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Development of Three PCR Assays for the Differentiation between *Echinococcus shiquicus*, *E. granulosus* (G1 genotype), and *E. multilocularis* DNA in the Co-Endemic Region of Qinghai-Tibet plateau, China

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Abstract. To investigate echinococcosis in co-endemic regions, three polymerase chain reaction (PCR) assays based on the amplification of a fragment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene were optimized for the detection of *Echinococcus shiquicus*, *Echinococcus granulosus* G1, and *Echinococcus multilocularis* DNA derived from parasite tissue or canid fecal samples. Specificity using parasite tissue-derived DNA was found to be 100% except for *E. shiquicus* primers that faintly detected *E. equinus* DNA. Sensitivity of the three assays for DNA detection was between 2 and 10 pg. Ethanol precipitation of negative PCR fecal samples was used to eliminate false negatives and served to increase sensitivity as exemplified by an increase in detection from 0% to 89% of *E. shiquicus* coproDNA using necropsy-positive fox samples.

INTRODUCTION

Human cystic and alveolar echinococcosis caused by infection with the metacestodes of the tapeworms *Echinococcus granulosus* (sensu stricto) and *Echinococcus multilocularis*, respectively, are among the most pathogenic helminth zoonoses.[1] These species occur sympatrically on the Qinghai-Tibet plateau of western China and as a result human cystic (CE) and alveolar (AE) echinococcosis are co-endemic in Tibetan pastoral communities.[2,3] In addition a new species, *Echinococcus shiquicus* was recently described in wildlife (Tibetan fox, *Vulpes ferrilata* and plateau pikas, *Ochotona curzoniae*). However, the zoonotic potential (if any) of this species is currently unknown.[2,4] The occurrence of these three species in this particular part of the world highlights the importance of studies on transmission ecology and epidemiology and the need for the development of species-specific diagnostic assays for the detection of these *Echinococcus* species within both intermediate and definitive hosts.

Although protocols for the detection of *E. granulosus* and *E. multilocularis* have been published by many authors,[5–10] only three polymerase chain reaction (PCR)-based assays have previously been developed and validated for the detection of *E. granulosus*[11,12] and *E. multilocularis* DNA[13,14] from dog or fox fecal samples, respectively. A recent assessment of the *E. granulosus* PCR assays failed to fully reproduce the species and/or subspecies specificity reported by the original authors.[15] Moreover, the species-specific optimization of the *E. granulosus* PCR tests[11,12] pre-dates the description of *E. shiquicus*[4] and thus reduces their diagnostic value within the *Echinococcus* multi-species co-endemic regions of western China.

In the case of *E. multilocularis*, primers optimized for the detection of *E. multilocularis* DNA from feces[13,14] were found to cross-react with DNA of *E. shiquicus* (B. Boufana, unpublished observations). The specificity of other primers optimized for the detection of *E. multilocularis* in definitive host feces[12] was not assessed against DNA of *E. shiquicus.* Recently, a PCR-restriction fragment length polymorphism protocol was described for the differential diagnosis of tissue-derived DNA of *E. shiquicus, E. granulosus* (G1 and G6 genotypes), and *E. multilocularis*.[16] However, in that study, the uniqueness of the restriction sites and specificity compared with those of other *E. granulosus* genotypes (sensu lato) or *Taenia* species was not assessed. The currently available PCR assays for detection of *Echinococcus* species have not been assessed in relation to potential cross-reactions with *E. shiquicus* in China and PCR tests using feces rather than DNA from purified eggs have not been described. We now report on the development of three species-specific PCR (uniplex) assays for the detection of *E. shiquicus, E. granulosus* (G1), and *E. multilocularis*.

MATERIALS AND METHODS

Primer design. Nucleotide sequences of the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene of *E. shiquicus* (GenBank accession no. AB208064), *E. granulosus* genotype 1 (G1) (GenBank accession no. AF297617), and *E. multilocularis* (GenBank accession no. AB018440) as well as those of related species namely *E. equinus* (G4) (GenBank accession no. AF346403), *E. ortleppi* (G5) (GenBank accession no. AB235846), *E. canadensis* (G6, GenBank accession no. HM563036; G7, GenBank accession no. AB235847, G8, GenBank accession no. AJ237643, and G10, GenBank accession no. AF525297), *E. vogeli* (GenBank accession no. AB208546), *E. oligarthrus* (GenBank accession no. AB208545), *Taenia multiceps* (GenBank accession no. GQ228818), *T. hydatigena* (GenBank accession no. GQ228819), *T. pisiformis* (GenBank accession no. AJ239109), *T. ovis* (GenBank accession no. AJ239103), and *T. crassiceps* (GenBank accession no. AF216699) were retrieved from sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) and aligned using ClustalW (http://align.genome.jp). Forward and reverse primers were designed manually within the sequences of the three relevant target *Echinococcus* species to amplify a

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species-specific diagnostic fragment within the ND1 gene. Primers were validated through the use of BLAST biosoftware (www.ncbi.nlm.nih.gov/BLAST/). The high-performance liquid chromatography purified primers were synthesized by MWG-Biotech (Ebersberg, Germany).

DNA extraction from parasite tissue. All tissue-derived DNA used in this study was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA extracted from adults of *E. shiquicus* (Tibetan fox, *V. ferrilata,* China), protoscoleces of *E. granulosus* (G1 sheep genotype, Tunisia), and adult tapeworms of *E. multilocularis* (dogs, China) was verified by sequencing and used as controls in this study.

DNA extraction from host feces. CoproDNA was extracted from dog and fox fecal samples using the QIAamp DNA Mini Stool Kit (Qiagen) implementing the procedure recommended to process 1–2 g of feces with adjustment of lysis buffer volume. The suspension was heated in a water bath for ~25–30 minutes and then processed according to the manufacturer's instructions.

***Echinococcus shiquicus* PCR protocol (Es PCR).** For the amplification of *E. shiquicus* DNA, 0.3 μM of each primer (EsF50, 5′ TTA TTC TCA GTC TCG TAA GGG TCC G 3′ and EsR73, 5′ CAA TAA CCA ACT ACA TCA ATA ATT 3′) was used in a 50 μL reaction volume containing 5× manufacturers Flexi reaction buffer (Promega Ltd., Southampton, UK), 200 μM of each deoxynucleoside triphosphate (dNTPs; Bioline, London, UK), 2 mM $MgCl_2$ and 2.5 U GoTaq polymerase (Promega Ltd.). The mastermix was overlaid with mineral oil and the cycling profile was as follows: 5 min at 94°C for 1 cycle, and then 36 cycles each consisting of 30 s at 94°C, 50 s at 60°C, and 30 s at 72°C. The diagnostic product was 442 bp in size.

***Echinococcus granulosus* PCR protocol (G1 PCR).** Amplification was performed in a 50 μL reaction volume with 5× manufacturers Flexi reaction buffer (Promega Ltd.), 200 μM of each deoxynucleoside triphosphate (dNTPs; Bioline), 0.3 μM of each primer (Eg1F81, 5′ GTT TTT GGC TGC CGC CAG AAC 3′ and Eg1R83, 5′ AAT TAA TGG AAA TAA TAA CAA ACT TAA TCA ACA AT 3′), 2 mM $MgCl_2$ and 2.5 U GoTaq polymerase (Promega Ltd.). The mastermix was overlaid with mineral oil and the reaction was subjected to an initial incubation of 5 min at 94°C for 1 cycle, followed by 36 cycles each consisting of 30 s at 94°C, 50 s at 62°C, and 30 s at 72°C to amplify a species-specific 226 bp fragment.

***Echinococcus multilocularis* PCR protocol (Em PCR).** The 50 μL reaction volume contained 5× manufacturers Flexi reaction buffer (Promega Ltd.), 200 μM of each deoxynucleoside triphosphate (dNTPs; Bioline), 0.3 μM of each primer (EmF19/3, 5′ TAG TTG TTG ATG AAG CTT GTT G 3′and EmR6/1, 5′ATC AAC CAT GAA AAC ACA TAT ACA AC 3′), and 2 mM $MgCl_2$. 2.5 U of Hotstart GoTaq polymerase (Promega Ltd.) was used to overcome primer multimerization. The mastermix was overlaid with mineral oil and the thermal cycling profile consisted of 5 min at 94°C for 1 cycle, followed by 35 cycles as follows: 30 s at 94°C, 50 s at 53°C, and 30 s at 72°C to amplify an *E. multilocularis*-specific 207 bp fragment.

The PCR procedures were carried out in fully equipped molecular laboratories using dedicated equipment to prevent amplification of extraneous DNA. Negative controls (PCR grade water) were included in all experiments to monitor for contamination. A Stratagene (La Jolla, CA) Robocycler was used for all cycling profiles. The PCR products were resolved on a 1.5% (w/v) agarose gel (Bioline) in 1× Tris-Borate-EDTA buffer (Severn Biotech, Kidderminster, UK) at 110 V, stained with gel red DNA dye (Cambridge BioSciences, Cambridge, UK), and visualized using Syngene G:Box gel documentation system (Cambridge Biosciences). Validation of the PCR tests was made against defined panels of parasite tissue-derived DNA and using DNA extracted from infected canid fecal samples as described below.

Cloning and sequencing. In an attempt to ascertain the identity of the amplified products, representative DNA fragments amplified by each PCR assay were prepared for sequencing. These were Tris-acetate-EDTA (TAE)-gel purified using PurLink™ quick gel purification Kit (Invitrogen, Paisley, UK) and cloned into the TOPO TA cloning vector (Invitrogen) according to manufacturer's instructions and subsequently transformed into competent *Escherichia coli* cells. Plasmid minipreps were prepared using the Wizard Plus DNA purification system (Promega Ltd.) and DNA from the recombinant plasmids was commercially sequenced using M13 vector-specific primers (Beckman Coulter Genomics, Essex, UK).

DNA sequence analysis. Nucleotide sequences were analyzed using the FinchTV software package (Geospiza, Seattle, WA) and compared with those in the GenBank database through the use of BLAST biosoftware (www.ncbi.nlm.nih.gov/BLAST/).

TABLE 1

Parasite tissue DNA used for the assessment of the performance of the *Echinococcus shiquicus*, *E. granulosus* G1, and *E. multilocularis* PCR assays

PCR assay	Parasite stage	Host	Origin (*n*)	Confirmed by sequencing
E. shiquicus	Adult tapeworms	Tibetan foxes (*Vulpes ferrilata*)	China (5)	Yes
E. granulosus	Hydatid protoscoleces	Sheep	Tunisia (6)	Yes
	Germinal layer	Sheep	Falkland Islands (2)	Yes
	Adult *E. granulosus* tapeworms	Jackal (*Canis aureus*)	Tunisia (2)	Yes
	Adult *E. granulosus* tapeworms	Necropsied dog	China (1)	Yes
	Adult *E. granulosus* tapeworms	Necropsied dogs	Kazakhstan (2)	Yes
	Hydatid tissue	Surgically confirmed CE patients	China (3) Tunisia (9)	Yes
	E. granulosus hydatid cyst	Guenon monkey	UK, translocated (1)	Yes
	Adult *E. granulosus* tapeworms	Necropsied dogs	Tunisia (6)	–
E. multilocularis	Adult *E. multilocularis* tapeworms	Necropsied dogs	China (2)	Yes
	Adult *E. multilocularis* tapeworms	Necropsied foxes	France (6)	–
	Metacestode tissue	Surgically confirmed AE patients	China (4)	Yes
	Alveolar echinococcosis lesion	Macaque monkey	UK, translocated (1)	Yes

TABLE 2

DNA extracted from feces of naturally infected dogs, red (*Vulpes vulpes*) and Tibetan *(Vulpes ferrilata)* foxes used in assessment of coprodetection of *Echinococcus shiquicus, E. granulosus* G1 and *E. multilocularis* PCR assays

PCR assay	Host	Origin (n)
E. shiquicus	Necropsied Tibetan foxes (*V. ferrilata*)	Tibet (8)
E. granulosus	Purged dogs*	Wales, UK (5)
	Necropsied dingoes	Australia (4)
	Necropsied dog	Libya (1)
	Necropsied dogs	Kenya (2)
	Necropsied dogs	Kazakhstan (2)
E. multilocularis	Purged dogs*	Tibet (6)
	Necropsied dog	China (1)
	Necropsied foxes (*Vulpes vulpes*)	France (10)

*Feces collected before purge.

Parasite tissue samples. The performance of the *E. shiquicus* PCR, *E. granulosus* G1 PCR, and *E. multilocularis* PCR was assessed using DNA extracted from parasite material as shown in Table 1.

Canid fecal samples. The DNA extracted from feces of naturally infected canids was used to assess the performance of the *E. shiquicus*, *E. granulosus* G1, and *E. multilocularis* primers in detecting coproDNA (Table 2). The DNA extracted from feces collected from negative Tibetan foxes (*V. ferrilata*) ($N = 4$), from endemic necropsied negative dogs from Libya ($N = 6$) and Kenya ($N = 1$) and from necropsied negative red foxes (*Vulpes vulpes*) from France ($N = 4$) was included in the panel tested by the *E. shiquicus, E. granulosus* G1, and *E. multilocularis* primers, respectively. Three to 10 μL of DNA was used as template and 20 μL of the amplified PCR product were loaded onto agarose gel for electrophoresis.

Evaluation of PCR specificity. Specificity of the three PCR assays (Es PCR, G1 PCR, Em PCR) was assessed using parasite tissue DNA extracted from cestodes (Table 3). The presence of cestode-specific DNA (*Taenia* species, *Echinococcus* species, *Dipylidium caninum*) used in evaluation of assay specificity was ascertained through the amplification of a 373 bp fragment within the 12S rRNA gene using cestode-specific primers.[8,17] In addition, tissue-derived DNA of *E. granulosus* G1 and *E. multilocularis* was used to check the *E. shiquicus* primers, whereas that of *E. shiquicus* and *E. multilocularis*, and *E. shiquicus* and *E. granulosus* G1 were used to ascertain specificity of the *E. granulosus* G1 and *E. multilocularis* PCR assays, respectively. An identical set of samples were tested at least three times for each PCR assay with identical results observed each time. Representative results are shown here.

Assessment of specificity using coproDNA. A subset of the coproDNA extracted from canid fecal samples was used to assess the copro-specificity of the three PCR assays. CoproDNA amplified using the *E. granulosus* G1 ($N = 10$) and *E. multilocularis* ($N = 11$) PCR was used to determine the copro-specificity of the *E. shiquicus* primers. The *E. granulsous* G1 primers were checked for specificity using coproDNA extracted from feces of *E. multilocularis* ($N = 11$) or *E. shiquicus* ($N = 3$) parasitologically infected hosts that had also amplified using the *E. multilocularis* and *E. shiquicus* PCR assays, respectively. In a similar manner *E. shiquicus* ($N = 3$) and *E. granulosus* G1 ($N = 5$) coproDNA positive samples amplified using the *E. shiquicus* and *E. granulosus* G1 PCR was used to test specificity of the *E. multilocularis* primers. In addition, DNA extracted from feces of dogs experimentally infected with *T. multiceps* and verified by sequencing was used. Furthermore, sequenced coproDNA extracted from dogs naturally infected with *D. caninum* and *Mesocestoides corti* collected at necropsy (Tunisia) was used to assess the copro-specificity of the three PCR assays.

Evaluation of PCR detection sensitivity. Detection sensitivity for the PCR assays was determined by using two-fold serial dilutions (2,500–0.6pg) of tissue DNA extracted from adult tapeworms of *E. shiquicus*, protoscoleces of *E. granulosus*, and adults of *E. multilocularis*. Three separate sets of dilutions were tested per PCR assay. In addition, the detection limit of the *E. granulosus* G1 PCR was evaluated using a negative fecal sample (1 gram) spiked with 1–5, 10, 100, or 1,000 *E. granulosus* eggs isolated from worms retrieved from a naturally infected Chinese dog. *Echinococcus granulosus* DNA extracted from feces collected 28–37 days post-infection from experimentally infected Australian dingoes ($N = 10$) was also used to determine copro-sensitivity and to investigate prepatent DNA detection. These dingoes formed part of a study that was approved by the Animal Experimentation Ethics Committee (AEEC) of the Australian National University, Canberra, Australia. They were given a standard dose of 40,000 protoscoleces from cysts contained in livers and lungs

TABLE 3

Parasite tissue DNA extracted from *Echinococcus* and *Taenia* spp. used to assess the specificity of *Echinococcus shiquicus*, *E. granulosus* G1, and *E. multilocularis* PCR assays

Parasite	Stage	Origin (n)	Confirmed by sequencing
T. multiceps	Adult	Powys, Wales, UK	Yes
T. hydatigena	Adult	Powys, Wales, UK	Yes
T. pisiformis	Adult	Powys, Wales, UK	Yes
T. pisiformis	Cysts	Malham, England	Yes
T. ovis	Adult	Powys, Wales, UK	Yes
T. crassiceps	Cysts, experimental mice	Belfast, UK	Yes
Dipylidium caninum	Adult	Powys, Wales, UK	Yes
E. granulosus G3 (buffalo strain)	DNA	Italy	Yes*
E. equinus G4	Protoscoleces	Bristol, UK	Yes
E. ortleppi G5	Cysts removed from a captive Philippine deer	UK	Yes
E. canadensis G6	Camel protoscoleces	Iran	Yes
E. canadensis, G7 pig strain	Germinal layer	Slovak Republic	Yes*
E. canadensis, G8 cervid strain	Protoscoleces	Minnesota, USA	Yes*
E. canadensis G10, Fennoscandian strain	Protoscoleces	Finland	Yes*

*DNA of *E. granulosus* G3, *E. canadensis* G7, G8, and G10 was verified by individuals who kindly provided the material (see Acknowledgments).

TABLE 4

Sensitivity of *Echinococcus multilocularis* ND1 primers in detecting coproDNA extracted from fecal samples of Chinese purged dogs and French necropsied red foxes

	Tibetan purged dogs*						French necropsied foxes														
Worm burden	114	261	800	5000	10000	NA	5	18	20	25	42	45	51	104	115	136	299	1025	1040	1055	3665	30012†
Echinococcus multilocularis ND1 PCR assay	+	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+

*Feces collected before purge; + = positive; − = negative; NA = not available.
† Adults not gravid.

of sheep slaughtered in an abattoir located in Goulburn NSW, Australia (DJ). Copro-sensitivity of the *E. multilocularis* PCR assay was assessed using DNA extracted from fecal samples of naturally infected Tibetan dogs ($N = 6$) and French red foxes (*V. vulpes*) ($N = 16$) from which worm burdens of *E. multilocularis* had been counted after purgation or at necropsy, respectively (Table 4). In the case of the Tibetan dogs, fecal samples were collected before purging.

Ethanol precipitation of coproDNA. Ethanol precipitation was carried out whenever coproDNA amplification was negative. It was assumed that there was no target DNA, too little target DNA in a given fecal sample or that the presence of inhibitors and/or non-target DNA may have interfered with the amplification. To illustrate the effect of ethanol precipitation on PCR amplification, nine necropsy-confirmed coproDNA samples of *E. shiquicus* infected Tibetan foxes (*V. ferrilata*), which were initially negative by PCR were tested. CoproDNA was precipitated using Pellet Paint Co-precipitant (Merck, Merck Chemicals, Nottingham, UK) according to the manufacturer's instructions. Concentrated DNA pellets were re-eluted in Qiagen Stool Kit elution buffer (elution volume depended on the initial sample volume and concentration factor) and between 3 and 10 μL were used as template in a new PCR reaction. If samples were still negative following ethanol precipitation they were diluted and retested to remove PCR-inhibitory substances.

Validation of ND1 PCR assays. The *E. granulosus* G1 PCR and the *E. multilocularis* PCR tests developed at the University of Salford and were assessed by our collaborator (G. Umhang) in their independent laboratory for specificity and detection sensitivity. This was carried out to overcome inter-laboratory discrepancies that may be generated as a result of the use of different PCR machines and/or reagents and allow for final optimization if necessary.

The DNA extracted from *E. granulosus* G1 protoscoleces and *E. multilocularis* adults was used as controls in the two PCR assays (G1 PCR, Em PCR). A Veriti thermal cycler (Applied Biosystems, Inc.) was used and PCR products were resolved on a 1% (w/v) agarose gel (Promega Ltd.) in 5× Tris-Borate-EDTA buffer (Promega Ltd.) at 110 V, stained using SYBR safe DNA stain, and visualized using Gel Doc XR (Invitrogen). Independent panels of parasite species-derived DNA were used by the French laboratory. Specificity of the *E. granulosus* G1 and *E. multilocularis* PCR assays was assessed using tissue DNA extracted from the following species (stage, concentration, host): *E. equinus* (protoscoleces, 59 ng/μL, horse), *E. canadensis* G6 (protoscoleces, 5.69 ng/μL, camel), *T. hydatigena* (cysts, 12.2 ng/μL, sheep), *T. pisiformis* (adult, 1.73 ng/μL, *V. vulpes*), *T. taeniaeformis* (adult, 36.9 ng/μL, *V. vulpes*), *Mesocestoides* sp. (adult, 7.27 ng/μL, *V. vulpes*) and *Toxocara* sp. (adult, 5.5 ng/μL, *V. vulpes*). The DNA of the previous parasites was obtained from laboratory panels that had been verified by sequencing. The level of detection of the *E. granulosus* G1 PCR and the *E. multilocularis* PCR was assessed using tissue-derived DNA ranging from 10 ng to 10 pg.

RESULTS

Sequencing of ND1 gene product. Sequencing of cloned fragments for each of the three uniplex PCR assays was undertaken to verify the identity of the generated amplicons. A 99% homology to *E. shiquicus* (GenBank accession no. AB159137) ND1 mitochondrial fragment was obtained when the *E. shiquicus* amplified product was blasted against the database. The products amplified by the *E. granulosus* G1 and the *E. multilocularis* PCR assays showed 100% homology for a fragment within the *E. granulosus* G1 genotype (GenBank accession no. HM636643) and *E. multilocularis* (GenBank accession no. EU704124) ND1 mitochondrial gene, respectively. Nucleotide sequences of the fragments amplified by the *E. shiquicus*, *E. granulosus*, and *E. multilocularis* PCR assays were deposited into GenBank

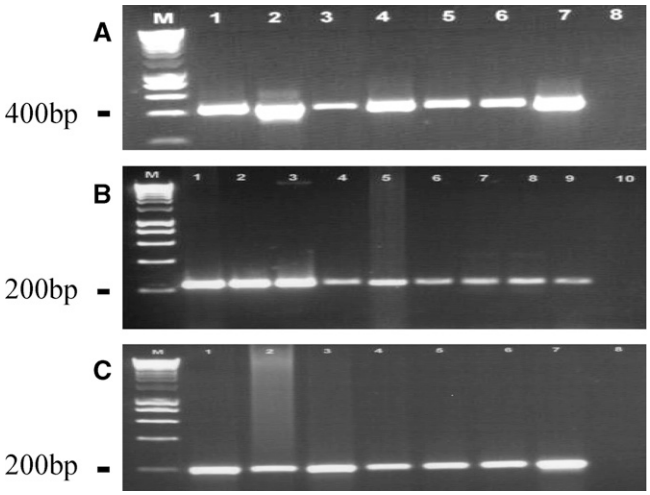

FIGURE 1. Performance of three uniplex polymerase chain reaction (PCR) assays, (**A**) *E. shiquicus* (Es PCR), Lane M, DNA marker, lanes 1–2, tissue-derived DNA from adults, lanes 3–6, coproDNA from necropsied Tibetan foxes, lane 7, positive control, lane 8, endemic negative control. (**B**) *Echinococcus granulosus* G1 (G1 PCR), Lane M marker, lanes 1–5, tissue-derived DNA from protoscoleces, germinal layer, human metacestode, Guenon metacestode and adults, respectively; lanes 6–8, coproDNA from Kazak, Welsh dogs and Australian dingos, respectively; lane 9, DNA extracted from a jackal, lane 10, endemic negative control. (**C**) *Echinococcus multilocularis* (Em PCR), Lane M, DNA marker, lanes 1–3, tissue-derived DNA from adults, human metacestode and macaque metacestode, respectively, 4–6, coproDNA from Tibetan purged dogs and necropsied French fox, respectively, lane 7, DNA extracted from necropsied Chinese dog, lane 8, endemic negative control.

under the following accession nos.: JN371772, JN371770, and JN371771, respectively.

Parasite tissue and coproDNA amplification. The performance of the three uniplex PCR assays (Es PCR, G1 PCR, Em PCR) was 100% in terms of the detection of parasite tissue-derived DNA from the confirmed respective *Echinococcus* species. The respective diagnostic products for *E. shiquicus* (442 bp), *E. granulosus* (226 bp), and *E. multilocularis* (207 bp) were generated when DNA extracted from metacestode cysts of intermediate hosts, adult worms from definitive hosts, or from human infection was used as template (Figure 1). A similar picture was observed with coproDNA extracted from fecal samples derived from purged or necropsied confirmed naturally infected canids. Representative examples of copro-amplification are shown in Figure 1.

Specificity of three uniplex PCR assays (Es PCR, G1 PCR, Em PCR) using parasite DNA and coproDNA panels. Using both parasite tissue-derived DNA and coproDNA as template, 100% specificity was observed when DNA from *E. shiquicus, E. granulosus*, or *E. multilocularis* was checked against each other (Figures 2 and 3). Furthermore, no signals were observed when DNA of *E. granulosus* G1 and *E. multilocularis* was used against the *E. shiquicus* primers (Figures 2A and 3A). The *E. granulosus* (G1) ND1 primers did not amplify DNA of *E. multilocularis* or *E. shiquicus* (Figures 2B and 3B). Similarly, the *E. multilocularis* uniplex PCR did not amplify DNA of *E. granulosus* G1 or *E. shiquicus* origin (Figures 2C and 3C). In addition, negative signals were obtained when tissue or coproDNA of *T. multiceps* and *D. caninum* or coproDNA of *M. corti* was used to check cestode genus specificity of the assays (Figures 2 and 3). The three sets of primers (Es PCR, G1 PCR, Em PCR) were also specific against tissue DNA extracted from *T. hydatigena, T. pisiformis,*

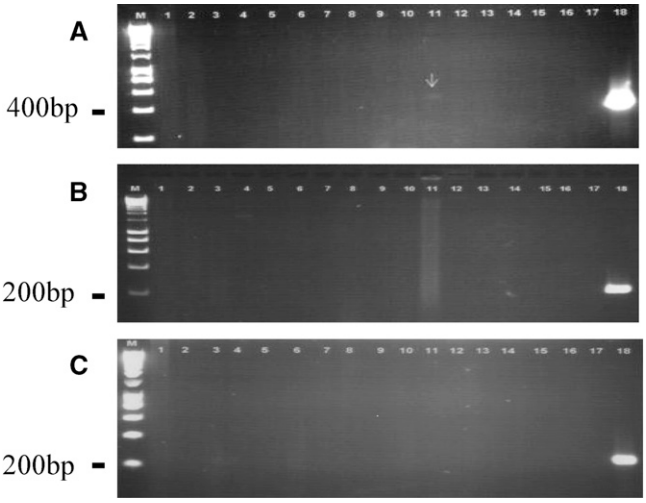

FIGURE 2. Specificity of three uniplex ND1 PCR assays; (**A**) *Echinococcus shiquicus* (Es PCR), (**B**) *E. granulosus* G1 (G1 PCR), and (**C**) *E. multilocularis* (Em PCR). Lane M, DNA marker, lanes 1–7, tissue-derived DNA of *Taenia multiceps*, *T. hydatigena*, *T. pisiformis adult*, *T. pisiformis cyst*, *T. ovis*, *T. crassiceps*, and *Dipylidium caninum*, lanes 8 and 9, tissue-derived DNA of *E. granulosus* and *E. multilocularis*, *E. multilocularis* and *E. shiquicus*, and *E. granulosus* and *E. shiquicus;* for A, B, and C, respectively; lanes 10-16, tissue-derived DNA of *E. granulosus* G3, *E. equinus*, *E. ortleppi*, *E. candensis* G6, G7, G8 and G10; lane 17, negative control, lane 18 positive control. Cross-reaction of *E. shiquicus* with *E. equinus* DNA, arrowed.

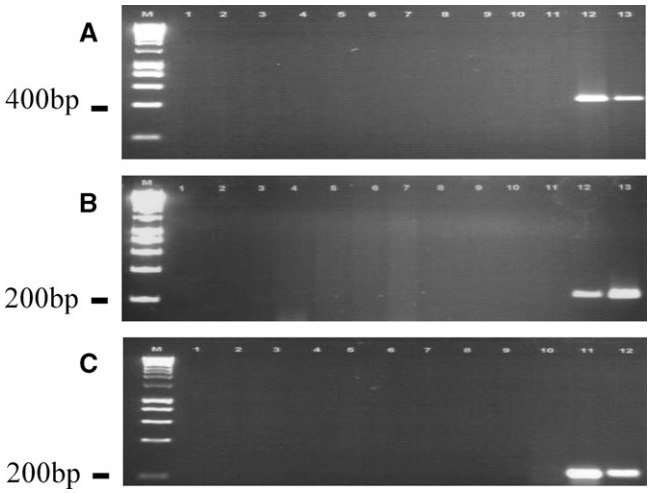

FIGURE 3. Copro-specificity of three ND1 PCR assays; (**A**) *Echinococcus shiquicus* (Es PCR), Lane M, DNA marker, lanes 1–4, *E. granulosus* coproDNA from positive Welsh, Australian, Kenyan, and Kazak dogs, respectively, lanes 5-6, *E. multilocularis*-positive dog purge DNA, lane 7, *E. multilocularis*-positive French fox DNA, lanes 8–10, *Taenia multiceps, Dipylidium caninum*, and *Mesocestoides corti* coproDNA, lane 11, endemic negative control, lane 12, tissue-derived positive control, lane 13, copro-positive control. (**B**) *Echinococcus granulosus* G1 (G1 PCR), Lane M, DNA marker, lanes 1–3, *E. multilocularis*-positive dog purge DNA, lane 4, *E. multilocularis*-positive French fox DNA, lanes 5–7, *E. shiquicus*-positive Tibetan fox DNA, lanes 8–10, *Taenia multiceps, Dipylidium caninum*, and *Mesocestoides corti* coproDNA, lane 11, endemic negative control, lane 12, tissue-derived positive control, lane 13, Welsh copro-positive control. (**C**) *E. multilocularis* (Em PCR), Lane M, DNA marker, lanes 1–4, *E. granulosus* coproDNA from positive Welsh, Australian, Kenyan, and Kazak dogs, respectively, lanes 5–6, *E. shiquicus*-positive Tibetan fox DNA, lanes 7–9, *Taenia multiceps, Dipylidium caninum*, and *Mesocestoides corti* coproDNA, lane 10, endemic negative control, lane 11, tissue-derived positive control lane 12, Chinese purge copro-positive control.

T. ovis or *T. crassiceps* (Figure 2A–2C). *Echinococcus shiquicus* primer specificity was compromised only against *E. equinus* (G4) DNA, when a faint positive signal was observed (Figure 2A). *Echinococcus granulosus* G1 and *E. multilocularis*

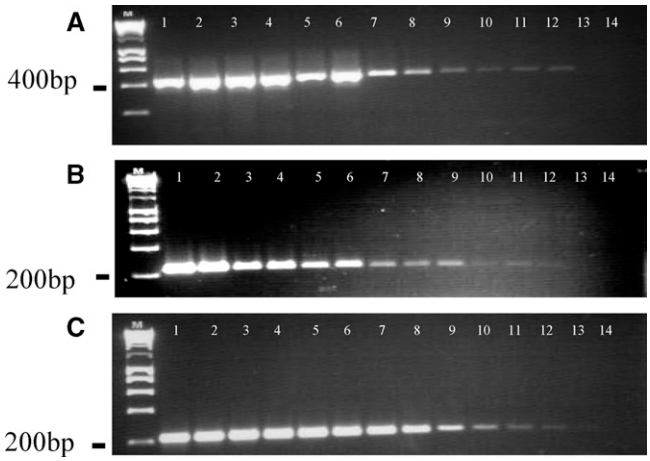

FIGURE 4. Sensitivity of three uniplex ND1 PCR assays. The DNA detection limit using a twofold serial dilution of DNA extracted from adult tapeworms of (**A**) *E. shiquicus* (Es PCR), (**B**) protoscoleces of *E. granulosus* G1 (G1 PCR), and (**C**) adults of *E. multilocularis* (Em PCR), respectively. Lane M, DNA marker, lanes, 1–13; 2500–0.6 pg, lanes 14, negative control.

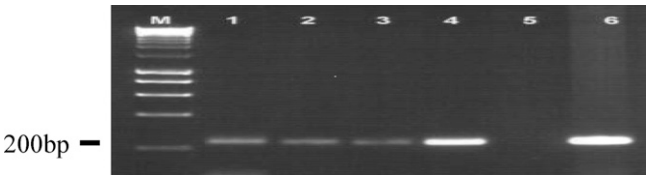

FIGURE 5. Detection of *Echinococcus granulosus* DNA in egg-spiked fecal samples using G1 PCR. Lane M, DNA marker, lanes 1–4, represent coproDNA extracted from 1 gram of feces spiked with 1–5, 10, 100, and 1,000 eggs, respectively, lane 5, negative control, lane 6, positive control.

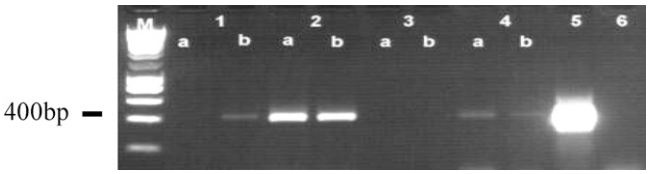

FIGURE 7. Effect of DNA ethanol precipitation and dilution on detection of *Echinococcus shiquicus* coproDNA (Es PCR). Lane M, DNA marker. CoproDNA extracted from Tibetan foxes, pre-ethanol (1a, 3a, and 3b) and post-ethanol precipitation (2a, 4a, and 4b); post-ethanol precipitation and dilution (1b and 2b), lane 5, positive control, lane 6, endemic negative control.

primers gave negative signals when tested against DNA extracted from *E. granulosus* G3 (Buffalo strain), *E. equinus* (G4), *E. ortleppi* (G5), or *E. canadensis* (G6, G7, G8, and G10) parasite tissue isolates (Figure 2B and 2C). The *E. granulosus* G1 and *E. multilocularis* primers were 100% specific in the hands of our collaborator (GU) including against tissue derived DNA of *T. taeniaeformis* and *Toxocara* sp.

PCR detection sensitivity. Sensitivity for DNA detection limit was checked using dilutions of *E. shiquicus, E. granulosus*, and *E. multilocularis* tissue-derived DNA ranging from 2500–0.6pg. The *E. shiquicus* and *E. multilocularis* assays detected 2–10 pg of DNA (Figure 4A and 4C). *Echinococcus granulosus* primers were capable of detecting between 5 and 10 pg of DNA (Figure 4B) This is in agreement with the results obtained by our collaborator (GU), which showed in his laboratory the detection level of *E. granulosus* and *E. multilocularis* to be 10 pg.

CoproPCR detection limit. *Echinococcus granulosus* PCR primers (G1 PCR) were able to amplify DNA from negative dog fecal samples spiked with at least 1–5 eggs of *E. granulosus* (Figure 5). They were also able to detect pre-patent *E. granulosus* infections in dogs from 30 to 37 days post experimental infection (Figure 6). The sensitivity of the *E. multilocularis* primers (Em PCR) in detecting coproDNA extracted from red fox fecal samples with known worm burdens is shown in Table 4. The *E. multilocularis* primers detected DNA from fox feces with at least 45 worms. They were also able to amplify DNA from feces of a French fox that harbored immature adult infections (> 30,000 non-gravid *E. multilocularis* worms).

Ethanol precipitation of coproDNA. To increase the concentration of target DNA in an extracted fecal sample, ethanol precipitation was used to maximize the chances of PCR amplification. Eight of the nine *E. shiquicus* fox necropsy positive coproDNA samples that were initially negative by PCR, gave positive signals with the amplification of the 442 bp diagnostic product following ethanol precipitation (Figure 7, 1a and 2a). In addition, when one of these ethanol precipitated fecal samples, which had only faintly amplified, was diluted and re-tested by PCR, a very bright band was obtained suggesting reduction of inhibition had occurred after dilution (Figure 7, 1b and 2b).

PCR reproducibility. The independent laboratory validation of the *E. granulosus* G1 and *E. multilocularis* ND1 PCR assays further confirmed the specificity of these two assays. Furthermore, the sensitivity level of both sets of primers was 10 pg of tissue-derived DNA.

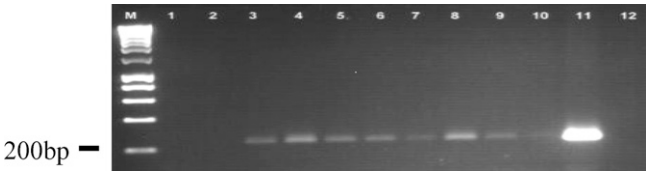

FIGURE 6. Sensitivity of *Echinococcus granulosus* PCR assay (G1 PCR) in detecting prepatent infections in experimentally infected Australian dingos. Lane M, DNA marker, lanes 1–10, coproDNA extracted 28–37days post infection, lane 11, positive control, lane 12, negative control.

DISCUSSION

Human cystic (CE) and alveolar (AE) echinococcosis are co-endemic in Tibetan communities and in extensive high pasture areas of Sichuan and Qinghai Provinces of western China where local prevalence levels range from < 1 to > 9% (mean 3%) for both CE and AE.[3] The Tibetan pastoral lifestyles are such that close associations with domestic or wild canid definitive hosts of *E. shiquicus, E. granulosus*, and *E. multilocularis* are maintained.[16] Although the infectivity (if any) of *E. shiquicus* to humans is unknown, the availability of a specific copro-detection test for this fox parasite and its differentiation from the other two *Echinococcus* species (*E. granulosus and E. multilocularis*) occurring on the Tibetan plateau is useful for epidemiological studies.

Diagnosis of *Echinococcus* species in definitive hosts is complicated by the fact that dog and fox hosts are frequently concurrently infected with *Taenia* species and/or other tapeworm species.[18,19] Eggs of *Taenia* species in particular are morphologically identical under the light microscope to those of *Echinococcus* species. In addition, Taeniidae family members of both *Echinococcus* and *Taenia* genera are genetically similar and hence specific molecular diagnosis can prove to be challenging. The detection of *Echinococcus* species DNA whether in tissue or feces has historically relied on the use of the 12S rRNA gene.[8,12–14] However, other genes such as the U1 snRNA gene,[5] cytochrome oxidase subunit 1,[7] and a tandem repeat region[11] have also been targeted. Our choice of the NADH mitochondrial gene (ND1) was taken to capitalize on the variability at the species level found within the *Echinococcus* genus.[20,21] The usefulness of the ND1 gene in differentiating between closely related species has been recently applied for the molecular detection of *Echinococcus* species and genotypes DNA from formalin fixed/paraffin

embedded clinical samples and for differentiation between cestode eggs.[22,23]

Using defined DNA panels extracted from parasite tissue or infected canid fecal samples, three PCR assays based on an ND1 gene sequence were developed for the detection of *E. shiquicus* (Es PCR), *E. granulosus* G1 (G1 PCR), and *E. multilocularis* (Em PCR). The number of samples used per panel in this work is comparable to those used in similar studies.[11–13] The *E. shiquicus* primers were shown to be highly species-specific with no PCR products detected when parasite DNA from the other related *Echinococcus* species or *Taenia* species were tested except for the amplification of *E. equinus* DNA. Although horses are frequent on the Tibetan plateau, to date *E. equinus* has not been described from either intermediate or definitive hosts from the plateau or indeed from any other part of China. Currently, the only *E. granulosus* species/genotypes recorded in China are *E. granulosus* G1 and G6 (*E. canadensis*).[24,25] The possibility of cross-reactions with *E. equinus* if it occurs, cannot be ruled out but is not considered a likely occurrence.

Initially, a multiplex assay for the concurrent detection of *E. shiquicus*, *E. granulosus*, and *E. multilocularis* was developed (data not shown). However, because of the limited sensitivity of this multiplex, the three uniplex ND1 assays described here were used. These PCR assays (Es PCR, G1 PCR, Em PCR) were capable of detecting at least 2–10 pg of *Echinococcus* DNA. Similar results were obtained by our collaborator's (GU) laboratory where the *E. granulosus* G1 and *E. multilocularis* assays were able to detect 10 pg of tissue-derived DNA. The *E. granulosus* G1 primers were able to detect at least 1–5 eggs, which would equate to 8–45 pg of DNA.[26] No such spiked fecal samples were available for *E. multilocularis* PCR primers, and instead we based our detection on fecal samples from necropsied red foxes with known worm burdens. This indicated that *E. multilocularis* primers appeared to require an infection of at least 45 adult *E. multilocularis* worms for a positive coproPCR signal to be observed. However, the sensitivity of the *E. shiquicus*, *E. granulosus*, and *E. multilocularis* PCR primers may differ when coproDNA rather than DNA derived from parasite tissue is assessed and would largely depend upon the quality and quantity of the template, the absence of inhibitors as well as the quantity of non-target DNA co-extracted with the *Echinococcus* species target DNA.[15]

Although no similar spiked fecal samples were available to test the copro-sensitivity of the *E. shiquicus* primers, the ethanol precipitation of PCR negative fecal samples from necropsy positive *E. shiquicus* foxes, increased the detection of *E. shiquicus* in fecal samples from 0% to 89%. The fecundity of *E. shiquicus* is known to be lower (< 100 eggs in a gravid segment)[4] than that of *E. granulosus* or *E. multilocularis* and thus the amount of DNA in fecal samples would likely to be significantly reduced. Many methods have been used to improve the recovery of DNA from feces through the extraction of DNA directly from *Echinococcus* species eggs.[27–29] It should be emphasized that the PCR assays developed here detected coproDNA, which includes egg and parasite tissue-derived DNA. The use of 1–2 grams of feces as starting material and the use of ethanol precipitation of initially negative DNA fecal samples helped increase assay sensitivity. Using this ethanol precipitation method we have documented the first record of *E. shiquicus* DNA in dogs from Shiqu County.[30]

In the current study, we have also shown that the *E. granulosus* ND1 primers were capable of detecting prepatent infections, i.e., without the need for the presence of eggs. Furthermore, although based on fecal samples from one fox with > 30,000 worms, a similar pre-patent detection was observed for the *E. multilocularis* ND1 assay. This has previously been documented for *Taenia* and *Echinococcus* DNA[15,31–33] and may be useful in the surveillance of control programs when definitive host exposure is reduced.[15] The development of these three uniplex ND1 PCR tests (i.e., Es PCR, G1 PCR, Em PCR) will serve to improve the detection and diagnosis of *E. shiquicus*, *E. granulosus*, and *E. multilocularis*, especially in definitive hosts. This will enable further investigation of the transmission biology of *E. shiquicus* and the epidemiology of cystic and alveolar echinococcosis in the unique highly endemic region of the eastern Tibetan Plateau, China.

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