Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran

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SUMMARY

Iran is an important endemic focus of cystic hydatid disease (CHD) where several species of intermediate host are commonly infected with *Echinococcus granulosus*. Isolates of *E. granulosus* were collected from humans and other animals from different geographical areas of Iran and characterized using both DNA (PCR-RFLP of ITS1) and morphological criteria (metacestode rostellar hook dimensions). The sheep and camel strains/genotypes were shown to occur in Iran. The sheep strain was shown to be the most common genotype of *E. granulosus* affecting sheep, cattle, goats and occasionally camels. The majority of camels were infected with the camel genotype as were 3 of 33 human cases. This is the first time that cases of CHD in humans have been identified in an area where a transmission cycle for the camel genotype exists. In addition, the camel genotype was found to cause infection in both sheep and cattle. Results also demonstrated that both sheep and camel strains can be readily differentiated on the basis of hook morphology alone.

Key words: *Echinococcus granulosus*, cystic hydatid disease, Iran, strains, genotypes, morphology, molecular characterization.

INTRODUCTION

Cystic hydatid disease (CHD), caused by the metacestode of *Echinococcus granulosus*, is medically and economically one of the most important of the zoonoses (Schantz, 1997; Thompson, 2001). Considerable genetic and phenotypic variation has been demonstrated in *Echinococcus*, with the recognition of a series of what appear to be host-adapted genotypes or strains (McManus, 1997; Thompson & McManus, 2001), which are likely to represent distinct species (Thompson, Lymbery & Constantine, 1995). Therefore there is a need to characterize the aetiological agents in different endemic areas using molecular epidemiological techniques, in order to determine transmission patterns, particularly where there is the possibility of interaction between cycles (Thompson, Constantine & Morgan, 1998; Thompson & McManus, 2001). For example, in endemic regions where usually a number of species of livestock may be infected with cystic echinococcosis, it is important to determine which species are responsible for maintaining the life-cycle. This provides the basis for implementing targeted control efforts and, more fundamentally, for developing a reliable surveillance system which is essential for assessing the success of intervention strategies (Gemmell & Roberts, 1995). Unfortunately, the degree of genetic variability in populations of *Echinococcus* is insufficiently characterized for many geographical regions (Schantz *et al*. 1995). Determining the geographical distribution and uniformity of host-adapted genotypes of *Echinococcus* is also important, so that formal taxonomic designation can be confirmed (Thompson *et al*. 1995).

CHD is a significant economic and public health problem in Iran (Dar & Alkarmi, 1997). High prevalences have been reported in various species of domestic food animals, including sheep, cattle, camels and goats, and at least 2 distinct cycles of transmission are thought to occur (Dar & Alkarmi, 1997). Human cases of CHD are also regularly reported from medical centres in different parts of the country (Noorjah, 1987; Mobedi, Arfaa & Farahmandian, 1971; Bastani & Dehdashti, 1995). However, the sources of infection to humans, and particularly the role of intermediate host reservoirs, such as the camel, remain to be determined (Dar & Alkarmi, 1997; Thompson, 2001).

In a preliminary study, molecular characterization, using mitochondrial DNA markers, of a

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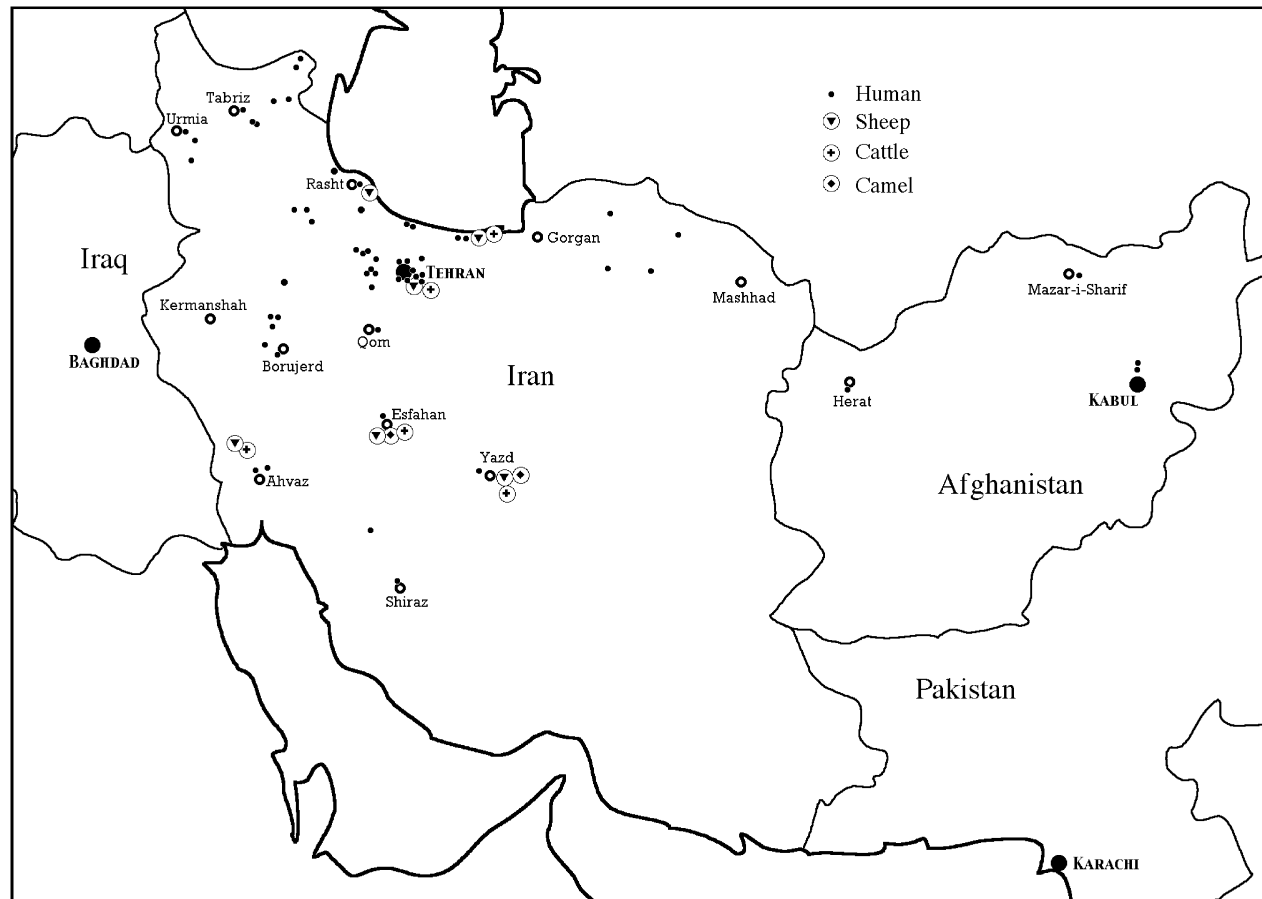

Fig. 1. Map of the study area showing geographical locations of all isolates collected.

small number (16) of isolates of *E. granulosus*, collected from various areas of Iran identified the occurrence of both the sheep and camel genotypes (Zhang *et al*. 1998). The aim of the present study was to undertake an extensive sampling programme of *E. granulosus* from humans and other animals collected from defined, geographical areas of Iran, and to characterize the isolates using both DNA and morphological criteria. Such information should allow a better understanding of the molecular epidemiology of CHD in Iran, particularly any evidence of interaction between cycles of transmission, identifying reservoirs of human infection, and the provision of a comprehensive data set for determining the nature and uniformity of the genotypes of *Echinococcus* endemic in Iran.

MATERIALS AND METHODS

Parasite materials

Hydatid cysts from naturally infected intermediate hosts were collected from several abattoirs in Iran (Fig. 1). A total of 213 *E. granulosus* isolates (all the above isolates except those from goats, plus an additional 70) were collected from humans, sheep, cattle and camels (*Camelus dromedarius*) in different geographical and ecological areas of Iran. Human isolates were obtained at surgery from different hospitals in Iran and Afghanistan (4 isolates) at the locations shown in Fig. 1. In the laboratory protoscoleces were collected from hydatid materials. Protoscoleces from each cyst were rinsed in physiological saline solution and then fixed and preserved in 80–95% (v/v) ethanol.

Morphological studies

Protoscoleces were mounted in polyvinyl lactophenol (RA Lamb) with sufficient cover-slip pressure to cause the hooks to lie flat. Details of the hook components measured are shown in Fig. 2 as in Hobbs, Lymbery & Thompson (1990). Measurements of the total length and blade length were made on 2 large and 2 small hooks per rostellum from each of 10 protoscoleces for each isolate (Kumaratilake, Thompson & Eckert, 1986). Measurements were made using an Olympus BX50 microscope with a 100× objective and an Optimas image analyser. Morphological differences between sheep and camel strains were analysed graphically by 2-dimensional scatterplots. Analysis of covariance was used to compare slopes and elevations of regression lines for sheep strain isolates between different host species (Zar, 1984).

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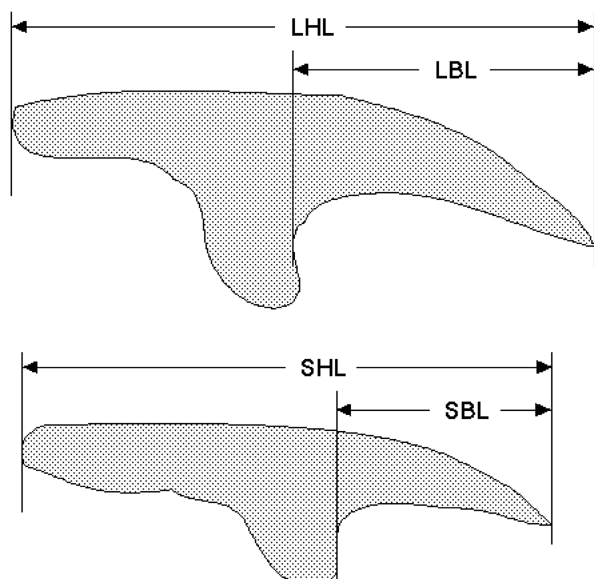

Fig. 2. Diagrammatic representation of hook dimensions as used in this study. Large hook length (LHL), large hook blade length (LBL), small hook length (SHL), small hook blade length (SBL).

Table 1. *Echinococcus granulosus* isolates according to host species analysed by PCR-RFLP of the ITS1 and their genotypic/strain identity

Host	Sheep strain	Camel strain
Sheep	40	3
Cattle	30	2
Goat	6	0
Camel	8	24
Human	33	3
Total	117	32

Molecular analysis

Total genomic DNA was extracted from ethanol preserved isolates. Several different extractions methods were trialed (Sambrook, Fritsch & Maniatis, 1989) and eventually a modified phenol-chloroform method (Bowles & McManus, 1993) was used with subsequent glass milk purification. *E. granulosus* DNA samples were characterized by means of restriction fragment length polymorphism (RFLP) patterns following polymerase chain reaction (PCR) amplification of an ~1 kb fragment spanning the non-coding internal transcribed spacer 1 (ITS1) of the ribosomal DNA (rDNA) repeats (Bowles & McManus, 1993).

A nested-PCR was designed using EGF1 (5′ CCAAACTTGATCATTTAGAGGAAG 3′) and EGR2 (5′ TATGGGCCAAATTCACTCATTA-CC 3′) as outside forward and reverse oligonucleotide primers, specifically designed for this study based on published *Schistosoma mansoni* rDNA sequence data, and BD1 (5′ GTCGTAAC-AAGGTTTCCGTA 3′) and 4S (5′ TCTAGATG-CGTTCGAARTGTCGATG 3′) as inside forward and reverse primers (Gibco BRL), which were previously designed by Bowles & McManus (1993) for this purpose.

PCR reaction volumes of 25 μl contained 1–5 ng of DNA template, 67 mM Tris–HCl (pH 8·8), 16·6 mM $(NH_4)_2SO_4$, 0·45% Triton X–100, 150 μM each of dCTP, d4TP, dTTP, dGTP, 2 mM $MgCl_2$, 12·5 pmol of each of the primers and 1 unit *Tth* Plus DNA polymerase (Fisher Biotech, Australia) and Taq Extender™ (Stratagene) proof reading DNA polymerase in reaction buffer.

In the first round of the nested-PCR (using primers EGF1 and EGR2), the temperature profile was as follows: 1 cycle of pre-PCR, 95 °C for 2 min (denaturation), 60 °C for 1 min (annealing), and 72 °C for 1 min (extension), followed by 45 cycles of 95 °C for 30 sec, 60 °C for 20 sec, 72 °C for 30 sec, and a final extension of 72 °C for 7 min.

The second round of the PCR (using primers BD1 and 4S) for each isolate was carried out by using the following temperature profile: 1 cycle of pre-PCR, 95 °C for 2 min, 55 °C for 1 min and 72 °C for 1 min, followed by 45 cycles of 95 °C for 30 sec, 55 °C for 20 sec, 72 °C for 30 sec, and a final extension of 72 °C for 7 min. PCR products were visualized on 1% (w/v) Tris-acetate-EDTA (TAE) agarose (Promega) gels and stained with ethidium bromide.

The PCR products were digested for 10–12 h with 5 units of each of the restriction endonucleases, *Alu*I, *Hha*I, *Msp*I, *Rsa*I and *Taq*I (New England BioLabs) according to the manufacturer's instructions using 20 μl digestion volume. Restriction fragments were separated by electrophoresis through 3% (w/v) TAE agarose gels, and then stained with ethidium bromide and detected on an UV transilluminator.

RESULTS

Molecular study

In total 143 *E. granulosus* isolates were studied by PCR-RFLP analysis of the ITS1 region of rDNA (Table 1). Molecular characterization of the isolates revealed 2 major banding patterns (Fig. 3A and B). Although some minor variation was detected in the relative intensity of some bands of the same size, the 2 patterns were readily distinguishable. Most of the isolates from sheep, cattle, goat and humans produced patterns similar to those obtained with the universal sheep strain including those from Afghanistan, and most of the camel isolates were characterized as the camel strain. However, 3 human, 2 sheep and 2 cattle isolates had patterns conforming to that of the camel strain and 8 camel isolates showed RFLP patterns similar to sheep strain (Fig. 3A and B).

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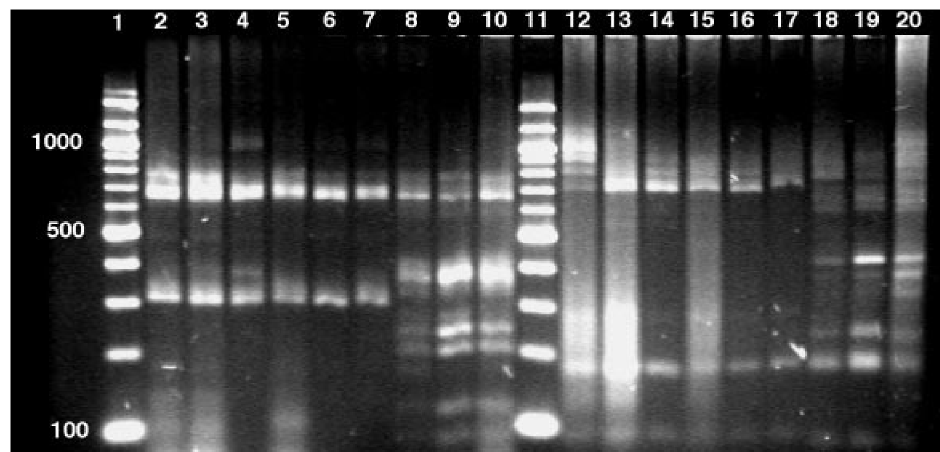
A



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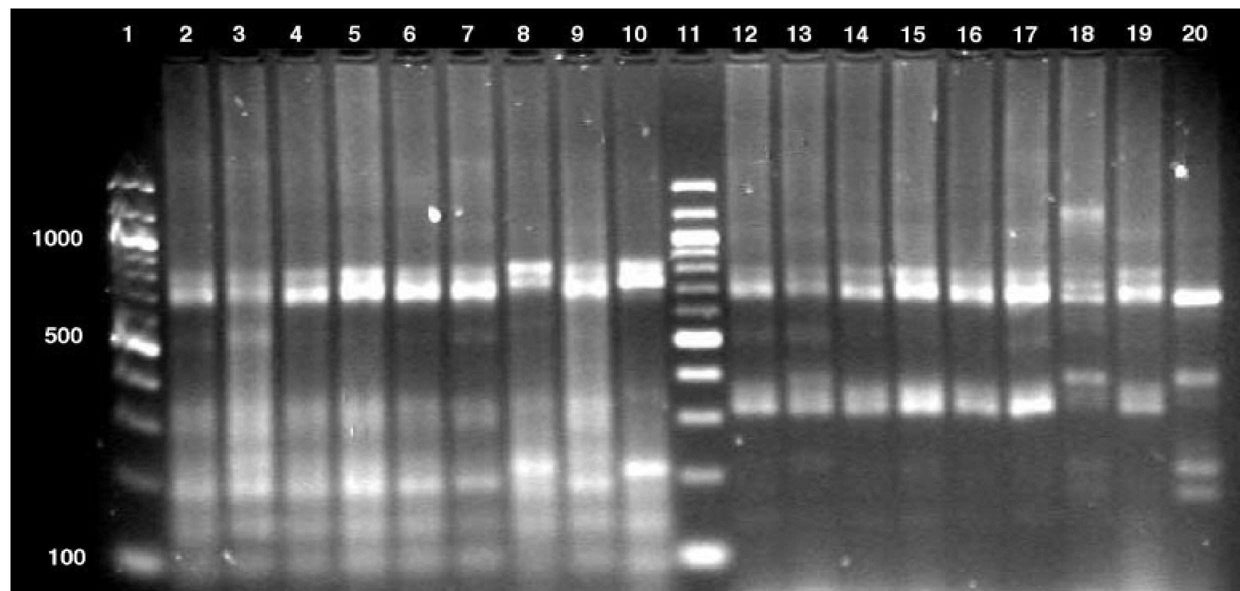

Fig. 3. ITS1-PCR-RFLP patterns for isolates of *Echinococcus granulosus* from Iran. ITS1-PCR products were digested with: (A) *Rsa*I 2 and 3 cattle, 4 goat, 5 and 6 sheep, 7 human, 8–10 camel; *Alu*I 12 and 13 cattle, 14 goat, 15 and 16 sheep, 17 human, 18–20 camel; (B) *Msp*I 2–4 cattle, 5–6 sheep, 7–9 human, 10 camel; *RsaI*I 12–14 cattle, 15–16 sheep, 17–19 human, 20 camel. (N.B. Lanes 8 and 18 of (B) show camel strain/genotype pattern.)

Morphology

A scatterplot of large blade length against large hook length (Fig. 4) clearly shows that hook morphology is influenced by genetic factors. Although other combinations of characters gave similar results, these 2 characters most clearly demonstrated the morphological differences between the 2 strains. Within each host species, there was a clear distinction between these hook measurements of sheep and camel strains. In sheep, cattle and humans, the single character of large hook length would be sufficient to predict the genetic strain of *Echinococcus*. All 21 isolates from camels which were not characterized by PCR-RFLP fell within the camel strain cluster. Similarly, all 18 isolates from sheep, 10 from cattle and 21 from human hosts which were not characterized by PCR-RFLP, fell within the sheep strain cluster for each host species.

It may also be seen graphically from Fig. 5 that the hook size is influenced by the host species. Although there were too few isolates of camel strain in non-camel hosts to conduct statistical analysis, hook measurements in these hosts were consistently at the lower end of the range found in camel hosts (Fig. 4). The sheep strain was found in a large number of non-sheep hosts, and a comparison of the regression lines for large blade length and large hook length indicated significantly different slopes (Fig. 5, $F = 3{\cdot}910$, $P < 0{\cdot}05$). Further analysis (Tukey test for multiple comparisons) indicated that the slope for human isolates was lower than all the others. The regression lines derived from the 3 remaining host species shared a common slope, but elevations were significantly different ($F = 9{\cdot}526$, $P < 0{\cdot}001$). A multiple comparison test consequently indicated that the regression line derived from cattle isolates was higher than that derived from sheep or camel isolates.

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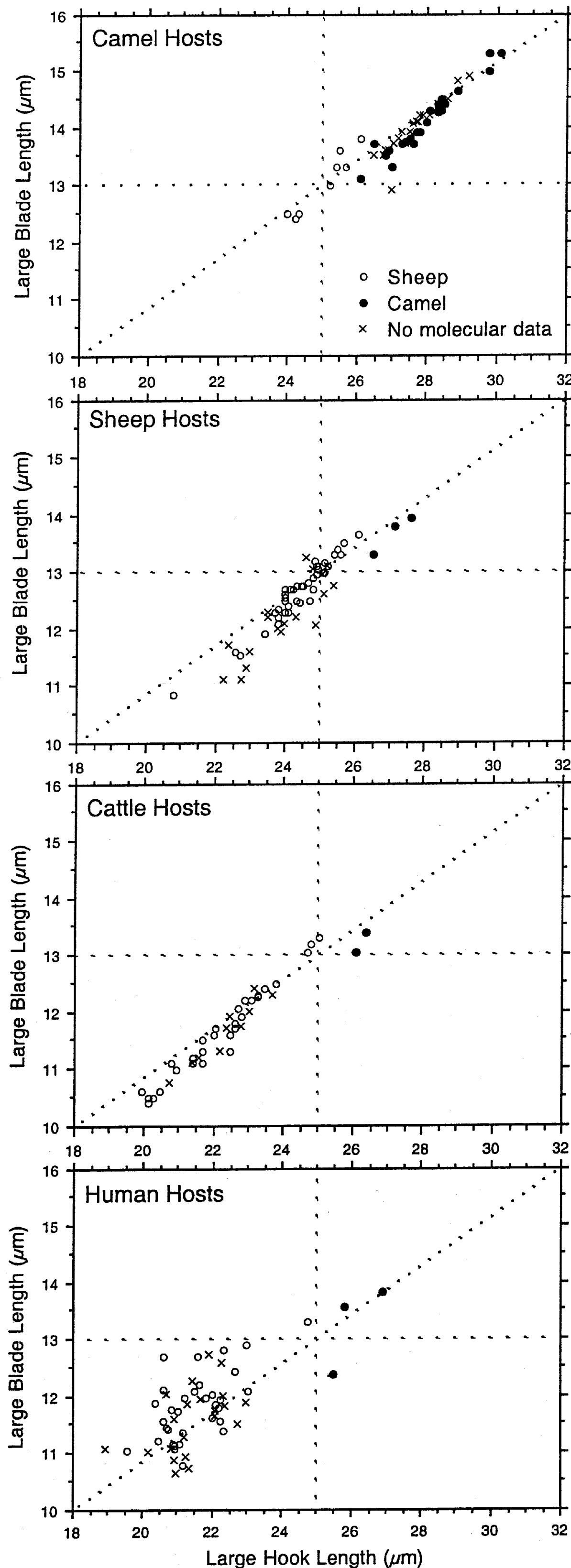

Fig. 4. Scatterplots of large blade length versus large hook length for each host species from which isolates were obtained. Each point represents the mean values of these 2 variables measured on 2 large hooks from each of 10 protoscoleces from a single isolate. Sheep strain isolates, camel strain isolates (as determined by molecular data), and those isolates for which strain was not determined are shown separately.

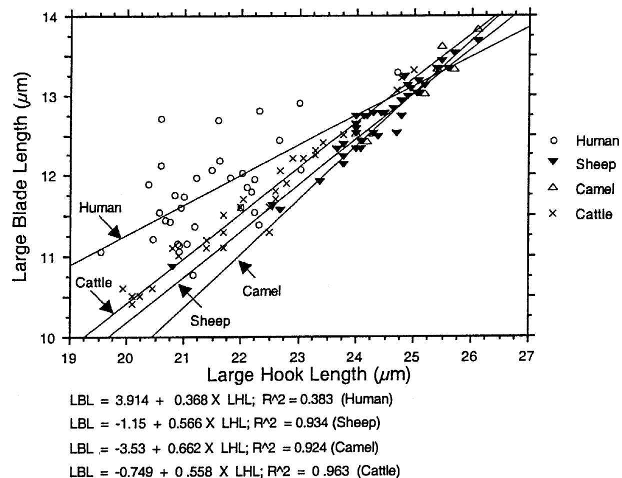

Fig. 5. Scatterplots and regression lines of large blade length–large hook length for the sheep strain isolates. Regression lines for each host are plotted separately.

DISCUSSION

There is now considerable evidence demonstrating the importance of determining the nature and extent of strain/genotypic variation in *Echinococcus* (Thompson, 1995, 2001; Thompson & McManus, 2001). This is particularly significant in endemic regions such as Iran, where more than one species of intermediate host is present and where there is the possibility of interaction between cycles of transmission (Thompson, 1995; Schantz *et al.* 1995). The design and implementation of control programmes is dependent upon such information and, in particular, which cycles of transmission are a risk to human health.

A variety of criteria has been used to characterize isolates of *Echinococcus* (Thompson & McManus, 2001). The practical value of using morphology for differentiating between forms of *Echinococcus* has been demonstrated on numerous occasions (Constantine *et al.* 1993), but the problems of host-induced morphological variation (Hobbs *et al.* 1990), and the lack of readily discernible morphological differences between some strains/genotypes of *E. granulosus* (Eckert *et al.* 1993) have served to emphasize the value of DNA characterization procedures.

In the present study, both morphology and DNA characterization were used to evaluate genotypic variation in a large sample size of *E. granulosus* isolates from sheep, camels, cattle and humans in Iran. Two genotypes, 'sheep' and 'camel' of *E. granulosus* were shown to occur in Iran. This confirms the results of an earlier study conducted using a restricted number of isolates collected from various areas of Iran (Zhang *et al.* 1998).

The sheep strain was the most common genotype of *E. granulosus* in Iran. The majority (92 %) of human cases of CHD were the result of infection with the sheep strain, including the 4 isolates

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collected in Afghanistan, as were most cases of CHD in non-human hosts. However, this study also demonstrated for the first time, in an endemic region where a transmission cycle for perpetuating the camel strain exists, cases of CHD in humans caused by infection with this strain. These results thus serve to reinforce a recent report from Argentina which provided evidence of CHD infection with the camel strain in 4 patients on the basis of molecular data (Rosenzvit *et al*. 1999). These results from Argentina were difficult to interpret in the absence of a local camel–dog cycle. However, the present study has demonstrated that host specificity is not necessarily applicable for all genotypes of *E. granulosus*. For example, the sheep strain infects a wide variety of host species apart from sheep, including camels (Thompson *et al*. 1995). In addition to humans, the camel strain is shown here to cause infection in sheep and cattle. Previous studies have also reported the isolation of the camel genotype from goats (Bowles, Blair & McManus, 1992; Wachira *et al*. 1993). Thus the proposal by Rosenzvit *et al*. (1999) that other species of intermediate hosts could represent reservoirs of the camel strain in Argentina appears likely.

The fact that fewer human cases of CHD were found to be due to infection with the camel strain than the sheep strain (3 of 38) raises questions about the epidemiology of echinococcosis in endemic regions such as Iran. Is the camel strain of less public health significance because humans are less susceptible to this strain, or is there less opportunity for human contact with definitive hosts infected with the camel strain in Iran? The latter seems more likely, since in Iran camels have a more localized geographical distribution than sheep. According to Government figures, the total number of camels in Iran was 143 000 in 1997 and they were distributed in 19 of the 27 provinces of the country (Anon, 1997), including all of those from which human isolates investigated in this study were obtained. Of the total camel population, 77·6% (111 000) is distributed in the eastern half of Iran (Khorassan, Kerman, Sistan-Baluchestan, Yazd and Semnan (Anon, 1997), which is the driest, hottest and least populated part of the country, and climatically represents a less favourable environment for the survival of *Echinococcus* eggs. Further, the prevalence of hydatid disease is higher in sheep than camels, and more sheep are slaughtered (Mobedi *et al*. 1970). However, it is evident from the present study that the 2 cycles must overlap and can interact, since infections caused by the sheep and camel strains were detected in camels and sheep, respectively, as well as in humans. Thus the camel–dog cycle should be targeted in any control campaign.

Results presented here demonstrate that the 2 strains present in Iran and Afghanistan may be differentiated by hook morphology alone. In human hosts, the single measurement of large hook length would be sufficient. All of the human isolates identified as the sheep strain by molecular methods had mean large hook lengths of $<25\ \mu$m, whereas those representing camel strain isolates were $>25\ \mu$m. All 70 isolates from humans and other animals which were not characterized by molecular methods could be 'strain-typed' morphologically.

Hook measurements of both strains were also found to be influenced by the host species. Although sample sizes were small, the hook measurements for the camel strain in all other hosts were consistently at the low end of the normal range found in camel hosts. Sample sizes of sheep-strain isolates in hosts other than sheep were large enough for statistical analysis. Hook measurements in protoscoleces derived from camel hosts were not different from those from sheep, but there were quite distinct differences in human and cattle hosts.

The results of this study lend further support for the revision of the species-level taxonomy of *Echinococcus*. Over the last 10 years, the discriminatory power of biochemical and molecular characterization techniques has provided the data to propose such a revision (Thompson *et al*. 1995). However, this required more detailed comparative studies to be undertaken in endemic regions in order to determine the geographical distribution and uniformity of the putative host-adapted species, as well as evidence of their sympatric occurrence. This and other recent studies (e.g. Zhang *et al*. 1998, and others reviewed by Thompson (2001) and Thompson & McManus, 2001) have provided a sound foundation on which to propose such a revised taxonomy.

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