Molecular study of *Echinococcus* in west-central China

Y. R. YANG[1,2], M. C. ROSENZVIT[3], L. H. ZHANG[1], J. Z. ZHANG[2] *and* D. P. McMANUS[1]*

[1] *Molecular Parasitology Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Brisbane, Q 4006, Australia*
[2] *Ningxia Medical College, Yinchuan, Ningxia Hui Autonomous Region, 75004, PR China*
[3] *Departamento de Parasitologia, Instituto National de Enfermedades Infecciosas (INEI), ANLIS 'Dr. Carlos G. Malbràn', Vélez Sarsfield 563, 1281 Buenos Aires, Argentina*

(*Received 24 February 2005; revised 15 March 2005; accepted 17 March 2005*)

SUMMARY

West-central China is an important endemic focus of both alveolar and cystic echinococcosis where several species of intermediate host are commonly infected with *Echinococcus granulosus* and *E. multilocularis*. Isolates of *E. granulosus* were collected from humans and other animals from different geographical areas of Qinghai, Ningxia, Gansu and Sichuan, and genotyped using the mitochondrial DNA marker ATP synthase subunit 6 gene (*atp6*). The sheep strain (G1 genotype) of *E. granulosus* was shown to be the only genotype present in sheep, cattle, goats, yaks and humans in the study areas. However, some heterogeneity in the *atp6* sequence was evident in a number of the isolates with the most frequent change being a silent substitution (G/A) at position 360 compared with the G1 reference sequence representing isolates collected from the majority of hosts except humans. Two *E. multilocularis* isolates examined also had sequences that varied from each other and from the reference *E. multilocularis atp6* sequence. The genotypic variation we report may reflect phenotypic differences with important consequences in terms of increased host infectivity for hosts by local *Echinococcus* strains, possibly impacting on the epidemiology and control of echinococcosis. Such adaptations may also result in different sensitivity to drugs or increased virulence for hosts that will impede control efforts and even affect vaccination strategies against *Echinococcus*.

Key words: *Echinococcus granulosus*, *Echinococcus multilocularis*, echinococcosis, mitochondrial *atp6* sequence, west-central China.

INTRODUCTION

Echinococcosis is not only one of the most widespread parasitic diseases, but it is also one of the most costly to treat and prevent in terms of public health. The canid intestinal tapeworms *Echinococcus granulosus* and *E. multilocularis* are the causative agents of this zoonosis. There is substantial evidence from many areas of the world that discrete strains of *E. granulosus* occur in different vertebrate hosts, and also 2 strains of *E. multilocularis* have been described (Bowles, Blair and McManus, 1992; Bowles and McManus, 1993*a*; Haag *et al*. 1997; McManus, 2002). Numerous studies have shown that genetic variation within the recognized species of *Echinococcus* spp. may be reflected in characters such as life-cycle pattern, host specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, impacting on the epidemiology, transmission dynamics and control of echinococcosis. Therefore, variation within certain species of *Echinococcus* is currently well-accepted and a number of strains of *E. granulosus* has been characterized using a range of procedures (Bowles *et al*. 1992; Bowles and McManus, 1993*a*, *b*; Rozenzvit *et al*. 1999; McManus *et al*. 2002; McManus, 2002). To date, 10 distinct genotypes within *E. granulosus* have been identified (Bowles, Blair and McManus, 1994; Scott and McManus, 1994; Scott *et al*. 1997; Lavikainen *et al*. 2003). In contrast, *E. multilocularis* appears far less variable than *E. granulosus* as molecular genetic studies have identified very little variation within the genomes of *E. multilocularis* isolates collected from various geographical locations (McManus and Thompson, 2003).

Two *E. granulosus* genotypes have previously been reported in China: the G1 (sheep strain) and G6 (camel strain) genotypes (Zhang *et al*. 1998). *E. granulosus* genotype G1 has widespread distribution globally, and its principal definitive host is the dog, although the fox, dingo, wolf, jackal and hyena have also been reported as definitive hosts (Eckert and Thompson, 1997). The sheep strain has been found to use sheep as its major intermediate host, though other hosts such as goats, camels, buffalo, yaks, moose, cattle, pigs and macropods are also involved (Bowles *et al*. 1992; McManus *et al*. 2002).

In the present study, we selected a fragment of the mitochondrial ATP synthase subunit 6 gene

* Corresponding author: Molecular Parasitology Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Brisbane, Q 4006, Australia. Tel: +61 7 3362 0401. Fax: +61 7 3362 0104. E-mail: donM@qimr.edu.au

Parasitology (2005), **131**, 547–555.
doi:10.1017/S0031182005007973 Printed in the United Kingdom

1, Ningxia Hui Autonomous Region

2, Gansu Province

3, Qinghai Province

4, Sichuan Province

Fig. 1. Study areas: Qinghai, Sichuan, Gansu provinces and Ningxia Hui Autonomous Region in west-central China.

(*atp6*) as a molecular marker to investigate a number of isolates of *E. granulosus* from a range of hosts (sheep, goats, cattle, yaks and humans); two *E. multilocularis* isolates, one from a naturally infected rodent and the other from a human clinical case were also analysed. The geographical origin of the isolates included Sichuan, Qinghai, Gansu and Ningxia, located in west-central China (Fig. 1). These are considered pastoral and semi-pastoral areas, and are recognized as highly endemic areas for both *E. granulosus* and *E. multilocularis* (see Li *et al*. 1985; Hong and Lin, 1987; Craig, Deshan and Zhaoxun, 1991; Craig *et al*. 1992; Shi, 1995; Qiu, Liu and Schantz, 1999).

MATERIALS AND METHODS

Parasites

Hydatid cysts of *E. granulosus* from sheep, goats, cattle and yak were obtained at abattoirs or from the field in Ningxia, Qinghai and Sichuan. Cysts of human origin were collected at surgery in Ningxia. An individual isolate represents parasite material collected from a single hydatid cyst. Cyst contents were aspirated and examined under light microscopy, rinsed in saline, then fixed in 70–95% (v/v) ethanol until used to isolate DNA.

An alveolar echinococcosis (AE) mass was obtained from the Pathology Department, Ningxia Medical College following operation of a patient with liver AE. Host tissues were carefully removed and the sample was cut into small pieces. The sample was ground in saline solution and injected into the abdominal cavity of mice. After several weeks, the mice were sacrificed and AE lesions obtained. Before rinsing, the host tissues were carefully removed and the parasite materials were preserved in 10% formalin until use. Another AE mass was collected from a naturally infected rodent in the field. After transplanting into laboratory mice, AE lesions were obtained several weeks later, rinsed in saline and preserved in 70% (v/v) ethanol until use.

DNA methods

Total *E. granulosus* and *E. multilocularis* genomic DNAs were prepared from 70–95% ethanol

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

Table 1. The host and geographical origin of *Echinococcus* isolates with genotypic identity determined by *atp6* sequencing

		E.g. (G1[a])		
Geographical origin	Host origin	No. of samples, NS[c]	No. of samples, S[d]	*E.m.* (M1[b]) No. of samples[d]
Ningxia	Sheep	13	1	
	Goat		1	
	Human	7	5	1
Gansu	Human	1		
	Mouse			1
Sichuan	Sheep	4	3	
	Yak	6	2	
Qinghai	Sheep	4	9	
	Cattle	4	8	
Total		39	29	2

[a] G1, sheep-dog strain of *Echinococcus granulosus*.
[b] M1, Eurasian strain of *E. multilocularis*.
[c] NS, without substitutions.
[d] S, with substitutions.

preserved isolates using the DNAeasy Tissue Kit (QIAgen, Hilden, Germany) following the kit protocol for DNA isolation from animal tissues. Total *E. multilocularis* genomic DNA was prepared from a 10% formalin-fixed tissue preserved isolate of *E. multilocularis* using the same kit as for the *E. granulosus* samples. However, the tissue sample was washed several times with PBS to remove fixative, after cutting and grounding the tissue with a tissue homogenizer. Then, the DNAeasy protocol for DNA extraction from animal tissues was followed. The genomic DNAs obtained were used as templates for polymerase chain reaction (PCR). A fragment of the *atp6* mitochondrial gene was amplified from each isolate using the following primer pairs: Forward primer EgMF (5′-AAACTGTAGGGTTCATG-TC-3′); reverse primer EgNO2R (5′-CAAAACCC-GAATAATCTATC-3′). The PCR was carried out in a 50 μl reaction mixture containing 50–100 ng of template DNA, 0·2 μmol of each primer, 100 μmol of each dNTP and 1·25 units of *Taq* polymerase (PCR Master Mix, Promega Corp., USA). For PCR amplification, 34 thermal cycles (95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) were used. Amplification products were purified from primers, nucleotides, polymerases and salts using the QIAquick™ Gel Extraction Kit (QIAGEN GmbH, Hilden, Germeny) or QIAquick™ PCR purification kit according to the manufacturer's recommendations. Then, the PCR amplicons were sequenced using an ABI Prism® BigDye™ terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) and gels were run on an ABI PRISM 377 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Sequences were aligned using the MacVector 7.2 program in a Macintosh computer. Reference *atp6* sequences from *E. granulosus* common sheep (G1 genotype, GenBank No. AF297617) and the camel (G6 genotype, GenBank No. AJ237637) strains and *E. multilocularis* (GenBank No. AF018440) were used for comparative purposes.

Phylogeny construction

Phylogenies were constructed by neighbor-joining and UPGMA, using the Kimura-2-parameter model, and tested by booststrap with 1000 replicates, using MEGA version 2.1 (Kumar *et al*. 2001), and by the maximum parsimony method using the program PAUP* version 4.0b4a.

RESULTS

Host and geographical origins of parasites

The *E. granulosus* samples examined by microscopy revealed that all isolates from sheep, goats, cattle, humans and yaks had protoscoleces and/or germinal membranes present. All samples were analysed. Two *E. multilocularis* isolates were also analysed. The geographical and host origins of all the isolates examined are listed in Tables 1 and 2.

Sequence analysis of the atp6 *gene*

In regards to *E. granulosus*, there were 21 sheep, 8 human, 6 yak and 4 cattle isolates that shared complete identity with the reference *atp6* sequence for the G1 genotype (Table 1). There were 29 isolates with minor variation in the *atp6* sequence compared with the reference G1 genotype; these substitutions

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

Table 2. Substitutions in the *atp6* gene for the *E. granulosus* sheep strain (G1 genotype) and *E. multilocularis* isolates investigated in this study

Genotype	Seq.[a]	Sam.[b]	Position of substitution	Host	Geographical origin
E.g. (G1)	G1V1	1	23(G/T); 24(T/G); 26(C/T)		
	G1V2	1	43(T/A); 73(T/C); 360(G/A)	Sheep	Sichuan
	G1V3	1	360(G/A)		
		2	360(G/A)	Yak	
		7	360(G/A)	Sheep	
		6	360(G/A)	Cattle	Qinghai
		1	360(G/A)	Goat	Ningxia
	G1V4	1	92(C/T) 360(G/A)		
	G1V5	1	265(T/C)	Sheep	Qinghai
	G1V6	3	252(G/A) 360(G/A)	Cattle	
	G1V7	1	43(T/A) 360(G/A)	Sheep	
	G1V8	3	91(G/A) 117(T/C)		
	G1V9	1	117(T/C)	Human	Ningxia
	G1V10	1	100(T/C)		
E.m. (M1)	EmV1	1	47(C/T); 60(T/G); 174(T/G); 258(A/G); 498(T/C)		
	EmV2	1	47(C/T); 60(T/G)	Mouse	Gansu

[a] Seq., Sequences with substitutions, coded from G1V1 to G1V10 for the *E. granulosus isolates*, and EmV1 and EmV2 for the *E. multilocularis* isolates.
[b] Sam., Number of isolates.

were distributed across the length of the 513 bp fragment. The sequence analysis allowed the definition of 10 different types of *atp6* sequence represented by variation at 13 different nucleotide positions listed in Table 2 and shown in Fig. 2. Of these, there were 23 isolates with variation at position 360 of the *atp6* gene, of which 17 isolates had a single point change (G/A). Six isolates also had substitutions at positions 43, 73, 92 and 252. Isolates with a silent substitution at position 360 originated from all hosts, except human, from all geographical locations investigated in this study. The remaining isolates had substitutions at random sites along the *atp6* gene. Nucleotide substitutions at positions 23, 24, 27, 43, 73, 91 and 92 were non-synonymous, while all other substitutions did not produce amino acid changes.

The same substitutions could be found in isolates from different host origins and geographical locations, such as sequence G1V3. Furthermore, the same sequence changes were evident in 3 human isolates (sequence G1V8) surgically removed from different anatomical locations but not in the two other human isolates from Ningxia (Table 2).

The two *E. multilocularis* isolates had different substitutions compared with the reference *E. multilocularis atp6* sequence in GenBank (Table 2). Five changes were apparent in 516 nucleotides of the *atp6* gene in the human isolate at positions 47, 60, 174, 258 and 498; two changes, at positions 47 and 60, were evident in the mouse isolate. Only the changes at positions 47 and 60 were non-synonymous; the other changes were silent.

All the *Echinococcus atp6* sequences obtained were translated into open reading frames thus eliminating the presence of pseudogenes.

Phylogenetic analysis

Phylogenetic analysis by the neighbor-joining, UPGMA and Maximum Parsimony methods, using the published *atp6* sequences from *E. granulosus* sheep strain (G1), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7); cervid strain (G8), *E. multilocularis* (Em), *E. oligarthrus* (Eo) and *Taenia solium* (Ts) showed that all the *E. granulosus* isolates analysed grouped with G1; there was strong (100%) bootstrap support for the clustering of all samples from China with a reference sample representing genotype G1, to the exclusion of all other genotypes and species of *Echinococcus* examined in the present study. Both *E. multilocularis* isolates clustered with the *E. multilocularis atp6* reference sequence, with high (100%) boostrap value. The topology obtained by the neighbor-joining method is shown in Fig. 3.

DISCUSSION

Four species are currently recognized in the genus *Echinococcus*, namely *Echinococcus granulosus*, *E. multilocularis*, *E. vogeli* and *E. oligarthrus* although revision of the genus has been recommended (Thompson and McManus, 2002). *E. granulosus* and *E. multilocularis* are of public health significance because of their world-wide distribution (Thompson

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

```
G1 ATP6.txt      1 ATGGTTGTTG TTAATGATTT TTGTTCTTTA ATTGGTTTGG TTTATATGTT  50
G6 ATP6.txt      1 ...A....G. .......... .G........ ..G...CGAT .....G....  50
G1V0             1 .......... .......... .......... .......... ..........  50
G1V1             1 .......... .......... ..TG.T.... .......... ..........  50
G1V2             1 .......... .......... .......... .......... ..A.......  50
G1V3             1 .......... .......... .......... .......... ..........  50
G1V4             1 .......... .......... .......... .......... ..........  50
G1V5             1 .......... .......... .......... .......... ..........  50
G1V6             1 .......... .......... .......... .......... ..........  50
G1V7             1 .......... .......... .......... .......... ..A.......  50
G1V8             1 .......... .......... .......... .......... ..........  50
G1V9             1 .......... .......... .......... .......... ..........  50
G1V10            1 .......... .......... .......... .......... ..........  50

G1 ATP6.txt     51 GGTGTTTGGT CGTGTGTCAT ATTACTATTT TGTTTTATTG GCGTTGGTAT 100
G6 ATP6.txt     51 ......AAAG G...C...G. ....T..... .........A AT......T. 100
G1V0            51 .......... .......... .......... .......... .......... 100
G1V1            51 .......... .......... .......... .......... .......... 100
G1V2            51 .......... .......... ..C....... .......... .......... 100
G1V3            51 .......... .......... .......... .......... .......... 100
G1V4            51 .......... .......... .......... .......... .T........ 100
G1V5            51 .......... .......... .......... .......... .......... 100
G1V6            51 .......... .......... .......... .......... .......... 100
G1V7            51 .......... .......... .......... .......... .......... 100
G1V8            51 .......... .......... .......... .......... A......... 100
G1V9            51 .......... .......... .......... .......... .......... 100
G1V10           51 .......... .......... .......... .......... .........C 100

G1 ATP6.txt    101 TAATGTGGTT TATGGTTTAT CGTTTGCCAT ATTGTTATAG GGTTTATTTG 150
G6 ATP6.txt    101 .GT.T..A.. .T.A...... .....A..T. .......... .T.......A 150
G1V0           101 .......... .......... .......... .......... .......... 150
G1V1           101 .......... .......... .......... .......... .......... 150
G1V2           101 .......... .......... .......... .......... .......... 150
G1V3           101 .......... .......... .......... .......... .......... 150
G1V4           101 .......... .......... .......... .......... .......... 150
G1V5           101 .......... .......... .......... .......... .......... 150
G1V6           101 .......... .......... .......... .......... .......... 150
G1V7           101 .......... .......... .......... .......... .......... 150
G1V8           101 .......... ......C... .......... .......... .......... 150
G1V9           101 .......... ......C... .......... .......... .......... 150
G1V10          101 .......... .......... .......... .......... .......... 150

G1 ATP6.txt    151 TTTTCCGTTT TTTTGTTTTG TGTGGTTTTT GTGATGTTTG TGTCGTTATT 200
G6 ATP6.txt    151 ....TT.... .......CG. .......... ..T....... ....A..G.. 200
G1V0           151 .......... .......... .......... .......... .......... 200
G1V1           151 .......... .......... .......... .......... .......... 200
G1V2           151 .......... .......... .......... .......... .......... 200
G1V3           151 .......... .......... .......... .......... .......... 200
G1V4           151 .......... .......... .......... .......... .......... 200
G1V5           151 .......... .......... .......... .......... .......... 200
G1V6           151 .......... .......... .......... .......... .......... 200
G1V7           151 .......... .......... .......... .......... .......... 200
G1V8           151 .......... .......... .......... .......... .......... 200
G1V9           151 .......... .......... .......... .......... .......... 200
G1V10          151 .......... .......... .......... .......... .......... 200
```

Fig. 2. (*cont.*)

and McManus, 2001). Epidemiological data have shown that geo-environmental factors and adaptations to different host species may induce the selection of genetic mutations in *Echinococcus*. Variation within certain species of *Echinococcus* is now a well-accepted phenomenon and that this intra-specific variation may play an important role with regard to differences in infectivity, especially to humans, epidemiology and control (McManus, Ding and Bowles, 1994; Eckert and Thompson, 1997; Thompson and McManus, 2001).

A number of previous studies have shown the existence of 10 distinct genotypes of *E. granulosus*, based on morphological characters, intermediate host specificity and/or genetic analysis of mitochondrial and nuclear DNA (Bowles *et al*. 1992; Bowles and McManus, 1993*a*, *b*; Thompson, Lymbery and Constantine, 1995; Eckert and Thompson, 1997; Thompson and McManus, 2001; Lavikainen *et al*. 2003). Several host-adapted strains of *E. granulosus* exist, the majority of which are geographically widely distributed (Thompson and McManus,

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

```
G1 ATP6.txt   201 TATGTGTCGT ATTTTTAATA AAGTTAATGG ATTTTTTGCT TGTTTTGTCC 250
G6 ATP6.txt   201 .......... G.G....G.. ........A. .......... A....C..T. 250
G1V0          201 .......... .......... .......... .......... .......... 250
G1V1          201 .......... .......... .......... .......... .......... 250
G1V2          201 .......... .......... .......... .......... .......... 250
G1V3          201 .......... .......... .......... .......... .......... 250
G1V4          201 .......... .......... .......... .......... .......... 250
G1V5          201 .......... .......... .......... .......... .......... 250
G1V6          201 .......... .......... .......... .......... .......... 250
G1V7          201 .......... .......... .......... .......... .......... 250
G1V8          201 .......... .......... .......... .......... .......... 250
G1V9          201 .......... .......... .......... .......... .......... 250
G1V10         201 .......... .......... .......... .......... .......... 250

G1 ATP6.txt   251 CGCTAGGAAC TCCTTTATGG ATATGTTTTT TAGTGTGCTT GGCCGAGTCT 300
G6 ATP6.txt   251 ..T.G..T.. A.....G.AC .........G .......TG. .......... 300
G1V0          251 .......... .......... .......... .......... .......... 300
G1V1          251 .......... .......... .......... .......... .......... 300
G1V2          251 .......... .......... .......... .......... .......... 300
G1V3          251 .......... .......... .......... .......... .......... 300
G1V4          251 .......... .......... .......... .......... .......... 300
G1V5          251 .......... ....C..... .......... .......... .......... 300
G1V6          251 .A........ .......... .......... .......... .......... 300
G1V7          251 .......... .......... .......... .......... .......... 300
G1V8          251 .......... .......... .......... .......... .......... 300
G1V9          251 .......... .......... .......... .......... .......... 300
G1V10         251 .......... .......... .......... .......... .......... 300

G1 ATP6.txt   301 ATTAGTTATG TTATACGTCC TGTTGTATTG GTTTTGCGTC CTTTTATTAA 350
G6 ATP6.txt   301 .......... ....T..... .A.A..G... A......... .......... 350
G1V0          301 .......... .......... .......... .......... .......... 350
G1V1          301 .......... .......... .......... .......... .......... 350
G1V2          301 .......... .......... .......... .......... .......... 350
G1V3          301 .......... .......... .......... .......... .......... 350
G1V4          301 .......... .......... .......... .......... .......... 350
G1V5          301 .......... .......... .......... .......... .......... 350
G1V6          301 .......... .......... .......... .......... .......... 350
G1V7          301 .......... .......... .......... .......... .......... 350
G1V8          301 .......... .......... .......... .......... .......... 350
G1V9          301 .......... .......... .......... .......... .......... 350
G1V10         301 .......... .......... .......... .......... .......... 350

G1 ATP6.txt   351 TATTAGGTTG GGGTGTTTTG GTGCAGTGGC ATTGGGTAAT TTGTGTTTTG 400
G6 ATP6.txt   351 .........A .......... ....T..T.. ...A...... .....C.... 400
G1V0          351 .......... .......... .......... .......... .......... 400
G1V1          351 .......... .......... .......... .......... .......... 400
G1V2          351 .........A .......... .......... .......... .......... 400
G1V3          351 .........A .......... .......... .......... .......... 400
G1V4          351 .........A .......... .......... .......... .......... 400
G1V5          351 .......... .......... .......... .......... .......... 400
G1V6          351 .........A .......... .......... .......... .......... 400
G1V7          351 .........A .......... .......... .......... .......... 400
G1V8          351 .......... .......... .......... .......... .......... 400
G1V9          351 .......... .......... .......... .......... .......... 400
G1V10         351 .......... .......... .......... .......... .......... 400
```

Fig. 2. (*cont.*)

2001; McManus, 2002). On the other hand, genetic diversity within *E. multilocularis* is comparatively very low, though there is some evidence of variation in morphology, pathogenicity and host specificity between strains from north America and Eurasia (Rinder *et al.* 1997; Thompson and McManus, 2002).

China is hyper-endemic for both cystic and alveolar echinococcosis, especially in the north-western region that includes Ningxia, Xinjiang, Gansu, Qinghia, Sichuan, Tibet and Inner Mongolia (Jiang, 2002; Vuitton *et al.* 2003). In these areas, the extensive range of environmental conditions, domestic livestock husbandry, and a variety of pastoral or semi-pastoral areas, provides inherent conditions whereby host adapted variants of *Echinococcus* could arise. Previous epidemiological data have demonstrated that the eco-environmental, social, religious and cultural background as well as the behaviour of local inhabitants (French and Nelson, 1982; MacPherson, Zeyhle and Romig, 1984; Craig, Zeyhle and Romig, 1986; Seimenis, 2003) may not only influence transmission, but also form or

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

```
G1 ATP6.txt   401 TTAATTGTTG ATGAGGGTTG GTTTTAGTTG GATTGTTTTT TTATGAAGTT 450
G6 ATP6.txt   401 .A.G...... G..G.T.... A........T T......... ......G... 450
G1V0          401 .......... .......... .......... .......... .......... 450
G1V1          401 .......... .......... .......... .......... .......... 450
G1V2          401 .......... .......... .......... .......... .......... 450
G1V3          401 .......... .......... .......... .......... .......... 450
G1V4          401 .......... .......... .......... .......... .......... 450
G1V5          401 .......... .......... .......... .......... .......... 450
G1V6          401 .......... .......... .......... .......... .......... 450
G1V7          401 .......... .......... .......... .......... .......... 450
G1V8          401 .......... .......... .......... .......... .......... 450
G1V9          401 .......... .......... .......... .......... .......... 450
G1V10         401 .......... .......... .......... .......... .......... 450

G1 ATP6.txt   451 TTTGTTGTAT TAATCCATTG GTACATTGTG TCTAGGATTT TAGATTTTTC 500
G6 ATP6.txt   451 .......CG. C......... A.TT...... A......... .G........ 500
G1V0          451 .......... .......... .......... .......... .......... 500
G1V1          451 .......... .......... .......... .......... .......... 500
G1V2          451 .......... .......... .......... .......... .......... 500
G1V3          451 .......... .......... .......... .......... .......... 500
G1V4          451 .......... .......... .......... .......... .......... 500
G1V5          451 .......... .......... .......... .......... .......... 500
G1V6          451 .......... .......... .......... .......... .......... 500
G1V7          451 .......... .......... .......... .......... .......... 500
G1V8          451 .......... .......... .......... .......... .......... 500
G1V9          451 .......... .......... .......... .......... .......... 500
G1V10         451 .......... .......... .......... .......... .......... 500

G1 ATP6.txt   501 AGTCGATCAT TAG 513
G6 ATP6.txt   501 ...T...... ... 513
G1V0          501 .......... ... 513
G1V1          501 .......... ... 513
G1V2          501 .......... ... 513
G1V3          501 .......... ... 513
G1V4          501 .......... ... 513
G1V5          501 .......... ... 513
G1V6          501 .......... ... 513
G1V7          501 .......... ... 513
G1V8          501 .......... ... 513
G1V9          501 .......... ... 513
G1V10         501 .......... ... 513
```

Fig. 2. Nucleotide sequences of a fragment (513 bp) of the *Echinococcus granulosus* mitochondrial *atp6* gene for 68 isolates analysed using ClustalW (multiple sequence alignment), aligned with the published reference *atp6* gene sequence of the G1 genotype (GenBank™/EBI Data Bank Accession number, AF297617) (top line). The published reference *atp6* gene sequence for the G6 genotype (second line) (Rosenzvit *et al*. 1999) is also shown as a comparison. A dot indicates a nucleotide that is conserved relative to the published G1 sequence. An isolate coded with the prefix G1V0 indicates that the sequence is identical to the published G1 sequence. Isolates coded G1V1–G1V10 indicate the genotype is designated as G1 but some substitutions are present (Table 2).

maintain local perpetuation of the life-cycle of the parasite by adaptive selection (McManus and Thompson, 2003; Rausch, 2003).

Polymerase chain reaction (PCR) combined with direct sequencing of mitochondrial genes, including *atp6*, have been successfully used by us and others for *Echinococcus* strain and species discrimination (see McManus, 2002; Lavikainen *et al*. 2003; Xiao *et al*. 2005). In the present study, differences in the *atp6* gene were used to evaluate genotypic variation in 68 *E. granulosus* isolates from sheep, goats, yaks, cattle and humans from west-central China including Qinghai, Sichuan, Gansu and Ningxia and two *E. multilocularis* isolates from Ningxia and Gansu. It is noteworthy that although the human isolate of *E. multilocularis* examined was analysed using formalin-fixed tissue, the 516 bp *atp6* fragment was successfully obtained by PCR and sequenced. Similar to previous reports from this area (McManus, Ding and Bowles, 1994; Zhang *et al*. 1998), the sheep strain (G1) of *E. granulosus* was shown to be present, although the camel strain (G6) that also occurs in Xinjiang (Zhang *et al*. 1999) was not identified in the current survey. It is noteworthy that almost half of the isolates examined had sequence substitutions at 11 different positions within the *atp6* gene within this strain. Of these, a substitution at position 360 occurred in 23 isolates from all geographical locations investigated and from the majority of hosts, except humans. Both *E. multilocularis* isolates examined also had sequences that varied from each other and from the reference (Eurasian strain) *E. multilocularis atp6* sequence. Minor genetic variation in the cytochrome c oxidase subunit 1 (*cox 1*) gene of the sheep strain

https://doi.org/10.1017/S0031182005007973 Published online by Cambridge University Press

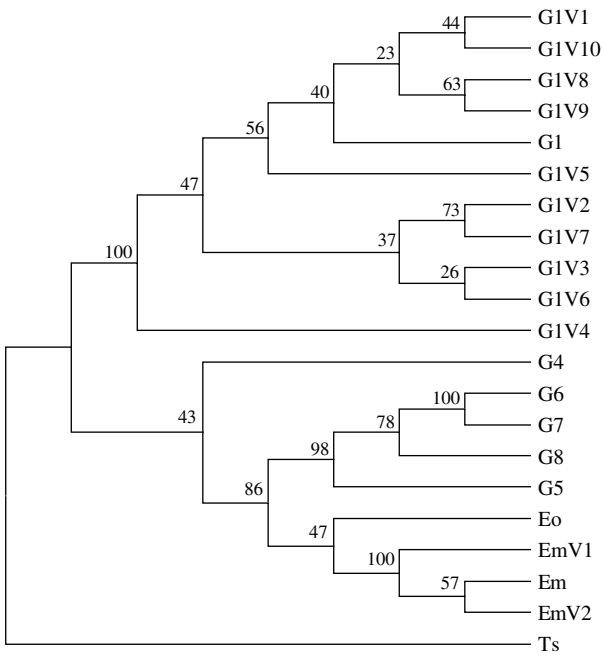

Fig. 3. Phylogenetic tree constructed using the neighbor-joining method on *atp6* sequences obtained from *Echinococcus granulosus* and *E. multilocularis* Chinese isolates and the reported *atp6* sequences from *E. granulosus* sheep strain (G1), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7); cervid strain (G8), *E. multilocularis* (Em), *E. oligarthrus* (Eo) and *Taenia solium* (Ts). Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of the data. G1V1-V10 are *atp6* sequences obtained for some of the analysed isolates differing in 1–3 positions from the G1 *atp6* reference sequence. EmV1 and EmV2 are the *atp6* sequences obtained from the *E. multilocularis* isolates, differing from *E. multilocularis* reference *atp6* sequence at 5 and 2 positions, respectively.

has been reported by Kamenetzky *et al*. (2002) following analysis of *E. granulosus* isolates collected from different host species and sites in Argentina. The existence of such sequence heterogeneity within isolates is in concordance with results of single-strand conformation polymorphism (SSCP) analysis of mitochondrial genes of *Echinococcus* (Gasser, Zhu and McManus, 1988). It is well accepted that genotypic variation may reflect phenotypic differences with important consequences in terms of increased opportunities for infectivity of hosts by local *Echinococcus* strains, possibly impacting on the epidemiology and control of echinococcosis (Thompson and McManus, 2001). Such adaptations may also result in different sensitivity to drugs or increased virulence for hosts that will impede control efforts and even affect vaccination strategies against *Echinococcus* (Bessonov *et al*. 1998).

We thank Dr Zhao Yu Ming, Lanzhou Medical College, Ms Li Yanbin and Dr Wei Yanqin. Ningxia Medical College and Dr Liu Xue Ting, the Second Provincial Hospital of Ningxia Hui Autonomous Region who kindly helped in the collection or provision of *Echinococcus* samples for analaysis. M.R. was recipient of a Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) fellowship.

REFERENCES

Bessonov, A. S., Gorokhov, V. V., Darchenkova, N. N., Romanenko, N. A., Zavoikin, V. D., Artamoshin, A. S., Zelia, O. P., Novosiltsev, G. I., Lebedeva, M. N., Khalilullina, A. S., Frolova, A. A. and Sergiev, V. P. (1998). [The epizootic situation in regions of the North in Russia and the risk of human infection with biohelminthiases.] *Meditsinskaia Parazitologiia i Parazitarnye Bolezni* **3**, 19–23.

Bowles, J., Blair, D. and McManus, D. P. (1992). Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology* **54**, 165–173.

Bowles, J., Blair, D. and McManus, D. P. (1994). Molecular genetic characterization of the cervid strain ('northern form') of *Echinococcus granulosus*. *Parasitology* **109**, 215–221.

Bowles, J. and McManus, D. P. (1993*a*). NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. *International Journal for Parasitology* **23**, 969–972.

Bowles, J. and McManus, D. P. (1993*b*). Molecular variation in *Echinococcus*. *Acta Tropica* **53**, 291–305.

Craig, P. S., Deshan, L., MacPherson, C. N., Dazhong, S., Reynolds, D., Barnish, G., Gottstein, B. and Zhirong, W. (1992). A large focus of alveolar echinococcosis in central China. *Lancet* **340**, 826–831.

Craig, P. S., Deshan, L. and Zhaoxun, D. (1991). Hydatid disease in China. *Parasitology Today* **7**, 46–50.

Craig, P. S., Zeyhle, E. and Romig, T. (1986). Hydatid disease: research and control in Turkana. II. The role of immunological techniques for the diagnosis of hydatid disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 183–192.

Eckert, J. and Thompson, R. C. (1997). Intraspecific variation of *Echinococcus granulosus* and related species with emphasis on their infectivity to humans. *Acta Tropica* **64**, 19–34.

French, C. M. and Nelson, G. S. (1982). Hydatid disease in the Turkana District of Kenya II. A study in medical geography. *Annals of Tropical Medicine and Parasitology* **76**, 439–457.

Gasser, R. B., Zhu, X. and McManus, D. P. (1988). Display of sequence variation in PCR-amplified mitochondrial DNA regions of *Echinococcus* by single-strand conformation polymorphism. *Acta Tropica* **71**, 107–115.

Haag, K. L., Zaha, A., Araujo, A. M. and Gottstein, B. (1997). Reduced genetic variability within coding and non-coding regions of the *Echinococcus multilocularis* genome. *Parasitology* **115** (Suppl.), S521–S529.

Hong, L. X. and Lin, G. Y. (1987). Studies on the development and histopathology of alveolar cestode of *Echinococcus multilocularis* in human and animal hosts. *Endemic Diseases Bulletin* (*in Chinese*) **2**, 51–53.

Jiang, C. (2002). Today's regional distribution of echinococcosis in China. *Chinese Medical Journal* **115**, 1244–1247.

Kamenetzky, L., Gutierrez, A. M., Canova, S. G., Haag, K. L., Guarnera, E. A., Parra, A., Garcia, G. E. and Rosenzvit, M. C. (2002). Several strains of *Echinoccocus granulosus* infect livestock and humans in Argentina. *Infection, Genetics and Evolution*, **2**, 129–136.

Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Lavikainen, A., Lehtinen, M. J., Meri, T., Hirvela-Koski, V. and Meri, S. (2003). Molecular genetic characterization of Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. *Parasitology* **127**, 207–215.

Li, W. X., Zhang, G. C., Lin, Y. G. and Hong, L. X. (1985). Natural animal hosts of adult *Echinococcus* multilocularis and morphological studies in China. *Acta Zoonologica Clinica* (*in Chinese*) **31**, 365–367.

Macpherson, C. N., Zeyhle, E. and Romig, T. (1984). An *Echinococcus* pilot control programme for northwest Turkana, Kenya. *Annals of Tropical Medicine and Parasitology* **78**, 188–192.

McManus, D. P. (2002). The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96** (Suppl. 1), S151–S157.

McManus, D. P., Ding, Z. and Bowles, J. (1994). A molecular genetic survey indicates the presence of a single, homogeneous strain of *Echinococcus granulosus* in northwestern China. *Acta Tropica* **56**, 7–14.

McManus, D. P. and Thompson, R. C. (**2003**). Molecular epidemiology of cystic echinococcosis. *Parasitology* **127** (Suppl.), S37–S51.

McManus, D. P., Zhang, L., Castrodale, L. J., Le, T. H., Pearson, M. and Blair, D. (2002) Short report: molecular genetic characterization of an unusually severe case of hydatid disease in Alaska caused by the cervid strain of *Echinococcus granulosus*. *American Journal of Tropical Medicine and Hygiene* **67**, 296–298.

Qiu, J. M., Liu, F. J. and Schantz, P. M. (1999). Epidemiology of echinococcosis in a Tibetan community in western Sichuan. In *Proceedings of the Workshop of Echinococcosis Control Strategy in China* (*in Chinese*). pp. 168, Institute for Endemic Disease Research and Control, Urumqi, Xinjiang.

Rausch, R. L. (2003). Cystic echinococcosis in the Arctic and Sub-Arctic. *Parasitology* **127** (Suppl.), S73–S85.

Rinder, H., Rausch, R. L., Takahashi, K., Kopp, H., Thomschke, A. and Loscher, T. (1997). Limited range of genetic variation in *Echinococcus multilocularis*. *Journal of Parasitology* **83**, 1045–1050.

Rozenzvit, M. C., Zhang, L. H., Kamenetzky, L., Canova, S. G., Guarnera, E. A. and McManus, D. P. (1999). Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina. *Parasitology* **118**, S523–S530.

Scott, J. C and McManus, D. P. (1994). The random amplification of polymorphic DNA can discriminate species and strains of *Echinococcus*. *Tropical Medicine and Parasitology* **45**, 1–4.

Scott, J. C., Stefaniak, J., Pawlowski, Z. S. and McManus, D. P. (1997). Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*. *Parasitology* **114**, 37–43.

Seimenis, A. (2003). Overview of the epidemiological situation on echinococcosis in the Mediterranean region. *Acta Tropica* **85**, 191–195.

Shi, D. (1995). The first report of *Echinococcus multilocularis* in domestic dogs in Zhang County of Gansu Province. *Chinese Medicine Journal* **108**, 615–617.

Thompson, R. C. A. and McManus, D. P. (2001). Aetiology: parasites and life-cycles. In *WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern* (ed. Eckert, J., Gemmell, M. A., Meslin, F. X. and Pawlowski, Z. S.), pp. 1–9. World Organisation for Animal Health, Paris, France.

Thompson, R. C., Lymbery, A. J. and Constantine, C. C. (1995). Variation in *Echinococcus*: towards a taxonomic revision of the genus. *Advances in Parasitology* **35**, 145–176.

Thompson, R. C. and McManus, D. P. (2002). Towards a taxonomic revision of the genus *Echinococcus*. *Trends in Parasitology* **18**, 452–457.

Vuitton, D. A., Zhou, H., Bresson-Hadni, S., Wang, Q., Piarroux, M., Raoul, F. and Giraudoux, P. (2003). Epidemiology of alveolar echinococcosis with particular reference to China and Europe. *Parasitology* **127** (Suppl.), S87–S107.

Xiao, N., Qiu, J., Nakao, M., Li, T., Yang, W., Chen, X., Schantz, P. M., Craig, P. S. and Ito, A. (2005). *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pike in China. *International Journal for Parasitology* (in the Press).

Zhang, L. H., Chai, J. J., Jiao, W., Osman, Y. and McManus, D. P. (1998). Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of *Echinococcus granulosus* in north-western China. *Parasitology* **116**, 29–33.

Zhang, L., Gasser, R. B., Zhu, X. and McManus, D. P. (1999). Screening for different genotypes of *Echinococcus granulosus* within China and Argentina by single-strand conformation polymorphism (SSCP) analysis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**, 329–334.