Table 1. Geographic origin and hosts of Peruvian isolates of *Echinococcus* identified by the DNA sequencing of mitochondrial *cox1* gene

Geographic origin	*E. granulosus* sensu stricto						*E. canadensis*					Total
							G6			G7		
	Sh	Ca	Pi	Go	Hu	Total	Go	Hu	Total	Pi	Total	
Cajamarca	-	7	-	-	-	7	-	-	0	-	0	7
Ancash	-	1	-	-	-	1	-	-	0	-	0	1
Ayacucho	2	4	-	-	-	6	-	-	0	-	0	6
Lima	13	2	4	1	1	21	-	1	1	8	8	30
Junín	-	-	-	-	1	1	-	-	0	-	0	1
Huancavelica	-	-	-	-	2	2	-	-	0	-	0	2
Ica	17	-	-	-	-	17	2	-	2	-	0	19
Moquegua	-	-	-	-	-	0	3	-	3	-	0	3
Puno	-	2	-	-	-	2	-	-	0	-	0	2
Total	32	16	4	1	4	57	5	1	6	8	8	71

Hosts are sheep (Sh), cattle (Ca), pigs (Pi), goats (Go), and humans (Hu).

Figure

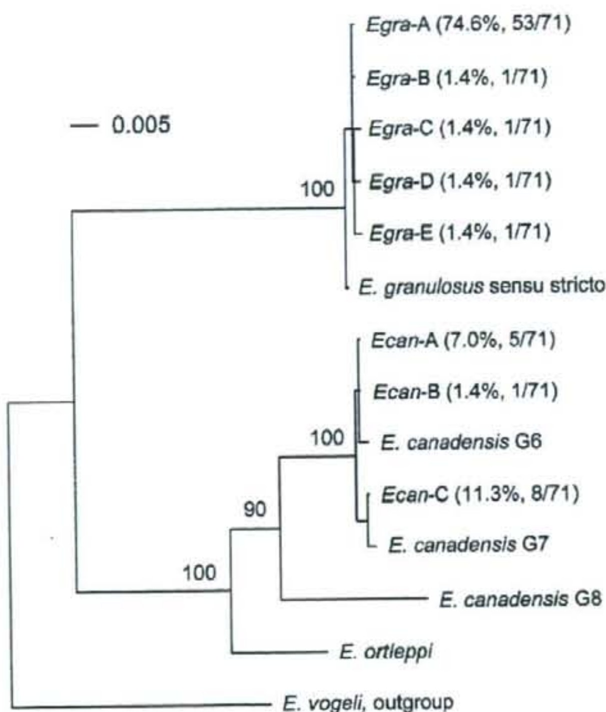

Fig. 1. The neighbor-joining tree of Peruvian *Echinococcus* spp. constructed by using partial DNA sequences of mitochondrial *cox1* gene. *Egra*-A to *Egra*-E and *Ecan*-A to *Ecan*-C are individual haplotypes found in Peruvian isolates. The frequencies of the haplotypes are shown in parenthesis. The tree includes the representative sequences of *E. granulosus* sensu stricto (database accession no. **AF297617**), the G6 genotype of *E. canadensis* (**AB208063**), the G7 genotype of *E. canadensis* (**AB235847**), the G8 genotype of *E. canadensis* (**AB235848**), *E. ortleppi* (**AB235846**) and *E. vogeli* (**AB208546**). Bootstrap values (%) are shown above branches. The scale bar represents the estimated number of nucleotide substitutions per site.

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Short Report: Genetic Analysis of *Echinococcus multilocularis* Originating from a Patient with Alveolar Echinococcosis Occurring in Minnesota in 1977

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Abstract. To date, only a single proven case of autochthonous human alveolar echinococcosis has been recorded in Minnesota in 1977. At that time, echinococcal lesions removed from the patient were experimentally inoculated into voles, and the parasite materials obtained from the voles were preserved as histopathologic specimens for 30 years. In this study, retrospective genetic analysis of larval *Echinococcus multilocularis* originating in the human case was performed using the histopathologic specimens. DNA was extracted from the hematoxylin and eosin–stained specimens, and mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was amplified by polymerase chain reaction. Subsequently, 20 small fragments (100–216 bp) covering almost the entire sequences (97%) of the *cox1* were successfully amplified, and the nucleotide sequence analysis showed that the *E. multilocularis* isolate from Minnesota was almost identical to an isolate from South Dakota rather than isolates from contiguous Alaska.

Alveolar echinococcosis (AE) in humans is a potentially lethal parasitic disease caused by the larval stage (metacestode) of the fox tapeworm, *Echinococcus multilocularis* and occurs in most of the northern hemisphere, including central Europe, most of northern and central Eurasia, and parts of North America.[1] The metacestode proliferates like a tumor in various organs, mainly liver, and develops into multivesiculated lesions. Clinical symptoms such as jaundice, epigastric pain, fatigue, and/or hepatomegaly may appear after an asymptomatic period of 5–15 years, and treatment may be difficult despite a variety of surgical and chemotherapeutic approaches.[2]

In North America, *E. multilocularis* is distributed in two distinct geographic regions: the northern tundra zone (western Alaska, Arctic islands such as St. Lawrence Island, and Canadian Arctic Archipelago), and northern central America, including three provinces of Canada and 13 contiguous states of the United States.[1,3,4] In Alaska, the tapeworm infections are perpetuated in a sylvatic cycle with carnivores, mainly arctic foxes (*Alopex lagopus*), red foxes (*Vulpes vulpes*) as definitive hosts, and small rodents such as voles (*Microtus oeconomus*) and brown lemming (*Lemmus trimucronatus*) as intermediate hosts.[3] Domestic dogs can also harbor the tapeworm. In central North America, red foxes, grey foxes (*Urocyon cinereoargenteus*), and coyotes (*Canis latrans*) as definitive hosts and red-backed voles (*Clethrionomys rutilus*), meadow voles (*Microtus pennsylvanicus*), bushy-tailed woodrat (*Neotoma cinerea*), and deer mouse (*Peromyscus maniculatus*) as intermediate hosts are involved in the completion of the parasitic cycle.[4]

Regarding human AE in North America, 73 cases were reported between 1951 and 1993: 71 were in Alaskan Eskimos,[5] and 2 cases were from Winnipeg, Manitoba, Canada in 1937[6] and Minnesota in 1977.[7,8] Regarding genotypes of *E. multilocularis* isolated from humans, there is no information about genotypes of *E. multilocularis* from the United States, although a few isolates from Canada have been genetically examined.[9,10]

In this study, to examine the genotype of *E. multilocularis* originating from the AE patient reported in Minnesota in 1977,[7] mitochondrial DNA analysis was performed using archival specimens prepared at that time. The case was autochthonous to Minnesota, and the patient was a 56-year-old woman. The patient complained of epigastric discomfort and malaise and was first suspected to have a malignancy. However, the clinical, serologic, and pathologic findings led to the diagnosis of AE. In addition, the identification of *E. multilocularis* was confirmed by metacestodes developed in red-backed voles (*C. rutilus*) inoculated intraperitoneally with tissue from the hepatic lesions of the patient. The parasite materials obtained from the voles were processed for histopathology and were preserved as hematoxylin and eosin (HE)-stained specimens for the past 30 years.

Template DNAs for polymerase chain reaction (PCR) were prepared by the method described previously[11]: the HE-stained sections were rinsed in xylene after removal of coverslips, washed in absolute ethanol, and air dried. At first, 10 μL of 0.05 N NaOH solution was placed onto some sections, and the sections were scalped, collected into Eppendorf tubes, and heated at 95°C for 1 hour. As an alternative method, a DNA Isolator PS kit (Wako Pure Chemicals, Osaka, Japan) was used for DNA extraction from the remaining sections. The PCR amplification of the cytochrome *c* oxidase subunit 1 gene (*cox1*) was performed in a 50-μL reaction mixture as reported previously.[12] Primer pairs used are shown in Table 1. F17 was used in combination with both R17 and R18. F20 was used with R18. The reaction was performed for 35 cycles of denaturation (94°C, 30 seconds), annealing (58°C, 30 seconds), and extension (72°C, 90 seconds) with a thermal cycler (GeneAmp 9700; PE Applied Biosystems, Foster City, CA). Direct DNA sequencing for the PCR amplicons was performed using a BigDye Terminator v. 3.1 Cycle Sequencing ready reaction kit (Applied Biosystems). Resultant sequence ladders were read with an ABI PRISM 310 or 3100 Genetic Analyzer (Applied Biosystems).

Because the *E. multilocularis* specimens used were fixed in formalin, and very limited amounts of the sections were available, only 20 small fragments (100–216 bp) were amplified (data not shown). Unfortunately, amplification of two regions using F1/R1 and F3/R3 primer pairs was not successful be-

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TABLE 1
PCR primer pairs used in this study

Primers*	Nucleotide sequences for *coxI*	Positions
F1	ATTTAGGGGCTGGTTGGTCATCTTAT	45–68 for *trnW*
R1	AACCAACAAAACCAGACCATA	100–80
F2	ATGAGAGTGGTGTGATTAGGTAG	1–23
R2	ATTATACCATGATTAGTCACCAAAAA	200–175
F3	TTAAGTTTTAGTTTGTTGATT	100–120
R3†	AATATAGGCATCAAAAAAAAAAA	230–208
F4	TGACTAATCATGGTATAATAATGATC	182–207
R4	GGCAAATTCAAATCAGACAAACCACC	293–268
F5	GTTTGGTAATTATTTATTGCCTTTG	240–264
R5	TGCAAAGAAAACATCAAAAAAATCAA	455–431
F6	TGCCACGTTTGAATGCTTTGAGTGCG	290–315
R6	ACAATGGAGGATAAAAAGTCCAACCA	400–375
F7	TCTTCTTCATATTTTTCTAGGAGTAG	400–425
R7	AGGCAACGTCACTAACAATAAAATA	600–576
F8	CTAGAGTTTTTAGTTCTATAAATTT	470–494
R8	CAAAAGCATAGTAATAGCAGCAGCC	630–606
F9	GTACTTTGTATAGTGTTTTTATGACT	500–525
R9	CCAAAAAAACCAAAACATATGCTGAA	716–692
F10	CGTTAGGTGGTGGTGATCCTATTCTA	665–690
R10	ACTCCCTAAACACACTATAGAAAACA	855–830
F11	ATGTTTTGGTTTTTTGGTCATCCGGA	700–725
R11	AAACAACAAACCATAAAACCCAAAC	825–801
F12	CGTTTGGGTTTTATGGTTTGTTGTTT	800–825
R12	TATTTACACTAGAATTAAGCAACAT	1,000–976
F13	GACTGGTATAAAGGTGTTTACTTGGT	945–970
R13	CACCACCAAACGTAAACAACACTAT	1,060–1,036
F14	AAGAGTGATCCTATTTTGTGGTGGGT	1,000–1,025
R14	CAACGGTCACCATCAAATAAACATAA	1200–1175
F15	TGTTATGTCGTTAGGTTCTTATATAA	1,140–1,165
R15	AATATTAGAAATTATACACTGACAT	1,260–1,236
F16	ATTACTGGTTTGAGGTTGAATAAGT	1,201–1,225
R16	CACCCACTAAACGCAGATATAAAAG	1,400–1,376
F17	ATTGGGTTAAAATGGTTTGTACTGT	1,346–1,370
R17	AGACCTCTTTCTTACTTACCATAGA	1,450–1,426
R18	CACCATAAGTATAATCAACACTATA	1,555–1,531
F19	TCTTTTATATCTGCGTTTAGTGGGTG	1,375–1,400
R19	TACAGGACTCATTAAATAATCCACTA	1,500–1,475
F20	GAAAGAGGTGTTGGGTTCATATAAA	1,440–1,464
F21	AGCTTGTCATAATGATTATTTTTGTT	1,500–1,525
R21	CTAACCAACAGCAAATACATAATTAC	1,608–1,583
F22	GATTATACTTATGGTGTATATTATAT	1,540–1,565
R22	ATCATAAACTTTAACTAACTAACC	24–1 for *trnT*

* Forward (F) and reverse (R) primers having the same number were basically used as primer pairs.
† Designed based on AB353729. Other primers from AB018440.

cause of the lack of DNA sample and positions 1–23 and 175–207 were not determined. The total number of nucleotide sites determined from the 20 *coxI* fragments was 1,552. Of these sites, 11 were substituted compared with known *coxI* sequences of *E. multilocularis* isolates (Table 2). The nucleotide sequences between two isolates from Minnesota and South Dakota were the same except for a nucleotide at position 688: C for Minnesota and T for South Dakota (Table 2). There were nine transitional substitutions between isolates from Minnesota and Alaska. Higher sequence homologies were shown with *E. multilocularis* from South Dakota (99.9%) compared with *E. multilocularis* from contiguous Alaska and from Japan (99.4%), indicating that *E. multilocularis* isolates from Minnesota and South Dakota belong to the same genotypic group.

There are some reports on genotypes of *E. multilocularis* isolates from Alaska and contiguous Canada and the United States; however, the number of *E. multilocularis* genotypes is variable (1–4) depending on the target genes and number of specimens examined.[9,10,13,14] The Minnesota isolate reported here seems to be identical to the genotype from Montana and Canada based on geographic locations.

Despite the widespread occurrence and high prevalence of *E. multilocularis* infections in definitive hosts in North America,[4,15–17] human cases seem to be few compared with the number of AE patients reported from Japan[18] and Europe,[19–21] where the prevalence of the *E. multilocularis* infection in definitive hosts is also high. In the extensive Arctic and sub-Arctic regions of Canada where *E. multilocularis* is endemic in definitive host animals,[4] cases of human AE have never been reported. To date only two cases of AE were diagnosed in the contiguous north-central region of Canada and the United States.[6,7] The reasons are not fully understood; however, human behavioral factors[21] and life cycles of the parasite involving synanthropic or sylvatic transmission[4,18] may be hypothesized. Genetic variations[9,10,13,14] and biological attributes[22] among *E. multilocularis* isolates and immunogenetic factors in humans[23–28] might play some roles in infectivity of *E. multilocularis* to humans. Because there are limited data on genotypes/haplotypes of *E. multilocularis* from North America except for Alaska, the data reported here may also provide useful information in considering the phylogeography of *E. multilocularis*.[29,30]

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TABLE 2
Base-substituted sites in cytochrome *c* oxidase subunit 1 genes among six *E. multilocularis* isolates

E. multilocularis isolates from	Accession numbers	Base-substituted sites										
		289	585	688	735	760	800	822	1,314	1,329	1,351	1,573
Minnesota	AB353729	C	A	C	G	G	T	G	G	A	A	G
South Dakota*	AB374425	C	A	T	G	G	T	G	G	A	A	G
Alaska*	Same as AB018440	T	G	C	T	A	C	G	T	G	G	A
Hokkaido, Japan	AB018440	T	G	C	T	A	C	G	T	G	G	A
Fukui, Japan†	AB385610	T	G	C	T	A	C	G	T	G	G	A
Slovak Republic	DQ013305	‡	‡	‡	‡	A	C	A	‡	‡	‡	‡

* The isolates from South Dakota and Alaska were derived from fox and vole, respectively (Nakao and others, unpublished data).
† This case was diagnosed at autopsy in a remote area from Hokkaido, Japan.[11]
‡ Data not available.

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Species identification of human echinococcosis using histopathology and genotyping in northwestern China

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Alveolar echinococcosis;
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Histopathology;
PCR;
Tibet

Summary Human cystic echinococcosis, caused by infection with the larval stage of *Echinococcus granulosus*, and alveolar echinococcosis, caused by the larval form of *E. multilocularis*, are known to be important public health problems in western China. *Echinococcus shiquicus* is a new species of *Echinococcus* recently described in wildlife hosts from the eastern Tibetan plateau and its infectivity and/or pathogenicity in humans remain unknown. In the current study, parasite tissues from various organs were collected post-operatively from 68 echinococcosis patients from Sichuan and Qinghai provinces in eastern China. The tissues were examined by histopathology and genotyped using DNA sequencing and PCR-RFLP. Histopathologically, 38 human isolates were confirmed as *E. granulosus* and 30 as *E. multilocularis*. Mitochondrial *cob* gene sequencing and PCR-RFLP with *rrnL* as the target gene confirmed 33 of 53 of the isolates to have the G1 genotype of sheep/dog strain of *E. granulosus* as the only source of infection, while the remaining 20 isolates were identified as *E. multilocularis*. No infections were found to be caused by *E. shiquicus*. Additionally, 5 of 20 alveolar echinococcosis patients were confirmed to have intracranial metastases from primary hepatic alveolar echinococcosis lesions. All these cases originated from four provinces or autonomous regions

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but most were distributed in Sichuan and Qinghai provinces, where high prevalence rates of human alveolar echinococcosis and cystic echinococcosis were previously documented.
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1. Introduction

Human echinococcosis refers to infection with the larval stages of any of the currently recognized four main species of *Echinococcus*, namely, *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (Kumaratilake and Thompson, 1982; Rausch and Bernstein, 1972). The latter two species occur only in Central and South America and may cause polycystic echinococcosis in humans (D'Alessandro, 1997), while *E. granulosus* and *E. multilocularis* are the causative agents of cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. *Echinococcus granulosus* has the greatest geographic distribution worldwide (Schantz et al., 1995), whereas *E. multilocularis* is a rare parasitic disease that is restricted to transmission in the northern hemisphere, with distribution from Alaska, across Canada and north central USA, through northern Europe and Eurasia to Japan (Craig, 2003). In China, both cystic and alveolar echinococcosis are highly endemic over large areas of the northwestern provinces and autonomous regions, mainly distributed in Qinghai, Sichuan, Xinjiang, Gansu and Ningxia (Chai, 1995; Craig et al., 1992; Li et al., 2005; Schantz et al., 2003; Yang et al., 2006). Recently, a new species of *Echinococcus*, *E. shiquicus*, was described from the Tibetan plateau. The Tibetan fox (*Vulpes ferrilata*) has been confirmed as the definitive host and the plateau pika (*Ochotona curzoniae*) as the intermediate host but its pathogenicity in humans, if any, remains unknown (Li et al., 2005; Xiao et al., 2005).

Substantial genetic diversity of *E. granulosus* has led to the description of 10 genotypes (G1-10) (Bowles et al., 1992, 1995; Bowles and McManus, 1993; Lavikainen et al., 2003; Nakao et al., 2007; Scott et al., 1997), and the G1 genotype has been proved to be cosmopolitan in its distribution and the major cause of human infection (McManus, 2002; Thompson and McManus, 2001). In China, previous genotypic analysis indicated the pathogenicity of the G6 genotype (dog/camel) in humans in Xinjiang autonomous region (Bart et al., 2006). However, the situation in other endemic regions of China is poorly understood. Furthermore, molecular confirmation of human AE due to *E. multilocularis* is rare in China.

2. Materials and methods

From 2004 to 2007, parasite tissues were collected post-operatively from patients clinically diagnosed with echinococcosis. Most cases were treated at the Aba Army Hospital (located in Maerkang, the capital of Aba Tibetan autonomous prefecture of Sichuan province), and hospitals in Chengdu (the capital of Sichuan province). General information about these cases including name, age, gender and region of origin was recorded. If possible, image data including ultrasound, computed tomography (CT) or magnetic resonance imaging (MRI) were reviewed. Part of the parasite biopsy tissue from each patient was stored in 99% ethanol for later DNA analysis, while the remaining part was fixed in 5% formalin and subjected to histopathological analysis. Formalin-fixed parasite tissue was embedded in paraffin wax and 3–5 μm sections were prepared and subsequently stained with hematoxylin and eosin. Genomic DNA from parasite tissues was extracted using a DNeasy tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Extracted DNA was examined by PCR-RFLP as described previously (Xiao et al., 2006). Briefly, the primer pair, Ech-LSU/F (5'-GGTTTATTTGCCTTTTGCATCATGC-3') and Ech-LSU/R (5'-ATCACGTCAAACCATTCAAACAAGC-3') was used to amplify a ~570bp DNA fragment of a mitochondrial gene (*rrnL*, large subunit of rRNA), within which species-specific *SspI* restriction site existed. A PCR cocktail contained 1 μl of template, 200 μmol/l of each dNTP, 0.2 μmol/l of each primer, 0.5 Unit of the *exTaq* DNA polymerase Hot Start (Takara, Tokyo, Japan) and the manufacturer-supplied buffer in 25 μl of a mixture reaction. Thermal reactions of PCR were conducted for 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s. Afterwards, the PCR product was cleaved with *SspI* (New England BioLabs, Ipswich, MA, USA) for 2 h at 37 °C. Electrophoresis was then performed on the restriction fragments in 2% agarose gel and stained with ethidium bromide, which led to specific restriction patterns for differentiation of *E. granulosus*, *E. multilocularis* and *E. shiquicus*. For further discrimination of the genotypes G1 and G6 of *E. granulosus*, cleavage of the PCR product with *BglII* (Takara) was necessary to obtain characteristic restriction maps.

PCR was conducted for amplification of a 549 bp DNA fragment of the mitochondrial cytochrome *b* (*cob*) gene as reported previously (Nakao et al., 2003; Xiao et al., 2005). The primer sequences used were 5'-GTCAGATGTCTTATTGGGCTGC-3' (forward) and 5'-TCTGGGTGACACCCACCTAAATA-3' (reverse). PCR was performed in a final volume of 50 μl containing 1 μl template, 200 μmol/l of each dNTP, 0.2 μmol/l of each primer, 1 Unit of Ex-*Taq* polymerase and the manufacturer-supplied buffer. The PCR protocol was composed of 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 60 s). The amplicons were purified using the NucleoSpin ExTract Kit (Macherey-Nagel, Düren, Germany) and directly sequenced using the Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Biosciences, Little Chalfont, UK) and an automated DNA sequencer (Applied Biosystems Model 377, Foster City, CA, USA).

3. Results

A total of 70 parasite tissue isolates from 68 operated echinococcosis cases were obtained and fixed in 5% formalin, of which 62 were removed from livers, five from brains, one from the abdominal cavity, one from a vertebra and one from the pelvic cavity. In one patient, parasite tissue was

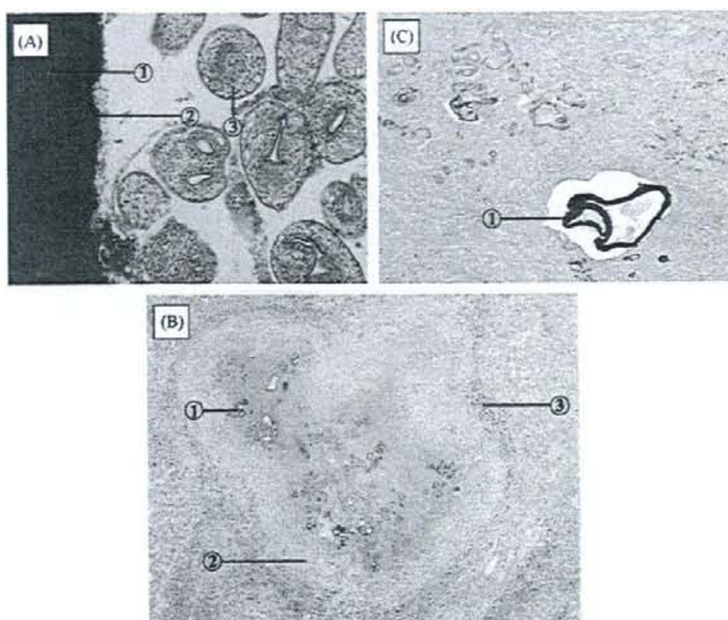

Figure 1 Histopathological characteristics of of cystic echinococcosis and alveolar echinococcosis lesions in humans. (A) Cystic echinococcal lesion in the liver (HE stain ×100); 1: laminated layer; 2: germinal layer; 3: protoscolex. (B) Alveolar echinococcal lesion in the liver (HE stain ×40); 1: vesicles; 2: hyperplasia of fibro-connective tissue; 3: cellular infiltration. (C) Alveolar echinococcal lesion in the brain (HE stain ×100); 1: vesicles.

obtained not only from the liver but also from the brain. In another patient, parasite samples were obtained from both the brain and bone (vertebra). In 38 cases, the structure of the lesions was characterized by a thick laminated layer and a germinal layer and brood capsules or protoscolices could be observed (Figure 1A), which were identified as *E. granulosus*, the cause of CE infection. Large numbers of vesicles of different sizes and shapes with a thin laminated layer were observed in 32 lesions from an additional 30 echinococcosis cases but protoscolices could not be confirmed. In these cases distinct hyperplasia of fibro-connective tissue and cellular infiltration of eosinophils, lymphocytes and plasma cells were observed (Figure 1B and Figure 1C), which resulted in a diagnostic confirmation of AE (Table 1).

Fifty-four parasite tissue samples from 53 of 68 patients were available after storage in 99% ethanol, of which 47 were excised from liver, four from brain, one from the abdominal cavity, one from the pelvic cavity and one from the vertebra. All the 54 isolates were examined by PCR-RFLP, identifying 33 as the sheep/dog strain of *E. granulosus* (G1 genotype) and 21 as *E. multilocularis* (Table 1). Furthermore, the amplicons of isolates from 54 parasite tissues were sequenced to identify the species; the results from

Table 1 Species confirmation of human echinococcosis by histopathological and molecular methods

Location	No. of samples			
	Histopathology		PCR-RFLP and DNA sequencing	
	AE	CE	AE	CE
Liver	26	36	16	31
Brain	5	0	4	0
Vertebra	1	0	1	0
Abdominal cavity	0	1	0	1
Pelvic cavity	0	1	0	1
Total	32	38	21	33

AE: Alveolar echinococcosis (*Echinococcus multilocularis*); CE: cystic echinococcosis (*E. granulosus*).

Figure 2 Geographic distribution of 56 confirmed human cases of echinococcosis in northwestern China. Locations of cases are not exact within counties.

the *cob* sequences were identical with PCR-RFLP typing data (Table 1).

In five of 68 patients, parasite lesions were removed from the brain and were subsequently identified to be *E. multilocularis* by histopathology and/or genotyping. Review of image data from CT or MRI of these cases indicated pathognomonic AE lesions in the liver and confirmation of intracranial metastases of AE was made in these five cases. In addition, one AE patient had metastasis to the vertebrae as well as the brain.

Among the 68 human echinococcosis cases confirmed in our study, 63 were Tibetans and five were Han Chinese, 41 were female and 24 were male (no information available for three cases). The youngest CE case was 7 years old and the youngest AE patient was 14 years old. The average age ($n = 59$, no information for nine cases) of echinococcosis cases was 33.0 years (35.1 years ($n = 31$) for CE cases and 30.6 years ($n = 28$) for AE cases). Information about case origin was obtained from 56 of 68 patient records, which showed a wide distribution in eight counties of Sichuan province including Seda, Yajiang, Ganzi, Shiqu, Maerkang, Dege, Hongyuan, Rangtang, Danba and Ruoergai. In addition, cases also originated from four counties of Qinghai Province, i.e. Banma, Gande, Dari and Maqin, and from Wen County, Gansu Province and Yili Prefecture of Xinjiang autonomous region (Figure 2).

4. Discussion

In the current study, histopathology and/or genotyping using PCR-RFLP and DNA sequencing were used to confirm the diagnosis of echinococcosis in 68 human cases from hospitals in Sichuan province, China. Of these, 38 cases were confirmed as CE due to *E. granulosus* and the remaining 30 as AE due to *E. multilocularis*. No infections with *E. shiquicus* were detected. Genetic analysis of cystic lesions in 33 of 38 cases revealed that the G1 genotype (sheep/dog strain) was the only source of *E. granulosus* infection. In addition, five patients with primary hepatic AE were shown also to have distant intracranial metastases; one of these had additional vertebral metastases. The majority of these human infections were ethnic Tibetans, distributed in four adjacent regions of China, i.e. Sichuan, Qinghai, Gansu and Xinjiang. Most of the cases originated from northwest Sichuan and southeast Qinghai, where both human CE and AE has previously been documented to be an important public health problem (Craig, 2006; Li et al., 2005; Schantz et al., 2003).

The sheep/dog strain of *E. granulosus* (G1) has been proved to be cosmopolitan in its distribution, as the predominant source of infection in both animals and humans (McManus, 2002; Thompson and McManus, 2001). The presence of *E. granulosus* genotypes G2, G5, G6 and G9 in humans was confirmed in Argentina and Poland (Guarnera et al., 2004; Rosenzvit et al., 1999; Scott et al., 1997), while in China previous studies from different areas revealed that human infections were caused by the G1 genotype (McManus et al., 1994; Yang et al., 2005; Zhang et al., 1998). However, in Xinjiang Uygur autonomous region the camel strain (G6) was also recently shown to infect humans (Bart et al., 2006). In our study, the G1 genotype was identified to be the only source of infection in all the 33 CE cases examined, with distribution in Sichuan and Qinghai provinces, where yaks, sheep and goats are used as the primary livestock by semi-nomadic herdsman. In that area of China, high prevalence of the larval stage of *E. granulosus* has been observed in domestic livestock and the infection source has been confirmed so far to be only the G1 genotype (Heath et al., 2005; Xiao et al., 2003; Yang et al., 2005). Therefore, in these areas humans probably acquire infection mostly through the sheep (yak)/dog cycle.

In China, the first human cases of AE were reported from western regions in the 1960s but most hospital records still remain fragmented. From the mid-1990s active mass screening surveys using portable ultrasound led to the discovery of the highest documented prevalence rates of human AE (Craig et al., 1992; Li et al., 2005; Yang et al., 2006). However, the application of molecular genetic analysis to human AE isolates in China is rare. In this study, histopathological examination of 32 parasitic lesions resected from 30 patients clinically diagnosed with AE supported the diagnosis of infection with *E. multilocularis*. In addition, 21 of the 32 lesions were further analyzed by both PCR-RFLP and DNA sequencing, which unequivocally identified the species as *E. multilocularis*. Metastases of AE in the brain were also demonstrated to have occurred in five patients, one of which had additional metastasis to the vertebrae. These cases indicate the serious public health problem of human AE in Tibetan communities of China and the need for early detection and treatment.

In addition to *E. granulosus* and *E. multilocularis*, a new species, *E. shiquicus*, was described recently on the eastern Tibetan plateau in Shiqu County, Sichuan, with the Tibetan fox as definitive host and the plateau pika as intermediate host (Xiao et al., 2005). For the first time we have used genotypic analysis to test parasite tissues from humans for the presence of DNA from *E. shiquicus*. In total, 54 isolates from individual parasite lesions were checked, however no infections were found to be caused by *E. shiquicus*. Dogs have been shown to play a crucial role in the transmission of *E. granulosus* and *E. multilocularis* on the Tibetan plateau (Li et al., 2005). However, no infections with *E. shiquicus*

have been found in dogs (Budke et al., 2005). The existence of large populations of dogs as companion animals facilitates the transmission of *E. granulosus* and *E. multilocularis* to humans. By contrast, small populations of foxes and limited contact with foxes may decrease opportunities for humans to be exposed to *E. shiquicus*. *Echinococcus shiquicus* has recently been demonstrated to be distributed in Shiqu and Seda counties of Sichuan province and Banma county of Qinghai province (T. Li et al., unpublished data), where human infections only with *E. multilocularis* and *E. granulosus* were identified in the present study. Although the three *Echinococcus* species sympatrically exist in these areas, *E. shiquicus* may have few chances to infect humans due to the unique relationship between the life cycles of Tibetan foxes and pikas or it may have poor infectivity in humans. This remains to be resolved in the future.

Authors' contributions: TL, AI, PSC and JQ designed the study protocol and drafted the manuscript; TL, PSC, AI and PG performed the analysis and interpretation of data; TL, MN, NX and XC conducted genetic analysis of all specimens; RZ did surgery; KN and MN did histopathology for morphological and molecular analysis; TL, XC and JQ collected tissue samples in the endemic areas. All authors read and approved the final manuscript. TL is guarantor of the paper.

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Evaluation of Three PCR Assays for the Identification of the Sheep Strain (Genotype 1) of *Echinococcus granulosus* in Canid Feces and Parasite Tissues

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Abstract. The performance of 3 PCR assays for the identification of the G1 sheep genotype of *Echinococcus granulosus* was evaluated using tissue and canid fecal samples. The "Dinkel" and "Štefanić" primers were the most sensitive in detecting *E. granulosus* DNA in feces of necropsied dogs (73.7% and 100%, respectively). The "Abbasi" primers detected 52.6% of *E. granulosus* infected dogs but were the most species-specific, cross-reacting only with *Echinococcus shiquicus* (tissue 90.9%; feces 75%). The Štefanić primers were the least specific (tissue, 27.3%; feces, 25%) for *E. granulosus*. The Dinkel primers also showed inter-species cross-reactivity (tissue, 63.6%; feces, 100%) but were found to be strain-specific for the *E. granulosus* G1 sheep genotype. Improvement of PCR tests for *Echinococcus* species and subspecific variants should rely on the use of less-conserved genes and development of protocols that improve the quality and quantity of DNA extracted from feces.

INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic infection caused by the larval stage of the dog tapeworm *Echinococcus granulosus*. Humans become infected through the accidental ingestion of eggs that give rise to hydatid cysts mainly in the liver and lungs. The life cycle is perpetuated between carnivores (especially the domestic dog) and a wide range of ungulates (particularly herbivorous livestock) that serve as definitive and intermediate hosts, respectively. In view of the impact on human health and economic losses to livestock,[1] there is a need for the development of reliable methods for the detection of infection in the dog definitive host.[2] This is particularly important when assessing transmission dynamics and the potential risk of human hydatidosis as well as for surveillance of hydatid control programs. Gold standard detection of *E. granulosus* in dogs has always been at post-mortem (necropsy) which, although highly sensitive and specific, is evidently laborious, raises ethical issues, and is biohazardous.[3] Coproantigen detection-based laboratory tests (usually ELISA) for canine echinococcosis have also been described by several groups.[4–8] Despite a lack of absolute species specificity, coproantigen ELISA is a useful tool for large-scale screening of dog populations.[3] There is a need for the development of more specific tests, particularly tests based on DNA detection in light of emerging genotypes and variants of *E. granulosus*, many of which are infective to humans.[9,10]

The first PCR method optimized for coprodiagnosis of *E. granulosus* relied on the detection of a tandem repeat unit within the genome of this cestode.[11] In addition, 2 PCR assays to amplify regions of the 12S rRNA gene for the identification of *E. granulosus* have been successfully developed.[12,13] Little information, however, is available on the specificity and sensitivity of these 3 PCR tests in routine laboratory practice. Also the genotypic specificity of PCR assays for isolates of *E. granulosus* has not been thoroughly assessed. The current study is an evaluation of the performance of these single-step PCR methods in detecting *E. granulosus* DNA from parasite tissue (hydatid cyst or adult tapeworm derived) including a number of *E. granulosus* strains/genotypes, and from fecal samples derived from naturally or experimentally infected canids.

MATERIALS AND METHODS

Fecal and parasite samples. Sensitivity of the coproPCR tests was determined using dog fecal samples from which *E. granulosus* worms were collected at necropsy and thus of known worm burden. These originated from China ($n = 7$), Libya ($n = 7$), Kenya ($n = 4$), and Jordan ($n = 1$). Fecal samples collected 21–37 days post-infection from experimentally infected Tunisian ($n = 15$) and Australian dogs ($n = 10$) were also included. PCR assay detection limit was evaluated using 1 g of a negative dog fecal sample spiked with 1, 10, 100, or 1000 *E. granulosus* eggs isolated from worms collected from dogs naturally infected with the sheep (G1) strain in China.

Parasite specificity of the *E. granulosus* PCR tests under evaluation was checked using tissue derived DNA from the following cestodes of canids (stage and place of origin): *Dipylidium caninum* (adult, Wales, U.K.), *Taenia crassiceps* (cysts, experimental mice, Belfast, U.K.), *Taenia hydatigena* (adult, Wales), *Taenia multiceps* (adult, Wales), *Taenia ovis* (adult, Wales), *Taenia pisiformis* (adult, Wales; cysts, Malham, U.K.), *Echinococcus multilocularis* (adult, China) and *Echinococcus shiquicus* (adult, Tibet, China). In addition, tapeworms of *Taenia solium* (adult and cysts, Peru), *Taenia saginata* (human derived adult, Tanzania), and *Hymenolepis diminuta* (adult, experimental rats, U.K.) were also used for DNA extraction.

Species specificity was also assessed using fecal samples

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from dogs after purgation ($n = 14$, China) and necropsied red foxes (*Vulpes vulpes*) ($n = 6$, France) from which *E. multilocularis* worms were collected. A fecal sample from 1 Tibetan fox (*Vulpes ferrilata*) from which the newly identified species *E. shiquicus* had been isolated was also available.[14] Fecal samples from necropsied dogs naturally infected with *T. crassiceps* ($n = 2$, Libya) and fecal samples from dogs experimentally infected with *T. multiceps* ($n = 2$) were also included.

Strain specificity was tested using DNA extracted from protoscoleces or the germinal layer from hydatid cysts of various *E. granulosus* genotypes,[15,16] namely, the sheep (G1, Libya, 4 isolates), horse (G4, UK, 3 isolates), cattle (G5, Switzerland, 2 isolates), camel (G6, Sudan), pig (G7, Slovak Republic, 3 isolates), cervid (G8, Minnesota), and Fennoscandian (G10, Finland) strains.

Fecal samples from 32 necropsied dogs (Libya, 28; Kenya, 4) and 17 necropsied red foxes from Franche-Comté, France, served as endemic-negative controls. Fecal samples ($n = 17$) from pet dogs in the Manchester area (U.K.) were used as "non-endemic" controls. DNA extracted from adults of the *E. granulosus* sheep strain (G1) (2 from Australia, 4 from China, 4 from Kazakhstan) as well as from protoscoleces (14 isolates from Libya) were used as positive DNA controls.

DNA extraction. Fecal DNA was retrieved from samples using the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with 1 minor adjustment: the fecal suspension was heated for 10 minutes at 85°C. In order to increase the amount of starting material and hence maximize the chance of detecting free *Echinococcus* DNA and/or DNA in eggs, an additional step was carried out prior to the use of the kit. One or two grams of fecal sample was emulsified in 0.01 M PBS and poured into a 100-μm cell strainer (BD Biosciences, Franklin Lakes, NJ) fitted onto a 50-mL tube containing 1.5 mL of Percoll (Sigma, U.K.) and 43 mL of 0.01 M PBS in order to remove light fecal debris (M. Campos-Ponce and P. S. Craig, unpublished). The tube was then centrifuged at 3600*g* for 30 minutes, and DNA extraction was performed using the pellet. DNA from parasite proglottids or metacestode tissue was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions.

Target DNA. The presence of DNA generic to all species and strains of *E. granulosus* used in this study was ascertained through the use of cestode-specific primers which amplified a fragment of the mitochondrial 12S rRNA gene.[13,17,18] The presence of *E. multilocularis* DNA in Chinese dog purges and French fox fecal samples was verified through the use of a species-specific PCR.[19] Detection of DNA of *E. shiquicus* extracted from feces of a Tibetan fox and generic cestode DNA derived from tissue and host fecal samples of a range of cestode species was achieved through the amplification of a fragment of the mitochondrial NADH dehydrogenase subunit 1.[14,20]

***E. granulosus* (G1) PCR assays.** Three published PCR tests optimized for the detection of the G1 strain of *E. granulosus* were evaluated. These were the "Abbasi,"[11] "Štefanić,"[12] and "Dinkel"[13] tests, respectively. PCR setups and cycling parameters were carried out as described by the respective authors with minor modifications as follows. The reagent concentrations quoted in the Abbasi protocol did not provide optimal results. Therefore, 250 μM (each) dNTPs, 1 μM of each primer, and 2% formamide were used instead. For the Štefanić test using Hotstart PCR, uracil DNA glycosylase and the internal control were not included and standard dTTP was used instead of the recommended dUTP. Finally the Dinkel test was used as specified by the authors with no modifications.

A Stratagene (La Jolla, CA) RoboCycler was used for all cycling profiles. The Hotstart procedure in the Štefanić test was carried out using a PCR Sprint Hybaid cycler (Thermo Electron Corporation, Waltham, MA). Go*Taq* (Promega U.K. Ltd., Southampton, U.K.) was used for DNA amplification of all protocols. Primers, as specified by the respective protocols were synthesized by MWG-Biotech AG (Ebersberg, Germany) and Invitrogen (Scotland, U.K.). PCR amplicons underwent electrophoresis in a 1.5% agarose TBE gel and were stained with ethidium bromide. A molecular weight marker (HyperLadder I, Bioline, London, England) was included on each gel for confirmation of amplicon sizes. Positive controls to monitor PCR success and negative controls to check for false-positive results that may have arisen from carry-over contamination were also included in all experiments. For all protocols, the same set of DNA samples within each group were systematically tested at least 3 times, and representative results are shown here. Gels were visualized under UV illumination using a Flowgen Alpha 1220 gel imaging system (Alpha Innotech Ltd., Staffordshire, U.K.).

Sequencing and analysis. DNA derived from both tissue and feces of all non-*Echinococcus* cestode species, *E. shiquicus* DNA from feces, and tissue DNA of *E. multilocularis* and *E. granulosus* genotypes G1 and G4 was verified by double-stranded sequencing (Cogenics, Norwich, U.K.). The sequence reports were analyzed using Chromas (http://www.technelysium.com.au/Chromas.html) and compared with the NCBI nucleotide database through the use of BLAST biosoftware (www.ncbi.nlm.nih.gov/BLAST/). Other genotypes of *Echinococcus* and parasite tissue of *E. shiquicus* had been molecularly typed by the individuals who kindly provided them.

Evaluation of reproducibility. The specificity of the Štefanić primers was further assessed by an independent laboratory (Departamento de Parasitología, Instituto Nacional de Enfermedades Infecciosas, Buenos Aires, Argentina). This was considered necessary in order to evaluate the reproducibility of results observed in the current work using the Štefanić assay as compared to the published data. This laboratory was not aware of our PCR data. Species specificity was assessed using a separate panel of tissue DNA derived from *E. granulosus* sensu stricto, *E. multilocularis* (cysts, Japan), *E. vogeli* (cysts, Venezuela), *E. oligarthrus* (cysts, Venezuela), and *T. hydatigena* (adult, Argentina). Strain specificity was also tested using DNA extracted from protoscoleces of *E. granulosus* genotypes (sheep G1, Tasmanian G2, horse G4, cattle G5, camel G6, and pig G7). Most *E. granulosus* samples tested were from Argentina; genotype G4 was from Spain. The uracil DNA glycosylase was not included, thus standard dTTP was used. An internal control was included for *T. hydatigena* test. DNA extraction was accomplished using the conventional phenol–chloroform method.

RESULTS

Sensitivity of PCR tests. Sensitivity was used to compare the limits of detection of each PCR assay for the same set of

samples and calculated as the percentage of *E. granulosus* samples correctly amplified. All 3 PCR tests performed well when DNA extracted from tissues of *E. granulosus* G1 adult worms or protoscoleces was tested, giving a 100% sensitivity with the amplification of the respective diagnostic products (data not shown).

All of the *E. granulosus* PCR protocols were capable of detecting DNA extracted from at least a single egg, yet test sensitivity for detection of *E. granulosus* in feces of naturally infected dogs ranged from 52.6% to 100% (Table 1). The Štefanić assay was the most sensitive with 100% copro-detection (worm burden from 4 to > 4000 worms/animal), although at times the PCR appeared to be performing sub-optimally, as manifested by the detection of very faint target bands for feces from 7 necropsy-positive *E. granulosus*-infected dogs. The Abbasi test did not detect DNA in 4 dogs with *E. granulosus* (worm burdens < 130 worms/animal). Fecal controls from endemic and non-endemic areas gave negative PCR signals for all 3 sets of *E. granulosus*-specific primers. Surprisingly, all 3 PCR tests demonstrated the ability for amplification of *E. granulosus* DNA in feces of prepatent experimentally infected dogs. The Abbasi and Štefanić tests detected parasite DNA with 100% sensitivity in dog feces from 21 to 27 days post-infection (dpi) and from 30 to 37 dpi, respectively. The Dinkel test detected *E. granulosus* DNA by 30 dpi (Table 2).

Species specificity of PCR tests. Specificity is used here to designate the correct amplification of target species (*E. granulosus* DNA) and the absence of a PCR product from nontarget species-derived DNA. All 3 protocols were tested using closely related *Echinococcus* and *Taenia* species (Figure 1). The Abbasi test was the most species-specific (90.9%), yielding a diagnostic band only from non-*E. granulosus* DNA with the amplification of a strong 133-bp band for *E. shiquicus* DNA Figure 1A; lane 10). The lowest specificity (27.3%) was recorded for the Štefanić primers, which amplified 2 bands for tissue DNA of *E. multilocularis*, one of which was the diagnostic 255-bp band and the other was probably of host origin (Figure 1B; lane 9). Furthermore, Štefanić primers amplified DNA extracted from tissue of *E. shiquicus*, *T. hydatigena*, *T. multiceps*, *T. ovis*, *T. pisiformis*, *D. caninum*, and *T. solium*. A diagnostic band of 254 bp was observed 50% of the time when the Dinkel primers were used to amplify tissue-derived DNA from *T. hydatigena*, *T. multiceps*, *T. ovis*, and *T. pisiformis*, giving a specificity of 63.6% (Figure 1C).

Specificity was also tested using *E. multilocularis* DNA extracted from fecal samples recovered from naturally infected purged dogs and necropsied foxes. Furthermore, *E. shiquicus* DNA from feces of one infected Tibetan fox, and fecal extracted DNA of *T. crassiceps* and *T. multiceps* from necropsied and purged dogs was tested (Figure 1; Table 3). The Abbasi and Dinkel PCR tests showed 100% specificity in respect of no cross-reactions with fecal extracted DNA of *E. multilocularis*, *T. crassiceps*, or *T. multiceps*. The Štefanić test was positive for 8 of 14 *E. multilocularis* dog-purge–derived fecal DNA samples as well as for *T. multiceps*-infected feces but remained negative for *E. multilocularis* and *T. crassiceps* DNA from necropsied foxes and dogs, respectively (Table 3). Both the Abbasi and Štefanić primers gave positive signals for *E. shiquicus* DNA extracted from fox feces, whereas the Dinkel assay remained negative when tested with the same sample. The overall specificity of the coproPCR tests for DNA extracted from feces of these 4 species (*E. multilocularis*, *E. shiquicus*, *T. crassiceps*, *T. multiceps*) was 25%, 75%, and 100% for the Štefanić, Abbasi, and Dinkel primers, respectively.

***E. granulosus* genotype specificity.** The 3 PCR assays were tested against a panel of 7 *E. granulosus* genotypes (G1 sheep, G4 horse, G5 cattle, G6 camel, G7 pig, G8 cervid, and G10 Finnish). The Dinkel test was found to be strain-specific to the *E. granulosus* G1 sheep genotype. In contrast, the Abbasi primers amplified DNA from G1, G4, G5, G7, and G10 genotypic isolates while the Štefanić PCR amplified DNA of all 7 *E. granulosus* genotypes tested (Figure 2).

Independent analysis. The results obtained by our collaborator (A.N.) with regard to the performance of the Štefanić primers were consistent with those observed in our laboratory. A 255-bp diagnostic product was amplified for *E. granulosus* G1 genotype, *E. multilocularis*, *E. vogeli*, *E. oligarthrus*, as well as for the G2, G4, G5, G6, and G7 genotypes of *E. granulosus*. In contrast, no such product was amplified using *T. hydatigena* DNA.

DISCUSSION

Human cystic echinococcosis (CE) is an important and widespread zoonotic disease. The causative cestode *E. granulosus* utilizes domestic dogs or wild canids as the main definitive host. CoproPCR testing is a potentially effective way of detecting species or even strain/genotype infections in living dogs and hence may provide an important confirmatory tool for epidemiologic studies and surveillance of hydatid-control programs. Species-specific detection of *E. granulosus* in the definitive host using coproPCR has to date been reported by

TABLE 1

Comparison of the sensitivity of 3 PCR tests for detection of *Echinococcus granulosus* DNA in egg-spiked fecal samples and from necropsied dogs with specific worm burdens from natural infections*

	Egg detection limit					*Echinococcus granulosus* worm burden																			
PCR assay	–	10	100	1000	Sensitivity (%)	4	5	90	95	130	< 200	< 200	< 500	< 500	< 500	500	> 500	1000	> 1000	> 1000	2342	> 2000	> 4000	Present†	Sensitivity (%)
Abbasi‡	+	+	+	+	100	–	–	–	–	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	52.6
Štefanić§	+	+	+	+	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Dinkel¶	+	+	+	+	100	+	+	+	–	+	+	–	+	+	+	–	–	–	+	+	+	+	+	+	73.7

* "+" target size product amplified, "–" target size product not amplified.
† Worms not counted.
‡ Abbasi test after Ref. 11.
§ Štefanić test after Ref. 12.
¶ Dinkel test after Ref. 13.

TABLE 2
Sensitivity of 3 PCR assays in detecting *Echinococcus granulosus* prepatent infections of experimentally infected dogs*

	Worm burden, days post-infection																									
	21	21	25	26	27	27	27	27	27	28	29	30	30	30	31	31	31	31	31	32	33	34	35	36	37	
PCR assay	2160	6040	100	3000	480	7700	150	500	10,000	Present†	Present†	Present†	2650	150	Present†	4918	110	60	80	Present†	Present†	Present†	Present†	Present†	Present†	Overall sensitivity (%)
Abbasi‡	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	92
Štefanić§	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	92
Dinkel¶	−	−	−	−	−	+	−	−	−	−	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+	52

* "+" target size product amplified; "−" target size product not amplified.
† Worms not counted.
‡ Abbasi test after Ref. 11.
§ Štefanić test after Ref. 12.
¶ Dinkel test after Ref. 13.

2 research groups.[11,12] A third assay for the amplification of *E. granulosus* from tissue has also been documented.[13] These 3 PCR tests, here referred to as the Abbasi,[11] Štefanić,[12] and Dinkel[13] tests, were assessed in the current study against panels of DNA directly extracted from tissue of *Echinococcus*

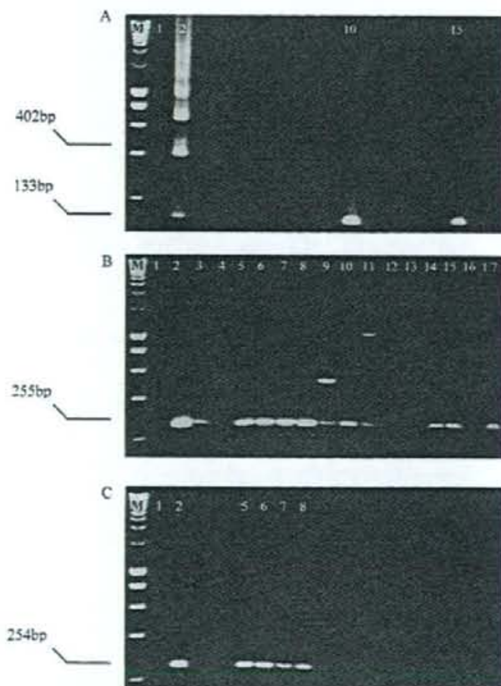

FIGURE 1. PCR amplification of tissue and fecal DNA from *Echinococcus granulosus* and related species; (**A**) Abbasi, (**B**) Štefanić, and (**C**) Dinkel assays, respectively. Lane M, 100-bp molecular DNA ladder; lane 1, negative control; lane 2, positive control; lanes 3–13, tissue-derived DNA of *Dipylidium caninum, Taenia crassiceps, Taenia hydatigena, Taenia multiceps, Taenia ovis, Taenia pisiformis, Echinococcus multilocularis, Echinococcus shiquicus, Taenia solium, Taenia saginata,* and *Hymenolepis diminuta;* lanes 14–17, fecal-derived DNA of *Echinococcus multilocularis, Echinococcus shiquicus, Taenia crassiceps,* and *Taenia multiceps.*

species (*E. granulosus, E. multilocularis,* and *E. shiquicus*), *E. granulosus* genotypes (G1, G4, G5, G6, G7, G8, and G10), as well as DNA from other cestode species (*D. caninum, T. crassiceps, T. hydatigena, T. multiceps, T. ovis, T. pisiformis, T. solium, T. saginata,* and *Hymenolepis diminuta*). In addition, a panel of fecal samples derived from canids with confirmed *E. granulosus* or *Echinococcus* species (*E. multilocularis, E. shiquicus*) or *Taenia* species (*T. crassiceps, T. multiceps*) was also used.

In terms of specificity, the Abbasi PCR test was the most species-specific for *E. granulosus.* Apart from *E. granulosus,* only DNA from either tissue or host feces of *E. shiquicus*[14] was detected. This latter result appears to indicate that the tandem repetitive unit described by Abbasi and others[11] is not unique to *E. granulosus.* The Štefanić *E. granulosus* primers were not species-specific in our hands as they cross-amplified DNA of *E. multilocularis, E. shiquicus,* and *T. multiceps* extracted from either tissue or feces; furthermore, DNA was amplified from 4 canid tapeworm species (*T. hydatigena, T. ovis, T. pisiformis, D. caninum*) and also from the human tapeworm *T. solium.* This lack of specificity with regard to *E. multilocularis* was confirmed by a second laboratory (A.N.). Additionally, *E. vogeli* DNA, which was not tested in the current study but was used to assess the specificity of the Štefanić protocol by the original authors, was positive in the hands of the collaborating researcher (A.N.). The Dinkel test primers also lacked specificity in our hands, amplifying tissue DNA from 4 canid *Taenia* species (*T. hydatigena, T. ovis, T. pisiformis, T. multiceps*) but not detecting DNA of *T. multiceps* from feces of infected dogs.

A 100% specificity for *E. granulosus* DNA was originally reported for the Štefanić and Dinkel tests,[12,13] although the previous assessment of the former test was primarily based on purged fecal samples from dogs naturally infected with this cestode. The lack of specificity we obtained for the Štefanić and Dinkel PCR tests in the current study may be a reflection of the fact that both sets of primers were designed within a highly conserved gene (12S rRNA).[12,13] In contrast, the high specificity we observed for the Abbasi primers for *E. granulosus* was only compromised by the new *Echinococcus* species (*E. shiquicus*), which was not available in the original study.[11] It should be noted that DNA samples from the neotropical species of *Echinococcus* (*E. vogeli, E. oligarthrus*) were not available for testing in the present assessment and were only used by the collaborating researcher (A.N.) for evaluation of the Štefanić assay.

TABLE 3

Species specificity of 3 PCR assays using *Echinococcus granulosus* "specific" primers in detecting DNA in fecal samples from dogs or foxes with natural infections of *Echinococcus multilocularis* or other heterologous species*

	Worm burden																						
	Echinococcus multilocularis purge														*Echinococcus multilocularis* necropsy						*Echinococcus shiquicus*†	*Taenia crassiceps*†	*Taenia multiceps*†
PCR assay	3000	31	114	5000	115	800	7	20,000	1500	261	2000	58	Present‡	Present‡	870	1025	755	4760	1660	46,250	> 200	Present‡	Present‡
Abbasi§	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Štefanić¶	+	−	+	+	−	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	+	−	+
Dinkel‖	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* "+" target size product amplified; "−" target size product not amplified.
† *E. shiquicus* and *T. crassiceps* DNA from naturally infected necropsied animals; *T. multiceps* DNA from experimentally infected purged dogs.
‡ Worms not counted.
§ Abbasi test after Ref. 11.
¶ Štefanić test after Ref. 12.
‖ Dinkel test after Ref. 13.

The Dinkel PCR was described essentially as a strain-typing PCR optimized to differentiate between the dominant G1 sheep–dog strain and the cattle (G5), camel (G6), and pig (G7) genotypes of *E. granulosus*.[13] The current assessment has both confirmed this and further shown the Dinkel test primers to be strain-specific against other genotypes of *E. granulosus* not evaluated in their original study.[13] Complete strain/genotype specificity, however, would need to include testing of the genotypes G2, G3, and G9 of *E. granulosus*, which were not evaluated in the present study. A rigorous molecular assessment of strain specificity was not included by the authors in the optimization of the Abbasi coproPCR assay; however, they reported a degree of cross-detection of the primers with DNA from other non-sheep (G1) strains of *E. granulosus*.[11] The current study confirmed this lack of strain specificity for *E. granulosus* with the Abbasi PCR test. Similar results to those obtained here were also recently reported in Argentina, in which the G1, G2, G4, G5, G6, and G7 genotypes of *E. granulosus* gave positive diagnostic products with the Abbasi primers.[21] This further emphasizes the probable occurrence of the tandem repeat units within the various strains or genotypes of *E. granulosus*. In fact, tandem-repeated sequences have also been described in the genome of the pig strain (G7) of *E. granulosus*.[22] The Štefanić primers were reported to be optimized for specific detection of the G1 strain of *E. granulosus* against control DNA isolates of the G4, G5, G6, and G7 genotypes.[12] The lack of *E. granulosus* genotype specificity we obtained for both the Abbasi and Štefanić PCR test primers may partly be due to the somewhat low annealing temperatures used in the respective protocols,[11,12] which we speculate were probably not sufficient to eliminate the amplification of DNA from other *E. granulosus* genotypes.

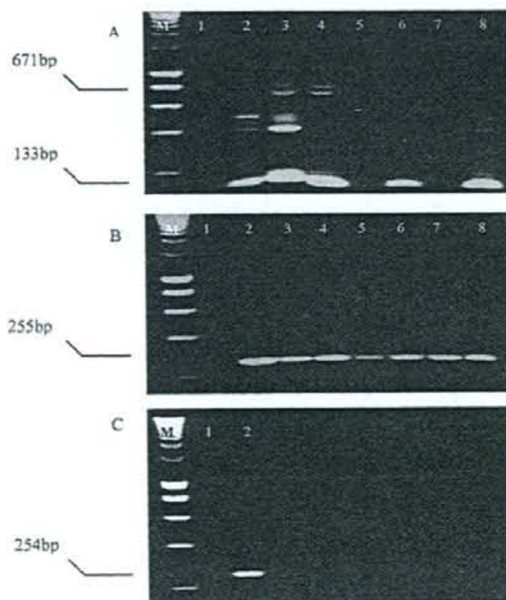

FIGURE 2. PCR amplification of *Echinococcus granulosus* genotypes; (**A**) Abbasi, (**B**) Štefanić, and (**C**) Dinkel assays, respectively. Lane M, 100-bp molecular DNA ladder; lane 1, negative control; lane 2, G1; lanes 3–7, G4–G8; lane 8, G10 genotypes.

Overall sensitivities for amplification of DNA from feces of dogs with prepatent experimental infections of *E. granulosus* were 92% for the Abbasi and Štefanić tests and 52% for the Dinkel assay. Positive DNA amplification from feces was obtained as early as 21 days post-infection in dogs experimentally infected with *E. granulosus* using either the Abbasi or Štefanić primers and by 30 days post-infection for the Dinkel assay. This indicates the ability of all 3 PCR assays to detect DNA in the absence of *E. granulosus* eggs because the prepatent period for the G1 strain is known to be about 45 days.[23] The ability of the Abbasi primers to detect prepatent *E. granulosus* from 25 to 33 days post-infection has also recently been reported in a separate study.[21] Tapeworm DNA has also been reported to be detected by coproPCR in human feces prior to patency.[24] These observations confirm that non-egg-derived DNA may also be present in feces of *E. granulosus*-infected canids and probably originates from proglottid breakdown, proglottid separation (apolysis), or tegumental turnover/disruption of tapeworms prior to patency. The abil-

ity of all 3 PCR protocols to detect prepatent *E. granulosus* infections is particularly relevant in follow-up of hydatid-control programs, where recently infected dogs could also be monitored as part of surveillance.

The overall sensitivities of the 3 PCR tests were 52.6% (Abbasi test), 73.7% (Dinkel test), and 100% (Štefanić test) for amplification of DNA from feces of naturally infected dogs with *E. granulosus* worm burdens ranging from 4 to > 4000. Only 2 of the PCR assays evaluated here were originally optimized to detect *E. granulosus* DNA in dog feces.[11,12] The Abbasi test was reported to be capable of detecting at least 1 fg of DNA, which was much less than the 8 pg calculated for 1 taeniid egg.[25] The high detection potential of the Abbasi test primers for *E. granulosus* DNA is derived from the amplification of a tandem repeat unit, with multiple targets for the primers to bind to.[11] Despite this, however, the Abbasi test was least sensitive when tested with feces from necropsied dogs infected with *E. granulosus*. The 100% sensitivity of coproPCR obtained by the Štefanić primers for *E. granulosus* may in part be due to the Hotstart procedure used and because the same DNA samples were used for all PCR reactions, results obtained using the Abbasi and Dinkel primers may not have been caused by PCR inhibitors but are rather a reflection of poor template quality and its reduced quantity. This factor may have affected the reaction dynamics of the primers and their ability to interact with the *Taq* polymerase and target DNA. Indeed, studies have shown that mammal host fecal DNA is of low concentration and is usually degraded.[26] This was clearly observed in this study because 100% sensitivity was recorded for all primers with DNA derived from *E. granulosus* (G1) tissue samples. Furthermore, some primers are affected more than others by impurities present in crude DNA samples.[27]

Sensitivity has also been noted to be affected by host and bacterial DNA.[21] Host DNA has been detected in the current study, particularly when generic cestode primers were used to ascertain the presence of *Echinococcus* DNA in fecal samples (data not shown). Chromatograms of the sequencing data of *E. granulosus* and *T. crassiceps* DNA amplified from infected host feces (as compared to that amplified from parasite tissue) had low signals and high background, indicating low DNA quantity as well as the presence of contaminants as manifest by other sequences probably pertaining to DNA of other organisms (data not shown). High concentrations of nontarget DNA may compete with target DNA for the primers, resulting in weak diagnostic signals or complete inhibition.[28] This may explain the occasional suboptimal performance of the Štefanić primers in detecting *E. granulosus* DNA in confirmed necropsy dogs. Primers are known to be affected by the presence of nontarget DNA which is known to be inhibitory to PCR.[29] Inhibition of amplification was more closely associated with natural as opposed to the experimental *E. granulosus* infections because all 3 PCR tests were capable of detecting 1 egg from spiked fecal samples. This suggests that prolonged exposure of parasite DNA to fecal matter may hasten its degradation, which would result in reduction in both quantity and quality of available DNA. The amplification of coproDNA of parasite origin may therefore require the implementation of specific procedures to maximize its detection when present. The importance of the use of good extraction methods for recovery of DNA from feces and its affect on PCR sensitivity has been stressed by many workers.[30,31]

In conclusion, the results obtained in the present *E. granulosus* PCR study show that, although the Abbasi PCR primers were not *E. granulosus* G1 strain-specific, they were highly species specific and thus most useful in confirmation of an *E. granulosus* infection in dogs. Conversely, the Dinkel primers appear to have the potential of being strain-specific for the *E. granulosus* G1 sheep strain and were 100% specific for *E. granulosus* in dog feces, but they showed evidence of cross-reaction with tissue-derived DNA from other canid tapeworm species. The Štefanić primers were 100% sensitive in detecting coproDNA from confirmed *E. granulosus* infections in dogs but in our assessment did not appear to be species-specific even when the recommended annealing temperature (53°C) was increased to 57°C. It is therefore evident that, although the Štefanić and Dinkel PCR tests for *E. granulosus* were reportedly specific in the hands of their original developers, the current assessment found them poorly reproducible. Finally, improvement in PCR tests for detection of *E. granulosus* (species and subspecies levels) should be directed to the identification of alternative, less-conserved gene targets to eliminate interspecies cross-reactions and on development of protocols to maximize and improve the quality of DNA extracted from host feces.

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Close relationship between clinical regression and specific serology in the follow-up of patients with alveolar echinococcosis in different clinical stages

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ABSTRACT

In this study, we compared for the first time four different ELISAs on 172 sera of 28 patients with alveolar echinococcosis in different clinical stages according to the WHO-PNM-staging system. The sequential antibody responses against Em^{2plus}, Em10, Em18, and a crude antigen extract were measured in cohorts with resected and unresected lesions. Antibody levels in all assays correlated with the PNM stage before treatment, and the highest correlation was shown for the Em18-assay. The PNM stage did not influence the antibody kinetics, but changes in antibody levels depended on the treatment. In patients after curative surgery, sero-reversion in the Em^{2plus}-ELISA signalled successful resection of lesions in more patients than any other assay, irrespective of the clinical stage. There were no significant differences in the time before assays which employ recombinant or purified antigens became unreactive. Antibodies directed against crude antigens were longer detectable than other antibodies in all patient cohorts and stages.