasites depends on carefully collected epidemiological data, including their precise identification and characterization from each endemic area. The recent application of molecular technology has opened up a new perspective for gathering such crucially important information by providing powerful new approaches, tools and assays whereby hydatid disease, cysticercosis and taeniasis can be more satisfactorily diagnosed. In addition, this advanced technology is already being employed for unambiguous species and sub-specific (strain) discrimination, in identification of taeniid eggs, in the assignment of function to important macromolecules, and in defining the evolutionary and phylogenetic relationships within the Taeniidae and between this group and other closely or distantly related parasites. Here we describe some of the recent progress made in our laboratory in several of these areas, principally with *Echinococcus* and *T. solium*. Two other reviews (McManus, 1990a,b), which describe some of our earlier work, compliment this paper.

SERODIAGNOSIS

Physical methods, especially ultrasound and computed axial tomography, can aid in diagnosis of cysticercosis and hydatidosis in man. Nevertheless, immunological methods can also play an important role, both in relation to seroepidemiological surveys and in corroboration of clinical diagnosis. Readily detectable levels of

antibody are produced in both diseases by the majority, but not all, infected individuals. Other problems with available serological tests are due to cross-reactivity of *Echinococcus* and *T. solium* antigen preparations with antibodies in sera from patients with other helminths, notably other taeniid, infections. In addition, there are difficulties with wide-scale application of serodiagnosis owing to limitations in the supply of parasite antigens for tests and in quality control of antigen preparations derived from different batches of parasites. Current research on application of recombinant DNA (rDNA) methods to production of appropriate antigens for diagnosis of infection may resolve some of these difficulties. Substantial problems remain in the development of serological tests for the diagnosis of hydatid disease in domestic animals and the application of rDNA technology may not provide an answer. Some recent results provide more hope for immunological detection of swine cysticercosis (Flisser *et al*, 1990). Three valuable reviews (Lightowlers, 1990a,b; Flisser *et al*, 1990) are available which describe some recent advances in immunological diagnosis of cysticercosis/hydatidosis and taeniasis/echinococcosis (adult worm infection). Some of the progress in our group aimed at producing serodiagnostic reagents, including recombinant antigens, for *E. multilocularis, E. granulosus* and *T. solium* is now described. Reference has been made, where appropriate, to important new information from other laboratories.

E. multilocularis

At the present time, a most satisfactory immunodiagnostic assay is available for alveolar hydatid based on ELISA and incorporating a specific component (termed EM2a), purified from *E. multilocularis* by affinity chromatography (see Gottstein *et al*, 1987). Nevertheless, interest has recently focussed on the production of recombinant *E. multilocularis* antigens and, to date, two laboratories have been successful. Vogel *et al* (1988) identified a clone expressing an antigen specific for *E. multilocularis*. The clone produced an unstable molecule, expressed as a fusion protein, but subclones from the initially identified cDNA were successful in improving the yield of parasite antigen (Müller, *et al*, 1989a; Müller *et al*, 1989b). ELISA with the stabilized protein provided excellent, but not absolute, sensitivity and specificity (Müller *et al*, 1989b).

A recombinant antigen with potential for use in serodiagnosis of alveolar hydatidosis has also been produced by our group (Hemmings and McManus, 1991). Using a similar approach to Vogel *et al* (1988), we screened an *E. multilocularis* lambda gt11 cDNA expression library with pools of human antisera and identified two potentially immunodiagnostic antigen gene clones, designated EM2 and EM4 (Hemmings and McManus, 1989). However, on further testing of the EM2 expressed antigen, it was found to cross-react with human antibody from several heterologous taeniid infections. Consequently, we then decided to concentrate on the protein encoded by EM4 as a diagnostic reagent. EM4 was thus expressed using the *Escherichia coli* expression vector pGEX–1 (Hemmings and McManus, in 1991). This vector is one of the pGEX1–3 series (Smith and Johnson, 1988) which express recombinant products as a fusion peptide with *Schistosoma japonicum* glutathione-S-transferase. These vectors have several potential advantages over other expressing systems, including relative ease of purification under non-denaturing conditions, and they are now being used widely by molecular parasitologists. The recombinant antigen expressed by EM 4 in pGEX–1 (now designated EM-GSTFP4) demonstrated 100% specificity on testing in ELISA with pools and then individual heterologous human antisera (Table 1). Unfortunately, only 18 (37%) of 49 individual human anti-*E. multilocularis* antisera reacted positively with EM-GSTFP4 (Table 1). This low diagnostic sensitivity may be improved by the addition of other cloned diagnostic antigens to form an antigen cocktail allowing a much broader spectrum of antibodies to be detected, offsetting the inherent heterogeneity in antigen recognition by individual hosts.

E. granulosus

Currently, there is no available immunodiagnostic test which can provide absolute sensitivity and specificity for unilocular hydatid disease in man or animals. A wide variety of different antigenic preparations, including protoscolex extracts and, especially, whole hydatid cyst

Table 1

The diagnostic specificity and sensitivity of a recombinant *E. multilocularis* antigen (EM-GSTFP4) in ELISA.

	Cystic hydatid		Cysticercosis	
Specificity	sera tested	sera positive	sera tested	sera positive
	11	0	11	0

= 100% specificity

	Alveolar hydatid	
Sensitivity	sera tested	sera positive
	49	18

= 37% positivity

fluid, have been incorporated in numerous tests. Only two antigenic molecules - Antigen B and Antigen 5 – have been defined with any precision. The characteristics of these two molecules have been described by a number of authors (see, for example, McManus, 1990b). Antigen B is heat stable, has a molecular weight estimated between 120 and 160 kDa in cyst fluid and breaks down into sub-units in the presence of SDS. We have estimated the lower molecular weight subunits in SDS-PAGE to be 12 kDa and 16 kDa (Shepherd and McManus, 1987) although, according to another group (Lightowlers *et al,* 1989b), Antigen B is comprised of an 8kDa subunit with additional subunits made up of multiple copies of the 8kDa monomer. In an earlier report (Shepherd and McManus, 1987), we suggested that the 12 kDa and 16kDa molecules might be species-specific for *E. granulosus*. However, a subsequent paper (Lightowlers *et al,*1989b) suggested that they may be *Echinococcus*-specific. Recently, we tested a substantial number of human sera from confirmed cystic hydatid (53 sera), alveolar hydatid (36) and cysticercosis (53) cases by immunoblot analysis using sheep hydatid cyst fluid as antigen. In particular, we scored the immunoreactivity of the sera with the smallest (12 kDa) subunit of Antigen B. We found that 48/53 (90.6%) cystic hydatid sera, 18/36 (50%) alveolar hydatid sera and 3/53 (5.7%) cysticercosis sera reacted with this molecule. Nevertheless, epitopes on the antigen may provide requisite specificity for detection of cystic hydatid and for this reason we have been applying recombinant technology to the problem.

Recombinant molecules can alleviate problems with quality control and supply of antigens derived directly from hydatid cysts and they may also, potentially, improve the specificity and sensitivity of diagnosis. cDNAs encoding *E. granulosus* antigens have now been cloned and, in some cases, sequenced (Shepherd *et al,* 1991; Lightowlers *et al,* 1989b) although the expressed

proteins have still to be assessed for diagnostic value. One of the molecules we have cloned encodes the carboxyl-terminal of the 12 kDa subunit of Antigen B described above. Unfortunately, we have encountered problems in inducing good expression of the molecule in the lambda gt11/*E. coli* system, possibly due to instability of the expressed fusion protein. We have good evidence (Shepherd *et al*, 1991) that the 12 kDa functions as a protease inhibitor and if the expressed fusion protein acts as an intracellular inhibitor of *E. coli* proteases, then this could account for the low tolerance of the host cell towards the fusion protein. At the present time we are attempting to subclone the insert cDNA into a different vector/host cell combination to obtain the recombinant parasite protein in quantity. We will then be able to assess the diagnostic value of the recombinant molecule against panels of human infection sera.

T. solium

A very effective test is currently available for diagnosing human cysticercosis (Tsang *et al*, 1989). The test also shows promise for diagnosing cysticercotic pigs (Flisser *et al*, 1990). The test detects antibodies to cysticercus antigens in an immunoblot assay incorporating a fraction of lentil-lectin affinity-purified cysticercus glycoprotein antigens. We are applying recombinant DNA techniques as an alternative approach to specific and sensitive diagnosis of cysticercosis. Firstly, we synthesized cDNA from the metacestode stage of *T. solium* and cloned it into the lambda gt11/*E. coli* expression system. Several gene libraries were prepared from different batches of parasites. Immunoscreening of the libraries was then carried out using a preabsorbed pool of human cysticercosis sera. This identified 40 sero-positive clones, of which a number were putatively shown to be expressing *T. solium*-specific polypeptides following differential testing with sera from humans infected with *E. granulosus, E. multilocularis* and *T. saginata*. Lysogens have been produced from these recombinant clones and beta-galactosidase fusion peptides ranging in Mr of approximately 135–150 kDa identified. Subsequently, these fusion peptides have been shown to be recognized by a pool of human sera from proven cysticercosis cases. Some of the characteristics of these clones and their expressed products are shown in Table 2. The cDNAs of several of these clones have been subcloned into the sequencing vector M13 and sequence information obtained. In future work, we will test the specificity and sensitivity of the expressed products of each of the clones with the ultimate aim of producing a very simple dot blot assay incorporating a cocktail of recombinant antigens for detection of circulating *T. solium* metacestode antibody in human serum.

MOLECULAR IDENTIFICATION

We have been developing a range of molecular techniques for distinguishing isolates and well characterized strains of *E. granulosus* and isolates of *T. solium*. For a review of earlier progress, see McManus (1990a). The techniques of PCR and direct sequencing, using primers homologous to evolutionarily conserved sequences, have recently been established in our laboratory. We have designed primers to amplify homologous genes or gene fragments from a variety of taeniid species, including *Echinococcus*. Two such sequences have proved of especial value for phylogenetic studies. The first, a portion of the 5′ terminal domain of large (28S) subunit rDNA has proved useful in the construction of phylogenies for the taeniids using two of the phylogenetic reconstruction programs available. The phylogenies produced using the two different methods are reasonably congruent. Furthermore, we have found that this sequence is of value in distinguishing between *T. solium* and *T. saginata* which differ in 13 out of 186 sequenced positions (93% homology). This sequence is identical in *T. saginata* and Taiwan *Taenia*, it cannot discriminate *E. granulosus* from *E. multilocularis* and it has not proved useful for distinguishing strains or isolates within species.

A second data set is also being collected for the same group of taeniids. In this instance, a protein-coding gene from the mitochondrial genome, cytochrome *c* oxidase 1, is being targeted. Again, this sequence shows promise for further distinguishing and ordering the species in question. The mitochondrial genome evolves more rapidly than the nuclear genome and many silent nucleotide substitutions are

Table 2

Characteristics of different cDNA clones putatively expressing *T. solium* antigens.

cDNA clone	Insert size[a] (bp)	β – gal fusion peptide[b] (kDa)
L1RH3	200	125
L1rRTS2	344	125
LSrNAB5*	140	120
L2H3	298	150
L1H6	285	130
L1HB1*	200	120
L1HR1	344	145
L4rrRTS1	150	120
L1RH5	350	145
L2rRHB5	150	120
9.2rHTS	300	135
L3B1*	220	130
L1rRTS4	394	145
L3r5	150	130
9.1rB*	200	130
L1rRH2	200	130
L1rRTS1	380	140

All clones reacted positively in plaque assays with a pool of sera from clinically identified cysticercosis patients but not with sera from patients infected with *Echinococcus granulosus* or *Taenia saginata*. Asterisked clones reacted with a rabbit antiserum prepared against purified Antigen B, an antigen known to be recognised by the majority of cysticercosis patients.

a = Determined both by PCR and restriction enzyme digestion
b = Determined by SDS-PAGE analysis

possible in a protein-coding gene. It is, therefore, possible that this sequence may prove more useful for detecting intra-specific variation, especially within *Echinococcus*. Of sequence obtained, to date, for cytochrome *c* oxidase 1, we have recorded some differences between *T. saginata* and Taiwan *Taenia* both in terms of nucleic acid sequence (96% homology) and derived amino acid sequence (98% homology). The sequences for *T.solium* and *T. saginata* are again quite distinct, exhibiting 86% and 91% nucleic acid and derived amino acid homology, respectively. We found no variation in the sequence for two isolates of *T. solium* (Zimbabwean and Mexican origin) or two African isolates of *T. saginata*. The data we have obtained corroborates biological findings (Fan, 1988) and DNA studies (Zarlenga *et al*, 1990) which have shown that Taiwan *Taenia* and *T. saginata*, although closely related, should be regarded, nevertheless, as distinct organisms.

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