Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA

D. TRACHSEL, P. DEPLAZES *and* A. MATHIS*

Institute of Parasitology, Vetsuisse and Medical Faculty, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland

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SUMMARY

A multiplex polymerase chain reaction (PCR) was evaluated for the identification of morphologically indistinguishable eggs of the taeniid tapeworms from carnivores using primers targeting mitochondrial genes. The primers for *Echinococcus multilocularis* (amplicon size 395 bp) were species-specific as assessed by *in silico* analysis and in the PCR using well-defined control samples. The design of primers that specifically amplify DNA from *E. granulosus* or *Taenia* spp. was not possible. The primers designed for *E. granulosus* also amplified DNA (117 bp) from *E. vogeli*, and those designed for *Taenia* spp. amplified products (267 bp) from species of *Mesocestoides*, *Dipylidium* and *Diphyllobothrium*. Nevertheless, as our diagnostic approach includes the concentration of taeniid eggs by sequential sieving and flotation, followed by their morphological detection, this non-specificity has limited practical importance. Sequence analysis of the corresponding amplicon can identify most of the described *E. granulosus* genotypes. *Taenia* spp. can be identified by direct sequencing of the 267 bp amplicon, or, for most species, by restriction fragment length polymorphism (RFLP) analysis. The multiplex PCR was readily able to detect 1 egg (estimated to contain 7000 targets, as determined by quantitative PCR). Having been validated using a panel of well-defined samples from carnivores with known infection status, this approach proved to be useful for the identification of taeniid eggs from both individual animals and for epidemiological studies.

Key words: *Echinococcus granulosus*, *E. multilocularis*, *Taenia* spp., multiplex polymerase chain reaction (PCR), genotypes, faecal samples, quantitative PCR, mitochondrial genes.

INTRODUCTION

The adult stages of tapeworms belonging to the family Taeniidae parasitize the intestine of humans (*Taenia*) and carnivores (*Taenia* and *Echinococcus*), causing little harm to these hosts. In contrast, the larval (metacestode) stages of some of these parasites can cause severe disease or even death in the intermediate mammalian hosts, including humans as accidental hosts. The infection of intermediate and aberrant hosts occurs by the ingestion of infective eggs which are morphologically indistinguishable among taeniid tapeworms. The specific identification of *E. granulosus* eggs using monoclonal antibodies has been described (Craig *et al*. 1986), but this method has not been utilized in further epidemiological studies. Molecular biological methods have also been used to determine species or genotypes of taeniids using 'pure' parasite DNA obtained from adult worms or metacestodes from intermediate hosts (Scott and McManus, 1994; Gasser and Chilton, 1995; McManus and Bowles, 1996; Gasser *et al*. 1999; Haag *et al*. 1999; von Nickisch-Rosenegk *et al*. 1999*b*). However, the potential of these approaches to identify or differentiate among species of taeniid eggs in faecal or environmental samples had not been evaluated. Several polymerase chain reaction (PCR) assays have been developed for the specific identification of *E. multilocularis* from such samples (reviewed by Mathis and Deplazes, 2006). A recent study describes a 'copro-PCR' for the simultaneous detection of the human tapeworms *T. solium* and *T. saginata* (see Yamasaki *et al*. 2004). Others reported oligonucleotide primers which have been evaluated in the PCR for the coprological diagnosis of *E. granulosus* infection in dogs (Abbasi *et al*. 2003; Stefanic *et al*. 2004). Both assays provide the possibility of identifying the 'sheep strain' of *E. granulosus*. In addition, 2 research groups have reported PCR-based tests for the identification of several *E. granulosus* strains (Cabrera *et al*. 2002; Dinkel *et al*. 2004). However, these tests have not been further validated for their diagnostic applicability to faecal or environmental samples.

Additional coprological tests with specificities for other taeniids are needed. However, this is not straightforward, as there are many different taeniid species, and there is significant genetic diversity within some species. For instance, within *E. granulosus*, some genotypes are proposed to represent species, whereas other, closely related ones are now

* Corresponding author. Tel: +41 (0)44 6358536. Fax: +41 (0)44 6358907. E-mail: alexander.mathis@access.unizh.ch

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Table 1. Origins of faecal samples of carnivores

	Origin [time of storage at −20 °C]
Group 1	5 Laboratory dogs from Switzerland that were kept in kennels and without cestode infections (controlled by microscopy, regular deworming with praziquantel) (negative control group [fresh samples]).
Group 2	Dogs ($n=4$), foxes ($n=4$), raccoon dogs ($n=4$) and a cat experimentally infected with *Echinococcus multilocularis* [2 years] (Kapel *et al.* 2006).
Group 3	5 Dogs experimentally infected with *Taenia multiceps* coenurosis cysts* [5 years].
Group 4	4 Naturally infected herding dogs from Nigeria, non-endemic area for *E. multilocularis* [1 year].
Group 5	17 Naturally infected rural dogs from Kazakhstan† [2 years].
Group 6	16 Naturally infected stray dogs from Spain diagnosed by necropsy (Deplazes *et al.* 1994), area non-endemic for *E. multilocularis* [15 years].

* Cysts from locally slaughtered sheep morphologically identified as *T. multiceps*; inoculation of dogs after deworming with praziquantel (Torgerson and Abdybekova, unpublished).
† Diagnosed by means of purgation; the purged taeniids were appointed to belong to either the genus *Echinococcus* or *Taenia* (Stefanic *et al.* 2004).

thought to constitute genotype clusters (Thompson and McManus, 2002; Eckert and Deplazes, 2004; Obwaller *et al.* 2004). Based on mitochondrial sequences, 5 genetically distinct clusters are presently recognized, namely cluster G1-3 ('sheep strain', 'Tasmanian sheep strain' and 'Indian buffalo strain'), G4 ('horse strain', *E. equinus*), G5 ('cattle strain', *E. ortleppi*), G6/7 ('camel strain' and 'pig strain') and G8/10 ('cervid strain') (see Thompson and McManus, 2002; Obwaller *et al.* 2004).

Echinococcus and *Taenia* spp. from carnivores vary in their infectivity to humans. Therefore, improved technology to differentiate animals infected with zoonotic tapeworms from those infected with non-zoonotic ones is required for both individual animals and population studies. The aim of this study was to develop a multiplex PCR which allows the differentiation among *E. multilocularis*, *E. granulosus* (all genetic variants) and *Taenia* spp. infections, with the option of being able to specifically identify the organism (*E. granulosus* strains/species and *Taenia* species) following additional analysis of the amplicons by sequencing or restriction fragment length polymorphism (RFLP).

MATERIALS AND METHODS

Parasite material

Samples of metacestodes (cyst fluid or parasite material from host tissue) or adult worms were collected from definitive or intermediate hosts and washed extensively in phosphate-buffered saline (PBS; pH 7·2). Cestodes included *E. granulosus* cluster G1-3 (3 isolates), genotype G4 ($n=2$), genotype G5 ($n=2$), cluster G6/7 ($n=5$), genotype G10 ($n=1$), *E. multilocularis* ($n=4$), *E. vogeli* ($n=1$), *Taenia multiceps* ($n=2$), *T. crassiceps* ($n=4$), *T. taeniaeformis* ($n=3$), *T. ovis* ($n=2$), *T. hydatigena* ($n=1$), *T. pisiformis* ($n=2$), *T. polyacantha* ($n=4$), *Diphyllobothrium latum* ($n=2$), *Dipylidium caninum* ($n=2$), *Mesocestoides* spp. ($n=2$). Nematodes included *Toxocara canis* ($n=3$), *Toxascaris leonina* ($n=1$), *Ancylostoma caninum* ($n=1$), *Uncinaria stenocephala* ($n=3$), *Angiostrongylus vasorum* ($n=1$), *Trichuris vulpis* ($n=4$) and *Capillaria plica* ($n=1$). Worms were identified morphologically by light microscopy and, if necessary, by PCR-based sequencing (Bowles *et al.* 1992; von Nickisch-Rosenegk *et al.* 1999*a*).

Faecal samples

Faecal samples from carnivores (Table 1) were chosen for the validation of the multiplex PCR. The samples were frozen at −80 °C for at least 3 days for safety reasons and kept at −20 °C, before being processed using a combination of sedimentation, flotation with zinc chloride and sequential sieving with sieves of different mesh sizes, allowing taeniid eggs to be concentrated and excluding the co-isolation of non-taeniid eggs (see Mathis *et al.* 1996). A panel of 55 taeniid egg-positive samples and 5 negative control samples was established (Table 1). The absence of eggs of other cestodes was verified by means of a bifocal, inverted microscope (Leica, Glattbrugg, Switzerland).

DNA isolation

Genomic DNA was isolated from parasites using a commercial kit, according to the manufacturer's instructions (QiAmp DNA mini kit, Qiagen, Hilden, Germany), and stored at −80 °C. The concentration of DNA in each sample was measured by a spectrophotometer (ND, Nano Drop Technologies, DE, USA), and the suitability for DNA amplification was confirmed by PCR using primers with a relatively broad specificity (Kocher *et al.* 1989; Liu *et al.* 1996). DNA extraction from individual faecal samples (Table 1) was carried out as described by Stefanic *et al.* (2004).

Table 2. Primer sequences and PCR conditions

Target Species	Gene	Primer designation	Sequences (5′-3′)	Amplicon size	Reference	Cycling conditions
Cestodes	*nad1*	JB11	AGATTCGTAAGGGGCCTAATA	529 bp	(Bowles and McManus, 1993)	30 sec/94 °C, 30 sec/50 °C,
		JB12	ACCACTAACTAATTCACTTTC			60 sec/72 °C, 40 cycles
Cestodes	*rrn*S	60.for.-mod	ATGTGGTACAGGATTAGATACCC	357 bp	(von Nickisch-Rosenegk *et al.* 1999*a*)[a]	30 sec/94 °C, 30 sec/61 °C,
		375.rev-mod	GGTGACGGGCGGTGTGTACC			45 sec/72 °C, 40 cycles
Echinococcus granulosus (‘sheep strain’)	*rrn*S	Eg1f	CATTAATGTATTTTGTAAAGTTG	255 bp	(Stefanic *et al.* 2004)	30 sec/94 °C, 30 sec/50 °C,
		Eg1r	CACATCATCTTACAATAACACC			45 sec/72 °C, 40 cycles
E. multilocularis	*rrn*S	EM-H15	CCATATTACAACAATATTCCTATC	200 bp	(Stieger *et al.* 2002)	30 sec/94 °C, 30 sec/55 °C,
		EM-H17	GTGAGTGATTCTTGTTAGGGGAAG			60 sec/72 °C, 40 cycles
E. multilocularis	*nad1*	Cest1	TGCTGATTTGTTAAAGTTAGTGATC	395 bp	This publication	multiplex PCR: 30 sec/94 °C,
		Cest2	CATAAATCAATGGAAACAACAACAAG			90 sec/58 °C, 10 sec/72 °C,
E. granulosus	*rrn*S	Cest4	GTTTTTGTGTGTTACATTAATAAGGGTG	117 bp		40 cycles
		Cest5	GCGGTGTGTACMTGAGCTAAAC			
Taenia spp.	*rrn*S	Cest3	YGAYTCTTTTTTAGGGGAAGGTGTG	267 bp		
		Cest5	GCGGTGTGTACMTGAGCTAAAC			
Taenia spp.[b]	*rrn*S	Cest5$_{seq}$	GATTCTTTTTAGGGGAAGG			

[a] Primers modified.
[b] Sequencing primer for the 267 bp amplicon of the multiplex PCR.

Single-target PCR, cloning and sequencing

Single target PCRs, using primer pairs and conditions listed in Table 2, were performed to identify additional mitochondrial sequences (Bowles *et al.* 1992; Bowles and McManus, 1993; von Nickisch-Rosenegk *et al.* 1999*a*) required for the primer design for the multiplex PCR. Also, single PCRs confirmed infections with *E. multilocularis* or *E. granulosus* ‘sheep strain’ (Stefanic *et al.* 2004; Stieger *et al.* 2002) based on the testing of faecal samples.

All PCRs were performed in 100 μl, as described previously (Stefanic *et al.* 2004) with 2 μl of template DNA (20–400 ng/μl) using the uracil DNA glycosylase (UDG) system (Sigma-Aldrich, Switzerland) to prevent carry-over contamination (Longo *et al.* 1990). If cloning was required, the PCR was repeated with dNTPs containing dTTP, and the purified (Qiagen PCR purification kit, Qiagen, Hilden, Germany) amplicon was cloned into the Topo-TA-cloning vector, according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA).

For automated DNA sequencing *via* a private service company (Microsynth, Balgach, Switzerland), amplicons were purified with the aforementioned kit, either directly from the PCR sample or after excision from agarose gels. Plasmids were purified using the Qiaprep spin miniprep kit (Qiagen, Hilden, Germany).

Multiplex PCR

Sequences of part of the mitochondrial genes for NADH dehydrogenase subunit 1 (*nad1*), cytochrome oxidase subunit 1 (*cox1*) and the small subunit of ribosomal RNA (*rrn*S) were either retrieved from GenBank, from the literature (von Nickisch-Rosenegk *et al.* 1999*b*) or were determined in this study (GenBank Accession numbers given in Table 3). The respective sequences were aligned (according to Corpet, 1988) and primer candidates of the desired specificity derived.

The multiplex PCR was conducted using a commercial kit (Qiagen multiplex kit, Qiagen, Hilden, Germany). The amplification reaction mixture (50 μl) consisted of 25 μl of master mix, 5 μl of primer mix (2 μM of primers Cest1, Cest2, Cest3, Cest4 and 16 μM of primer Cest5 in Tris-EDTA or H_2O; Table 2), 18 μl H_2O and 2 μl of template DNA. Initially, a 15 min *Taq* DNA polymerase activation step was performed at 95 °C. Further cycling conditions are given in Table 2. Amplicons were detected on 2% agarose gels, following staining with ethidium bromide.

The analytical specificity of the PCR was assessed by *in silico* analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and by PCR from DNA from the helminths listed above. To assess the analytical

Table 3. GenBank Accession numbers and references for mitochondrial gene sequences used for the design of primers for the multiplex PCR

Species	*nad1* gene	*rrn*S gene
Echinococcus granulosus (*EgG1*)	AJ237632, AF297617	AF297617
E. granulosus (*EgG2*)	AJ237633	[a]
E. granulosus (*EgG3*)	AJ237634	[a]
E. granulosus (*EgG4*)	AJ237635, AF346403	AF346403
E. granulosus (*EgG5*)	AJ237636	AY462127
E. granulosus (*EgG6*)	AJ237637	AY462126
E. granulosus (*EgG7*)	AJ237638	AY462128
E. granulosus (*EgG8*)	AJ237643	[a]
E. granulosus (*EgG10*)	AF525297	DQ408423[b]
E. multilocularis	AB018440	AB018440
E. vogeli	AJ237641	DQ408426[b]
Taenia hydatigena	AJ239102	AB031352 (von Nickisch-Rosenegk *et al.* 1999*b*)
T. pisiformis	AJ239109	AB031353
T. taeniaeformis	AJ239101	L49443, DQ408425[b]
T. crassiceps	NC_002547	NC_002547
T. serialis	AJ239105	(von Nickisch-Rosenegk *et al.* 1999*b*)
T. ovis	AJ239103	(von Nickisch-Rosenegk *et al.* 1999*b*), DQ408421[b]
T. polyacantha	DQ408420[b]	(von Nickisch-Rosenegk *et al.* 1999*b*), DQ408419[b]
T. multiceps	AJ239104	DQ408418[b]

[a] No sequence available.
[b] Determined in this study.

sensitivity of the PCR, single taeniid eggs from a sieved faecal sample were isolated with a pipette under microscopic control. DNA was isolated (Stefanic *et al.* 2004) and subjected to multiplex PCR. Eggs were identified by direct sequencing of the amplicon. Also, plasmids containing inserts of parts of the mitochondrial *nad1* and *rrn*S gene sequences (Bowles *et al.* 1992; von Nickisch-Rosenegk *et al.* 1999*a*) from *E. multilocularis*, *E. granulosus* (cluster G1-3) and *T. hydatigena* were produced and subjected to multiplex PCR (in a dilution series).

The multiplex PCR was validated using the DNA from the 55 faecal samples containing taeniid eggs and the 5 'negative controls'. In addition, the presence of *E. multilocularis* and of *E. granulosus* 'sheep strain' in these samples was investigated by specific, single target PCRs (Stieger *et al.* 2002; Stefanic *et al.* 2004).

Further analyses

The 117 bp amplicons representing *E. granulosus* derived from 5 faecal samples were cloned and sequenced. The 267 bp amplicons representing *Taenia* spp. produced from 7 faecal samples were sequenced directly using an internal sequencing primer $Cest5_{seq}$ (see Table 2). To evaluate whether restriction fragment length polymorphism (RFLP) analysis could be a useful additional tool to further characterize the 267 bp amplicon produced in the multiplex PCR, a NEBcutter analysis (Vincze *et al.* 2003) was performed.

Quantitative PCR

The PCR was conducted employing a commercial kit (Quantitect sybr green, Qiagen, Hilden, Germany). The amplification reaction mixture (25 μl) consisted of 12·5 μl of master mix, 0·3 μM of each primer (Cest3, Cest5) and 2 μl of template DNA. Initially, a 15 min *Taq* DNA polymerase activation step was performed at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 25 sec annealing at 58 °C and 35 sec at 72 °C. Melting curve analysis was carried out over a temperature range of 58 °C to 98 °C. The cycling was performed in an iCycler (Bio-Rad, Hercules, CA). DNA from target plasmids (in serial dilutions) and from 3 individual eggs were subjected to PCR. Reactions were performed in duplicate, and the experiment was repeated.

RESULTS

Defining primers for use in the PCR

Parts of 3 mitochondrial genes (*nad1*, *cox1* and *rrn*S) of a wide range of cestodes were analysed for

their suitability as primer targets for developing a multiplex PCR, yielding specific products for *E. multilocularis*, *E. granulosus* (all genotypes/species) and *Taenia* spp. from carnivores. The *cox1* was not further considered because of a lack of sufficient sequence divergence, as revealed based on preliminary analyses. Several primers candidates targeting the 2 other genes were evaluated using DNA from metacestodes, faecal specimens (including mixed infections of *Taenia/Echinococcus*), and plasmids containing the cloned target sequences. The results were compared with PCRs containing single primer pairs. The reaction conditions in relation to primer concentrations and cycling parameters were optimized. Eventually, the multiplex assay (as detailed in Table 2) was established utilizing primer concentrations of 0·2 μM (primers Cest1, Cest2, Cest3, Cest4) and 1·6 μM (primer Cest5).

Specificity of primers in the PCR

The specificity of the primer pair Cest1/Cest2 for *E. multilocularis* was 100% when a range of DNA samples from different species of cestodes and nematodes (see Materials and Methods section). The primer pair Cest3/Cest5 was able to detect all *E. granulosus* genotypes/species. Furthermore, the respective sequence of *E. vogeli*, which was determined towards the end of this study, revealed that the forward primer Cest3 has only 2 mismatches (4th and 10th positions from the 3′-end) with the target locus of this species from South America, whereas the reverse primer Cest5 had a perfect match. Multiplex PCR from *E. vogeli* DNA using the optimized conditions indeed yielded an amplicon of the size expected for *E. granulosus*. The primer pair Cest4/Cest5 detected all of the *Taenia* spp. tested but also some other cestodes from carnivores, including *Mesocestoides* spp., *Dipylidium caninum* and *Diphyllobothrium latum*.

Estimation of the analytical sensitivity of the multiplex PCR

The multiplex PCR was able to detect DNA from single eggs of *T. hydatigena*. In reactions spiked with cloned target sequences, the detection limit was $3{\cdot}3 \times 10^1$ for all targets (*E. multilocularis*, *E. granulosus* and different species of *Taenia*) (not shown). Quantitative PCR, using 10-fold dilutions of a cloned target sequence as a standard, predicted ~7100 (standard deviation: ~1900) mitochondrial targets to be present in a single egg of *T. hydatigena*.

In order to test the effectiveness of the multiplex PCR to detect mixed infections, cloned targets of *E. granulosus* or *E. multilocularis* were mixed in different ratios with the respective target of *Taenia* spp. Hence, 100 targets of both *Echinococcus* species yielded the expected amplicons in reactions containing the *Taenia* target in excess of a 100 or a 1000 times (not shown).

Confirmation of the specificity of amplicons from the multiplex PCR

Sequence analyses (after cloning) of the 117 bp diagnostic amplicon produced using primer pair Cest3/Cest5 revealed that *E. vogeli* can be discriminated readily from *E. granulosus*. Furthermore, within *E. granulosus*, it is possible to delineate cluster G1-3, genotypes G4 and G5. The corresponding sequence of the *E. granulosus* cluster G6/7 allows it to be discriminated from these genotypes but not from genotype G10. All sequences within a genotype or a genotype cluster, either retrieved from GenBank or determined in this study, were identical.

Sequence analyses (direct sequencing) of the 267 bp amplicon using the internal sequencing primer $Cest5_{seq}$ can identify unequivocally all target species. Furthermore, a NEBcutter analysis revealed that RFLP can be used identify most of these parasites (Table 4). Hence, the PCR products of *T. hydatigena*, *T. multiceps*, *T. serialis*, *T. crassiceps* and *Diphyllobothrium* spp. were exclusively cleaved by different commercially available endonucleases (Table 4A). Other enzymes cleaved the amplicons of several of the target organisms (Table 4B), and the digestion patterns using different enzymes can be used to deduce the identity of the organism. For example, *T. ovis* can be identified by combining an *Alu*I digestion (yielding fragments of 183 and 84 bp, which might not be distinguishable upon gel electrophoresis from the corresponding amplicons of 167 and 100 bp of *M. corti*) with an *Apo*I digestion (which cleaved the amplicon of *M. corti* but not the one of *T. ovis*). Finally, some enzymes cleaved the amplicons of all but 1 of the target organisms (Table 4C), allowing the identification of *T. taeniaeformis* (*Tse*I), *Diphyllobothrium* spp. (*Fok*I), *Dipylidium caninum* (*CviA*II, *Fat*I, *Nla*III) and *M. corti* (*Ssp*I) based on the absence of cleavage with the specific enzymes given in parentheses.

Validation of the multiplex PCR using DNA from faecal samples

In all 55 samples microscopically positive for taeniid eggs, the assay produced at least 1 band after gel electrophoresis and was able to detect mixed infections (Table 5; Fig. 1).

Multiplex PCR gave negative results using DNAs from faecal samples from 5 helminth-free dogs (group 1, Table 1). The expected results were obtained using samples from 2 groups of animals experimentally infected with *E. multilocularis* or *T. multiceps* (Table 1, groups 2 and 3). The results for

Table 4. Restriction fragment length polymorphism (RFLP) analysis of the 267 bp amplicon from multiplex PCR: useful restriction endonucleases which are commercially available and organisms identifiable

(Th: *Taenia hydatigena* ($n=3$): AB027135, AB031352, IPZ11 (adult, Kazakhstan, collection Institute of Parasitology, Zurich); To: *T. ovis* ($n=3$): *T. ovis*1, 2 (von Nickisch-Rosenegk *et al.* 1999*b*), DQ408421; Tm: *T. multiceps* ($n=1$): DQ408418; Tpo: *T. polyacantha* ($n=2$): *T. polyacantha* (von Nickisch-Rosenegk *et al.* 1999*b*), DQ408419; Ts: *T. serialis* ($n=1$): *T. serialis* (von Nickisch-Rosenegk *et al.* 1999*b*); Tt: *T. taeniaeformis* ($n=7$): AB120128, AB031354, AB027134, L49443, DQ408425, IPZ45, IPZ46 (metacestodes from *Arvicola terrestris*, Switzerland). Sequence L49442 which shows a considerable different restriction pattern than the other 7 available sequences of this species was omitted from the analysis; Tpi: *T. pisiformis* ($n=2$): AB031353, IPZ3 (adult, Kazakhstan); Tc: *T. crassiceps* ($n=2$): AB031358, NC002547; Diph: ($n=2$): *Diphyllobothrium ditremum* (AB031366), *Diphyllobothrium* sp. (L49458); Dc: *Dipylidium caninum* ($n=2$): L49460, AB031362; Meso: *Mesocestoides corti* ($n=1$): AB031363.)

Taxon	Th	To	Tm	Tpo	Ts	Tt	Tpi	Tc	Diph	Dc	Meso
A. Restriction sites (fragment sizes) unique for a taxon											
*Cac*8I									144/123		
*Cje*NII	164/103										
*Hinc*II					234/33						
*Hpa*I					234/33						
*Hpy*CH4V									142/125		
*Hpy*188I								190/77			
*Nsp*I									146/121		
*Rsa*I			193/74								
*Sca*I			193/74								
*Tat*I			191/76								
B. Restriction sites (fragment sizes) found in several taxa											
*Alu*I		183/84			153/114				141/126	139/128	167/100
*Apo*I							226/41		163/104		199/68
*Dde*I									150/93 93/34	138/129	
*Hpy*188III			205/62	201/66	203/64						
C. Restriction enzymes that do not cleave single taxons: size of largest fragment obtained in the other taxa											
*ApeK*1	139	137	135	138	137	—	137	138	138	136	138
*CviA*II	156	158	160	157	158	156	158	157	143	—	157
*Fat*I	155	157	159	156	157	155	157	156	142	—	156
*Fok*I	222	222	222	222	222	222	222	222	—	222	222
*Nla*III	159	161	163	160	161	159	161	160	146	—	160
*Ssp*I	111	182	182	112	182	103	182	104	112	134	—
*Tse*I	139	137	135	138	137	—	137	138	138	136	138

group 2 (*E. multilocularis*) were independently verified using an established, specific single-target PCR.

The multiplex PCR results achieved using DNA from faecal samples from 3 different groups of naturally infected dogs are given in Table 5. Four dogs from Nigeria (group 4) yielded amplicons of 267 bp only, and sequencing revealed the presence of *T. hydatigena* in all cases.

From the 17 taeniid egg-positive samples from the Kazakh dogs (group 5), multiplex PCR identified *Taenia* spp. in 16, and *E. multilocularis* or *E. granulosus* in each of 4 dogs. Except for 1 infection with *E. granulosus*, all *Echinococcus* spp. infections were double infections with *Taenia* spp. All 4 infections with *E. multilocularis* were confirmed using the single-target PCR. *E. granulosus* infection was verified in 3 of 5 samples by the single-target PCR specific for *E. granulosus* 'sheep strain'. Sequencing of the multiplex PCR amplicons of the other 2 samples revealed the presence of another genotype of *E. granulosus* (representing cluster G6/G7), which, to the best or our knowledge, is the first report of this genotype from Kazakhstan. The results of arecoline hydrobromide purgation (*cf.* Stefanic *et al.* 2004) correlated only weakly with the PCR results (Table 5). Hence, 6 of the 17 dogs investigated were negative for taeniids upon purgation, but did shed taeniid eggs based on microscopical examination after the sieving procedure. From 4 dogs diagnosed with *E. multilocularis* infection by the PCRs, this tapeworm had been diagnosed in only 2 of them by purgation. Also, *Taenia* spp. had been identified by purgation in only 4 of 16 dogs infected. In contrast, purgation revealed the presence of (most probably immature) *Taenia* spp. or *Echinococcus* spp. in one dog which tested negative by the PCRs.

As expected, all samples from Spain (group 6) were negative for *E. multilocularis* in the multiplex PCR. Of the 12 samples positive for *E. granulosus* in this PCR, 11 were positive in the single-target PCR for *E. granulosus* 'sheep strain', and another represented *E. granulosus* (cluster G6/7) by sequence analysis. These 12 samples originated from dogs known to be infected based on necropsy. Two

Table 5. Validation of the multiplex PCR with faecal samples from naturally infected dogs from Nigeria (group 4), Kazakhstan (group 5) and Spain (group 6): results of multiplex PCR, single target PCR, purgation/necropsy data and sequence analysis

Group[a]	No.[b]	Multiplex PCR			Single PCR[c]	Single PCR[d]	Purgation		Necropsy		Sequencing
		Em	Tae	Eg	Em	Eg	*Taenia*	*Echinococcus*	*Taenia*	*E. granulosus*	
4	4	Neg	pos	neg	nd	neg	nd	nd	nd	nd	*T. hydatigena*
5	2[e]	Neg	pos	neg	neg	neg	neg	neg	nd	nd	nd
	4	Neg	pos	neg	neg	neg	pos	neg	nd	nd	nd
	1	Neg	pos	neg	neg	neg	pos	pos	nd	nd	nd
	1	Neg	pos	neg	neg	neg	neg	neg	nd	nd	nd
	1	Pos	pos	neg	pos	neg	neg	neg	nd	nd	nd
	1	Pos	pos	neg	pos	neg	pos	neg	nd	nd	nd
	1	Pos	pos	neg	pos	neg	neg	pos	nd	nd	nd
	1[e]	Pos	pos	neg	pos	neg	pos	pos	nd	nd	nd
	1	Neg	pos	pos	neg	pos	neg	pos	nd	nd	nd
	1[e]	Neg	neg	pos	neg	pos	pos	pos	nd	nd	nd
	1[e]	Neg	pos	pos	neg	pos	neg	pos	nd	nd	nd
	2	Neg	pos	pos	neg	neg	neg	neg	nd	nd	*E. granulosus* G6/7 ($n=2$)
6	6	Neg	pos	pos	nd	pos	nd	nd	pos	pos	*E. granulosus* cluster G1-3[f]
	1	Neg	pos	neg	nd	neg	nd	nd	pos	neg	nd
	1	Neg	neg	pos	nd	pos	nd	nd	neg	pos	nd
	3	Neg	pos	neg	nd	neg	nd	nd	pos	pos	nd
	1	Neg	neg	pos	nd	pos	nd	nd	pos	pos	nd
	1	Neg	neg	pos	nd	neg	nd	nd	neg	pos	*E. granulosus* G6/7
	3	neg	pos	pos	nd	pos	nd	nd	neg	pos	*T. hydatigena* ($n=2$) *T. pisiformis* ($n=1$)

[a] See Table 1.
[b] Number of investigated dogs.
[c] Single target PCR specific for *E. multilocularis* (Stieger *et al*. 2002).
[d] Single target PCR specific for *granulosus* 'sheep strain' (Stefanic *et al*. 2004).
[e] Gel electrophoresis of 4 of these sample is shown in Fig. 1.
[f] Two samples were verified for checking purposes.
nd, not done.

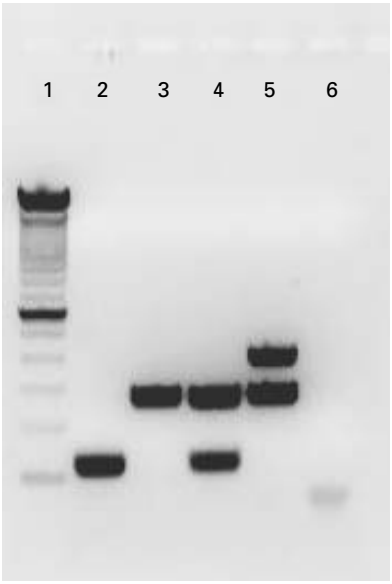

Fig. 1. Multiplex PCR using DNA from taeniid eggs from faecal samples from naturally infected dogs from Kazakhstan (primer sequences and reaction conditions; see Table 2). Lane 1, 100 bp ladder; lane 2, infection with *Echinococcus granulosus* only; lane 3, single infection with *Taenia* spp.; lane 4, mixed infection of *E. granulosus* and *Taenia* spp.; lane 5, mixed infection with *E. multilocularis* and *Taenia* spp. (for further information on these samples; see Table 5); lane 6, negative control reaction.

samples with (possibly immature) worms at necropsy did not show any band in the multiplex PCR. Three samples which were negative at necropsy were positive for *Taenia* spp. using the multiplex PCR, results which were confirmed by sequencing (*T. hydatigena* and *T. pisiformis* being detected).

DISCUSSION

Multiplex PCR is being widely used for the diagnosis of bacterial and viral diseases, but has infrequently been used for the diagnosis of parasitic helminth infections. Its applicability to simultaneously detect several organisms has, to the best of our knowledge, been demonstrated once for the diagnosis of cestodes from faecal material. Yamasaki and colleagues (2004) devised a multiplex PCR for differential diagnosis of taeniasis and cysticercosis of humans (*T. saginata*, *T. asiatica* and *T. solium*).

The principal aim of this work, namely the development of a multiplex PCR for unequivocal identification of taeniids (*Echinococcus* spp. and *Taenia* spp.) from carnivores was not entirely achieved. Both the primers for detecting *Taenia* spp. or *E. granulosus* are not strictly specific for their intended target organisms. The *Taenia* spp. primers also detect some non-taeniid cestodes, whereas the *E. granulosus* primers also amplify DNA from *E. vogeli*. In addition, we cannot exclude the possibility that DNA from *E. oligarthrus* or from the recently described *E. shiquicus* (Xiao *et al.* 2005) can be amplified, as neither sequence information nor DNA samples were available.

The mitochondrial genome was chosen as target, as the most information was available for its sequences (*cf.* McManus *et al.* 2004). As the mitochondrial genes of the species of the genus *Taenia* differ considerably, it was not possible, despite extensive *in silico* analyses and optimization of the reaction conditions, to derive primers which recognize all *Taenia* spp. but not other non-taeniid cestodes. DNA from *Mesocestoides* spp., *Dipylidium caninum* and *Diphyllobothrium latum* yielded fragments of the expected sizes in the multiplex PCR, despite the fact that the forward primer (Cest3) has a mismatch at the 3′-end and/or several internal ones and the reverse primer (Cest5) has 2 internal mismatches compared with the sequence for *Dipylidium caninum* (no sequence information is yet available about the region of this primer of the other organisms). Nonetheless, our diagnostic approach consists of an initial isolation of taeniid eggs by sequential sieving (Mathis *et al.* 1996) and subsequent, careful microscopical examination of the samples, with only taeniid egg-positive samples being tested further. This isolation procedure removes eggs of the other carnivore cestodes which would be positive by multiplex PCR (*Mesocestoides* spp., *Dipylidium caninum*, *Diphyllobothrium latum*). Nevertheless, in samples containing taeniid as well as other cestode eggs, a definitive diagnosis can be achieved by further analyses of the amplicons from the multiplex PCR (sequencing of cloned amplicons or RFLP in some instances; see Table 4). Similarly, sequence analysis can identify *E. vogeli*, which gives rise to an amplicon with the primers originally designed to be specific for *E. granulosus*.

The validation process with faecal samples of different origins showed that the multiplex PCR detected all samples positive in the single target PCRs specific for *E. multilocularis* or *E. granulosus* 'sheep strain'. The comparison with traditional diagnosis after purgation (Stefanic *et al.* 2004) proved to be very difficult in some cases. The present PCR approach (which is reliant on the amplification of DNA from taeniid eggs) does not detect pre-patent infections. Also, shedding of eggs does not occur consistently during patency, particularly for *Taenia* spp. Moreover, the sensitivity of purgation has been shown to be ~65% for *E. granulosus* (see Schantz, 1997) and has not been determined for *E. multilocularis*. In 6 of the 17 samples from Kazakhstan, which were positive for taeniid eggs, no worm had been found upon purgation. The multiplex assay detected *Taenia* spp. in all 6 samples and mixed infections with *Echinococcus* spp. in 3 of them. Necropsy, which served as a reference procedure in previous studies, yielded conflicting results in 3 of 16 samples. The 3 dogs negative for *Taenia* spp.

by necropsy revealed eggs of *T. hydatigena* and *T. pisiformis* based on multiplex PCR and sequence analyses. In such cases, an intestinal passage of eggs after coprophagy is most likely to have occurred, as the presence of adult *Taenia* spp. cannot be overlooked upon necropsy of fresh carcases.

The optimized multiplex PCR is highly sensitive, as single taeniid eggs could be detected, in accordance with other single-target PCR-based tests (Dinkel *et al*. 1998; Abbasi *et al*. 2003). As few as $3{\cdot}3 \times 10^1$ targets could be reliably amplified in the multiplex PCR, as assessed by spiking reactions with cloned targets. In order to determine how many of the mitochondrial target sequences are present in a single taeniid egg, a quantitative PCR was performed revealing ~7000 copies for *T. hydatigena*. Correspondingly, ~140 target copies are present in a multiplex PCR sample using 1/50 (2 of 100 μl) of the DNA isolated from a single egg. This number is somewhat higher than the detection limit. Therefore, a single egg should be readily identified with our approach. These data corroborate our diagnostic strategy to sieve all faecal samples, allowing to both concentrate taeniid eggs from large amounts of faeces or environmental samples and also to remove PCR-inhibitory components which are of major concern when investigating such samples (*cf*. Mathis *et al*. 1996). Indeed, all the 55 samples positive herein for taeniid eggs based on microscopical examination were also positive by multiplex PCR.

This assay, combining sieving and PCR, is relatively labour intensive and may not be suitable for routine diagnostic or large-scale purposes. However, the present multiplex PCR assay might serve as the method of choice for identification of taeniid eggs recovered from faecal specimens by the classical methods performed in diagnostic laboratories. The presence of eggs is directly related to the risk of infection and, therefore, egg identification is of particular interest in the context of diagnosis in individual carnivores as well as for epidemiological studies. Eggs are resistant and can be identified by PCR after prolonged storage at −20 °C (as shown in this study using samples stored for more than 10 years) as well as from environmental specimens (Stieger *et al*. 2002). Coproantigen tests have a low positive predictive value in populations with a low prevalence of *Echinococcus* spp. (Christofi *et al*. 2002; Hegglin *et al*. 2003). Therefore, in successful control programs, PCR is of significant value for confirmatory purposes. The present multiplex PCR can detect mixed infections of *E. granulosus*, *E. multilocularis* and *Taenia* spp. and, thus, is of great value in areas of co-endemicity.

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