MOLECULAR TAXONOMY AND EPIDEMIOLOGY OF CYSTIC ECHINOCOCCOSIS

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Abstract. *Echinococcus granulosus* exhibits substantial genetic diversity that has important implications for the design and development of vaccines, diagnostic reagents, and drugs effective against this parasite. DNA approaches are now being used routinely for accurate identification of these genetic variants and in molecular epidemiological surveys of cystic echinococcosis in different geographical settings and host assemblages. The recent publication of the complete sequences of the mitochondrial (mt) genomes of the horse and sheep strains of *E. granulosus* and of *E. multilocularis*, and the availability of mt DNA sequences for a number of other *E. granulosus* genotypes, has provided additional genetic information that can be used for more in depth strain characterization and taxonomic studies of these parasites. This very rich sequence information has provided a solid molecular basis, along with a range of different biological, epidemiological, biochemical and other molecular-genetic criteria, for revising the taxonomy of the genus *Echinococcus*. This has been a controversial issue for some time. Furthermore, the accumulating genetic data may allow insight to several other unresolved questions such as confirming the occurrence and precise nature of the *E. granulosus* G9 genotype and its reservoir in Poland and whether it occurs elsewhere, more precise delineation of the host and geographic ranges of the genotypes characterized to date, and whether additional genotypes of *E. granulosus* remain to be identified.

INTRODUCTION

An important feature of the biology of *Echinococcus granulosus* is the fact that it comprises a number of intra-specific variants or strains that exhibit considerable variation at the genetic level (Thompson and McManus, 2001; McManus *et al*, 2003). The term strain is used to describe variants that differ from other groups of the same species in gene frequencies or DNA sequences, and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis (Thompson and Lymbery, 1988; McManus and Bowles, 1996; Bowles *et al*, 1995). The extensive intra-specific variation in nominal *E. granulosus* may influence life cycle patterns, host specificity, development rate, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents and pathology (Thompson and Lymbery, 1988; Thompson and McManus, 2001; 2002). This may have important implications for the design and development of vaccines, diagnostic reagents and drugs impacting on the epidemiology and control of echinococcosis.

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A number of well-characterized strains are now recognized that all appear to be adapted to particular life cycle patterns and host assemblages (Thompson and McManus, 2001; McManus, 2002). Genetic variation in *Echinococcus* has been investigated in both the nuclear and mitochondrial genomes. Mitochondrial DNA (mtDNA) has proved particularly useful for the discrimination of closely related organisms because of its relatively rapid rate of evolution. The advent of the polymerase chain reaction (PCR) has provided a highly sensitive approach that is now widely used for *Echinococcus* identification purposes, including discrimination of eggs. A detailed description of the various techniques and approaches that have been used for the molecular genetic analysis of *Echinococcus* isolates can be found in McManus (2002) and McManus and Thompson (2003). To date, molecular studies, using mainly mitochondrial DNA (mtDNA) sequences, have identified 9 distinct genetic types (genotypes G1-9) within *E. granulosus* (McManus, 2002). This categorization follows very closely the pattern of strain variation emerging based on biological characteristics. Applications of DNA-based approaches for identification of these genetic variants and their use in selected molecular epidemiological studies of echinococcosis are summarized here. Sequence comparisons of segments of the mitochondrial *cox1* and *nad1* genes have proved especially valuable in these studies, but other mitochondrial genes such as those coding for cytochrome b (*cob)* or ATPase 6 (*atp6)* should prove equally as useful.

GENETIC DIFFERENCES BETWEEN THE HORSE-DOG AND SHEEP-DOG STRAINS OF *E. GRANULOSUS* ARGUE FOR SEPARATE SPECIES

As the result of extensive study, instigated by Professor Des Smyth in the 1970s, discrete horse/dog and sheep/dog forms of *E. granulosus* have been shown to be present in the United Kingdom that differ in a wide spectrum of biological criteria (Smyth, 1977; Thompson and Lymbery, 1988). Conventional DNA and PCR-based analysis (see McManus, 2002) confirmed the distinctiveness between, but uniformity within, these two forms of *E. granulosus.* Furthermore, analysis of isolates collected world-wide using these approaches indicated that the sheep-dog strain is cosmopolitan in its geographical distribution, that it is remarkably uniform genetically, and that the horse-dog form is genetically similar to that infecting equines in other countries. This early DNA sequence data indicated that these 'strains' were as distinct as the accepted species of *Echinococcus* (Bowles *et al*, 1992a) suggesting that they may be more appropriately regarded as sibling species, especially in light of the considerable biological and biochemical differences that were shown to exist between them.

No evidence of gene exchange was found in examination of their rDNA sequences (Bowles and McManus, 1993), implying that the sheep and horse strain parasites do not interbreed despite the fact that they use the same definitive host and occur sympatrically. Careful phylogenetic analysis of the mitochondrial sequence data, in combination with additional nuclear sequence data (Bowles *et al*, 1995), formally demonstrated the evolutionary distinctiveness of the sheep and horse strains of *E. granulosus*. Confirmation of species identity was obtained after analysis of the complete mitochondrial genomes that were recently obtained for both strains and another taeniid cestode, *Taenia crassiceps* (Le *et al*, 2002). The complete circular mitochondrial genome of the sheep-dog strain (G1 genotype) is shown in Fig 1. Pair-wise comparisons of concatenated protein-coding genes indicated that the sheep-dog and the horse-dog forms were almost as distant from each other as each was from *E. multilocularis.* In addition, sequences for the variable genes *atp6* and *nad3* were obtained from additional genotypes of *E. granulosus*, from *E. vogeli* and *E. oligarthrus*. Again, pair-wise comparisons showed the distinctiveness of the G1 and G4 genotypes. Phylogenetic analyses of concatenated *atp6*, *nad1* (partial) and *cox1* (partial) genes from *E. multilo-cularis*, *E. vogeli*, *E. oligarthrus*, 5 genotypes of *E. granulosus*, and using *T. crassiceps* as an outgroup, yielded the same results (Le *et al*, 2002).

These data and a range of different biological, epidemiological, biochemical and other molecular-genetic criteria provide an overwhelming argument in favor of separate species status for the horse-dog and sheep-dog strains. Of public health significance is the fact that the sheep strain is infective to humans but, probably, non-infective to horses. The horse strain appears to be poorly infective to sheep and may prove to be non-infective to humans. This is borne out by the DNA data as, to date, the horse strain (G4 genotype) has not been reported in sheep or humans, and the sheep strain (G1 genotype) has not been identified by DNA analysis in horses.

The fact that the genetic characteristics of the horse-dog and sheep-dog forms of *E. granulosus* are maintained in sympatry in endemic areas where the life cycles overlap [*eg* UK, Spain, and Jordan; (Kamhawi and Hijawi, 1992; Siles Lucas *et al,* 1994)] reinforces the argument that the two forms are separate species. Rausch (1967) quite correctly identified the problem of recognizing the form in horses as a sub-species since Williams and Sweatman (1963) provided no evidence of a segregating mechanism, since subspecies by definition can interbreed. Consequently, if Williams and Sweatman (1963) had proposed species status for the form in horses, its taxonomic status is unlikely to have been questioned as rigorously. Considering the additional evidence that has accumulated in the intervening 40 years, we have proposed (Thompson and McManus, 2002) that *E. equinus* be recognized as a distinct species, following the description given by Williams and Sweatman (1963).

DNA ANALYSIS OF CYSTIC ECHINOCOCCOSIS CASES FROM POLAND

It had been suspected, on epidemiological grounds, that *E. granulosus* from pigs has low infectivity to humans (Pawlowksi, 1985; Eckert *et al,* 1993; Pawlowski *et al,* 1993) but this needed to be confirmed by identification of isolates taken from humans residing in an area (Poznan) where sheep were rarely bred, where pig hydatidosis was highly prevalent, and where the pig strain of *E. granulosus* was the most common form found in domesticated animals. Nuclear (ribosomal ITS1) and mitochondrial (*nad1)* DNA sequences were compared for human isolates of Polish origin (Scott *et al,* 1997) collected by fine needle aspiration biopsy (FNAB). The data indicated clearly that the Polish patients were not infected with the common sheep strain (G1 genotype) of *E. granulosus*, normally associated with human cystic echinococcosis. Instead, the form of *E. granulosus* infecting the Polish patients shared very similar *nad1* sequence with the

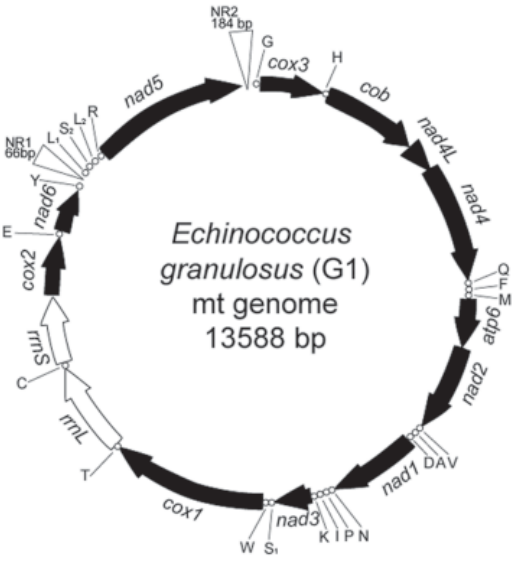

Fig 1- The circular mitochondrial genome of the sheep-dog strain (G1 genotype) of *Echinococcus granulosus.* See Le *et al* (2002) for full details. This genome (13,588 bp) is typical in organization to those of most animals. Filled arrows indicate protein-encoding genes and open arrows represent ribosomal RNA genes. All genes are transcribed in the same direction. The 12 protein-coding genes fall into the following categories: nicotinamide dehydrogenase complex (*nad1-6* and *nad4L* subunits); cytochrome *c* oxidase complex (*cox1-3* subunits); cytochrome *b* (*cob*) and adenosine triphosphatase subunit 6 (*atp6*). Unlike the situation in most other metazoans, there is no *atp8*. Two genes encoding ribosomal RNA subunits are present: the large subunit (*rrnL* or 16S) and small subunit (*rrnS* or 12S). NR1 and NR2 are long non-coding regions. As is common in mitochondrial genomes, there are 22 transfer RNA (tRNA) genes that are indicated by the single-letter conventional code for the amino acid they specify. L1 = *trnL(CUN)*, L2 = *trnL(UUN)*, S1 = *trnS(AGN)*, S2 = *trnS(UCN)* .

previously characterized pig (G7) genotype but it exhibited some clear DNA differences. In particular, a single ITS1 fragment of 1.04 kb in size was amplified by PCR (the G7 genotype produces 2 distinct bands of 1 kb and 1.1 kb) and unique restriction fragment length polymorphism (RFLP) patterns were obtained after restriction digestion.

Accordingly, it was proposed that these human isolates represented a distinct *E. granulosus* genotype (designated G9). A subsequent study of human and pig isolates from Poland, Slovakia, and Ukraine (Kedra *et al,* 1999) failed to confirm the existence of this genotype. This second study suggested that, based on *nad1* sequences, pigs (56 isolates examined) and humans (4 isolates examined) were infected with the G7 genotype or pig strain. Separate isoenzyme and DNA-based investigations of *E. granulosus* isolates (Snabel *et al,* 2000; Turcekova *et al,* 1998; 2003), using RAPDs, *nad1* sequence comparisons and PCR-RFLP analysis of the nuclear ITS1 region, have provided additional evidence for the almost exclusive presence of the G7 genotype in Slovakia. DNA from an isolate of *E. granulosus* taken from a wild boar (Sumy region, Ukraine) had identical *nad1* sequence to the G7 genotype earlier found in pigs from the same region (Kedra *et al,* 2000).

Major questions that are outstanding concern confirmation of the existence of the G9 genotype and the reservoir(s) of human hydatid disease in Poland and other countries in Central and East Europe. It is unlikely to be sheep in Poznan Province in Poland as ovine infections with *E. granulosus* are rarely seen there, whereas the prevalence of echinococcosis in pigs is higher than in other parts of the country (Pawlowski *et al,* 1993). In Poland in 1985, the national figures for cystic echinococcosis in slaughtered animals showed prevalences of 5.35% in pigs, 1.08% in sheep and 0.04% in cattle (see Scott *et al,* 1997). Furthermore, the G7 genotype had not, until recently, been shown by molecular analysis to definitively infect sheep (McManus, 2002). However, Gonzalez *et al* (2002) showed that two of four Spanish pig isolates had identical molecular characteristics to the G1 genotype whereas the other two conformed to the G7 genotype (pig strain). Scott *et al* (1997) speculated that in Poland pigs naturally habor the G9 genotype although, unlike in humans, it may develop poorly, producing small, yet viable cysts, in this host. Clearly this is an important epidemiological question that needs to be further addressed. Examination of additional *E. granulosus* isolates from Poland and surrounding countries from humans, pigs, and other potential intermediate hosts is clearly warranted to resolve this controversial issue. Interestingly, an isolate of *E. granulosus* obtained from a wild European beaver, *Castor fiber,* from North-Eastern Poland was typed, on the basis of identical *nad1* sequence, as the G7 genotype (Tkach *et al,* 2002). This is the first report of *E. granulosus* from the European beaver but it is unlikely that this host plays any significant role in the transmission of echinococcosis.

YAKS AND *ECHINOCOCCUS* INFECTION

Echinococcosis is widespread and endemic in western parts of China, particularly among Tibetan pastoral herders inhabiting grazing lands above 3,800

meters. In Tibet, animal infection rates of 54% in yaks (*Bos grunniens*) and 81% in sheep have been recorded. Human hydatid infection rates in exclusively stock-raising areas were 8-27%, of which 50% were due to *Echinococcus granulosus* and 50% to *E. multilocularis* (He *et al*, 2000). Advanced cases of hydatid disease require surgery and due to limited availability of treatment in rural Western China, the disease is often fatal.

To initiate a demonstration of hydatid control for the area, 100 four-year-old yaks from the four townships of the Datangba Flatlands, Ganzi County, Sichuan were necropsied to provide baseline data (Heath *et al*, 2004). A further 25 older yaks slaughtered for meat and identified as originating from Datangba, were examined at slaughter. Visually, cysts found were classified as multilocular (possibly *E. multilocularis*) or unilocular (certainly *E. granulosus*). Often, both types of cysts were found in the same animal. Qiu *et al* (1989) had previously described atypical *E. multilocularis* infections in the liver of yaks and sheep. These cysts did not contain brood capsules or protoscoleces, but had a strong resemblance to *E. multilocularis* infections in humans. If the multilocular cysts contained infective protoscoleces, this would be the first record of large grazing animals acting as an intermediate host for *E. multilocularis*. This might be expected, because dogs in this area have been shown to be heavily infected with both *Echinococcus* species (He *et al*, 2000).

Cysts were cut open and then dissected from liver or lung tissue, fixed immediately in ethanol and portions of unilocular and multilocular cysts were subjected to histological and molecular (genomic DNA isolation and purification, and polymerase chain reaction (PCR)-amplification, automatic sequencing and alignment analysis of fragments of the mt *cox1* gene) analysis. The DNA analysis of unilocular and multilocular cysts showed that both types were caused by the sheep strain (G1 genotype) of *E. granulosus* and not *E. multilocularis* (Health *et al*, 2004). This was supported by examination of hematoxylin and eosin stained histological sections. The convoluted laminated membranes in the multilocular cysts were lined on the inside by germinal membrane, but showed no budding to the exterior, and there were no protoscoleces present. These multilocular cysts were probably a manifestation of an immune response to *E. granulosus* that walls off the developing cyst so that the laminated and germinal membranes continue to proliferate within a confined space. The unilocular cysts also had no evidence of protoscoleces or developing brood capsules.

A Ganzi Hydatid Control and Community Health Project in Sichuan is intended to provide guideline information for the development of future hydatid control programs in China, and is focused on interrupting the lifecycle of the hydatid parasite by dosing dogs with praziquantel and vaccinating (Heath *et al*, 2003) the animals that host the cystic stage of the tapeworm. An understanding of the *Echinococcus* life-cycle and how people can avoid becoming infected with hydatid disease is included as part of the community and health education activities.

At the beginning of the project, it was thought that Datangba Flatlands yaks, sheep, and goats could all produce cysts that would be able to reinfect dogs with the parasite. Vaccination of all these animals would prevent infections becoming established and reduce the chances of dogs becoming reinfected by eating animal organs containing hydatid cysts.

Emerging technology has shown that not all types of grazing animals are involved in the transmission to dogs of cystic hydatid disease caused by *E. granulosus* (McManus, 2002). Zhang *et al* (1998) reviewed previous work in China showing the predominance of the sheep strain (G1 genotype) and a report of this genotype in a sample of hydatid material from yaks. An echinococcal lesion in the liver of a yak from a neighboring region (Shiqu county) in the Qinghai-Tibet plateau region was shown by mt DNA typing to be the result of infection by the G1 genotype of *E. granulosus* (Xiao *et al*, 2003).

We have now shown that this local Datangba sheep strain of *E.granulosus* usually only produces protoscoleces in sheep and goats, and not in yaks. There are reports of yaks contributing to human hydatid disease in a population of yaks around Qinghai Lake in Qinghai and in cattle in Xinjiang. The Qinghai yaks and Xinjiang cattle are now thought to actively host a different (G5) genotype (Bowles *et al*, 1992b) which does produce cysts that are infective for dogs but this cycle may not occur elsewhere in western China. The G5 (cattle-dog strain) has been shown by genotyping human cyst material to be infective to humans (Bowles *et al*, 1992b)

We are now proposing to collect human hydatid cyst material from Datangba people undergoing hydatid surgery at the Ganzi Hospital in order to determine the infective hydatid genotype. If it is solely or predominantly G1, as we predict, the control of hydatid disease caused by *E. granulosus* will concentrate there on sheep and goats, while putting less emphasis on yaks.

CONCLUDING COMMENTS

Molecular techniques have validated the genetic basis of important morphological differences that can now be used with confidence as a reliable and simple means of identifying and differentiating between strains and species of *Echinococcus* (*eg* Harandi *et al,* 2002; Tashani *et al,* 2002). The recent publication of the complete sequences of the mt genomes of the horse and sheep strains of *E. granulosus* (Le *et al,* 2002) and *E. multilocularis* (Nakao *et al,* 2002) and mt DNA sequences for a number of other *E. granulosus* genotypes (Pearson *et al,* 2002; Le *et al,* 2002), has provided additional genetic information that can be used for even more in depth strain characterization and phylogenetic study of the hydatid organisms. Already, the availability of this very rich sequence information has provided a solid molecular basis for revising the taxonomy of the genus *Echinococcus* (Le *et al,* 2002; Thompson and McManus, 2002), a controversial issue for decades. Furthermore, the accumulating genetic data may allow insight to several other unresolved questions such as confirming the presence and precise nature of the G9 genotype and its reservoir in Poland, whether it occurs elsewhere, why the camel strain (G6 genotype) appears to affect humans in certain geographical areas but not others, more precise delineation of the host and geographic ranges of the genotypes characterized to date, and whether additional genotypes of *E. granulosus* remain to be identified.

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