Molecular tools for studies on the transmission biology of *Echinococcus multilocularis*

P. DEPLAZES[1]*, A. DINKEL[2] *and* A. MATHIS[1]

[1] *Institute of Parasitology, University of Zürich, 8057 Zürich, Switzerland*
[2] *Department of Parasitology, University of Hohenheim, 70599 Stuttgart, Germany*

SUMMARY

Two novel approaches for diagnosis of intestinal *Echinococcus multilocularis* infection, the detection of *E. multilocularis*-specific coproantigens in ELISA and of copro-DNA by PCR, have been successfully implemented. These methods have proven their value for the *post mortem* and the *intra vitam* diagnosis of *E. multilocularis* in definitive hosts. They have also made novel approaches possible to study the transmission biology of the parasite as they allow detection of the infection in faecal samples collected in the environment. Coproantigen detection is the diagnostic method of choice as it is sensitive, fast and cheap. Studies on faecal samples collected in the field revealed that coproantigen detection did reflect the different prevalences in fox populations as assessed from foxes at necropsy and also the effect of deworming efforts in foxes as achieved by long-term distribution of praziquantel-containing baits. The use of PCR for routine diagnostic or large-scale purposes is hampered by the fact that DNA extraction from faecal material is a very laborious task. Therefore, PCR is rationally used for confirmatory purposes of copro-antigen-positive samples. As taeniid eggs cannot further be differentiated morphologically, PCR is the method of choice to identify *E. multilocularis* infections in faecal or environmental samples containing taeniid eggs. In intermediate rodent hosts, PCR is routinely used in epidemiological studies for identifying *E. multilocularis* from liver lesions which are often very small, atypical or calcified.

Key words: *Echinococcus multilocularis*, transmission, diagnosis, coproantigen, PCR.

INTRODUCTION

The life cycle of *Echinococcus multilocularis* is perpetuated by rodents as intermediate hosts and foxes as definitive hosts in a predominantly wildlife cycle. A semidomestic or synanthropic cycle with domestic carnivores (dogs) as the definitive host may occur and was documented to be of major importance in a few geographic areas (such as parts of Alaska and China). For studying the transmission biology of this helminth, different methods to detect it both in the definitive carnivore hosts and the rodent intermediate hosts can be applied. Hence, parasite transmission can be assessed by detecting egg contamination of faecal and environmental specimens or by diagnosing the infection in intermediate or aberrant hosts such as pigs (Deplazes & Eckert, 2001).

For diagnosing the intestinal infection in definitive hosts after necropsy, the sedimentation and counting technique (SCT) is the 'gold standard' (Eckert *et al.* 2001*a*). This polyspecific method also allows a quantitative analysis of all intestinal helminths and to determine their developmental stages (e.g. larval, preadult, gravid stages). For mass screening of foxes, the intestinal scraping technique (IST), a somewhat less laborious technique with a sensitivity of 78% as compared to the SCT (Hofer *et al.* 2000), has been widely used. The obvious disadvantages of both these parasitological methods are the high logistical requirements. For example, about 4–10% of all fox carcasses delivered by hunters are unsuitable for *post mortem* examination due to decomposition of the intestine (personal communication T. Romig). The methods are also time consuming and require special safety precautions due to the infection risk for the investigator. Furthermore, a drawback of these methods is the fact that they can be applied to dead animals only. This renders these methods unsuitable for diagnosis in pet animals. Data collection by these strategies is strongly influenced by hunting regulations, and an increased hunting pressure can influence the structure of wild animal populations.

Serological screening using crude parasite antigens or affinity-purified Em2 antigen has been considered unsuitable for reliable diagnosis of intestinal *E. multilocularis* infections because of a poor correlation between the presence of antibodies and worms (Deplazes & Eckert, 1996). However, serology might be of value under certain circumstances to detect exposure of animals to the parasite.

The microscopical detection of worm eggs in faecal samples by routine coprological methods suffers from a low methodology-related sensitivity and is limited by the variable intensity of egg shedding.

* Corresponding author: Institute of Parasitology, University of Zürich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland. Tel: +41 01 635 85 01. Fax: +41 01 635 89 07. E-mail: pdeplaze@access.unizh.ch

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Furthermore, eggs of *E. multilocularis* cannot be differentiated morphologically from those of other taeniid worms. As outlined below, the analysis of such eggs by the polymerase chain reaction (PCR) can overcome this limitation.

Two alternative approaches, the detection of *E. multilocularis*-specific coproantigens and copro-DNA, have been successfully developed and evaluated. These allow the reliable diagnosis of *E. multilocularis* using faecal material from both necropsied and living definitive hosts (Table 1). An important additional advantage of these alternative methods is that they can also be applied to samples from the environment. This facilitates new approaches to investigate parasite transmission dynamics without interacting with the populations of wild or domesticated definitive hosts. Of crucial importance in this approach is the correct identification of the origin of field faecal samples. The biochemical analysis of bile acids by thin-layer chromatography has allowed the identification of fox faeces (Major *et al*. 1980) but this is a rather laborious approach. Morphological analysis of hair in faeces should in principle determine the species of large carnivores, as for example fox hair is not present in dog faeces. However, such an approach has not yet been evaluated for this purpose. In recent field studies, fox faeces were identified by assessing physical parameters such as size, shape, typical fox smell and the presence of food remnants. The reliability of this latter strategy was demonstrated by the different parasite spectra of putative fox and dog faecal specimens in a study on urban transmission of *E. multilocularis* which required the discrimination of fox faeces from faeces of dog origin (Stieger *et al*. 2002).

Another possibility to identify fox faeces could be the genetic analysis of faecal samples by PCR. Such an approach has been developed, for example for the Iberian lynx (Palomares *et al*. 2002), and has proven to be a reliable technique that can be used in large-scale surveys. To the knowledge of the authors no such approach is available yet for the identification of fox faeces. Such a PCR could be combined with an *E. multilocularis*-specific PCR into a diagnosis system designed for investigating faecal samples from the environment.

MOLECULAR DIAGNOSIS OF *E. MULTILOCULARIS* IN DEFINITIVE HOSTS AND IN SPECIMENS FROM THE ENVIRONMENT

Detection of coproantigen

Several groups have independently developed coproantigen tests for diagnosis of intestinal *Echinococcus* infections (reviewed by Craig, Rogan & Allan, 1996; Deplazes & Eckert, 1996). Tests originally developed for the diagnosis of *E. granulosus* showed cross-reactivity with *E. multilocularis* but lacked sensitivity (Allan *et al*. 1992; Deplazes *et al*. 1992). ELISAs using antibodies produced against *E. multilocularis* antigens have subsequently been developed and evaluated (Table 1) (Kohno *et al*. 1995; Sakai *et al*. 1998; Deplazes *et al*. 1999). One test-kit for the detection of both *E. multilocularis* and *E. granulosus* coproantigen has been commercialised (Chekit® Echinotest; Dr. Bommeli AG, 3097 Bern, Switzerland). Extended evaluations of this test for detecting *E. multilocularis* are currently being carried out in independent laboratories.

Although cestode coproantigens have not fully been characterized, they have been shown to be heat resistant (Nonaka *et al*. 1996) and predominantly protein-, carbohydrate- and lipid-rich molecules (Craig *et al*. 1996). Further chemical analyses are under way in different laboratories.

Coproantigens are detectable during the prepatent and the patent periods in dogs, foxes and cats, and disappear within a few days after the elimination of the cestodes from the host (Sakai *et al*. 1998; Deplazes *et al*. 1999). In a study with experimentally infected dogs and cats, coproantigens were first detectable 6–17 days p.i. in samples of 8 dogs (worm burdens at necropsy: 6330–43 200) and from 11 days p.i. onward in samples of 5 cats infected with 20–6833 worms (Deplazes *et al*. 1999). The sensitivity of this ELISA was 83·6% in 55 foxes infected with 4–60 000 *E. multilocularis*, but reached 93·3% in the 45 foxes harbouring more than 20 worms. This test identified those animals harbouring approximately 99·6% of the total number of adult *E. multilocularis* in a fox population investigated. The specificity of the ELISA with regard to other non-*Echinococcus* helminths was 95·0%–99·6% as shown by the examination of faecal samples from 32 foxes, 658 dogs and 262 cats. The specificity was also surprisingly high (84%) with samples of 32 dogs naturally or experimentally infected with *E. granulosus* (Deplazes *et al*. 1999).

A prerequisite for the usefulness of the detection of coproantigens not only in fresh but also in weathered samples from the environment is their stability. Such a stability of helminth coproantigens has been documented in two experimental studies. *T. hydatigena* antigens were stable for at least 5 days at room temperature (Deplazes *et al*. 1990), and the detection of *E. granulosus* coproantigens was not influenced by exposing the faeces for six days and nights to sun-exposed places in the Australian Capital Territory (Jenkins *et al*. 2000). Field studies have confirmed coproantigen stability. No significant differences have been found in the percentage of *E. multilocularis* coproantigen-positive field faecal samples of different estimated ages and of different composition (Sakai *et al*. 1998; Raoul *et al*. 2001; Stieger *et al*. 2002).

A number of studies have confirmed the usefulness of coproantigen detection in faecal samples

Table 1. Characteristics of test systems for diagnosis of *E. multilocularis* in definitive hosts

Test system	Test characteristics (SE: sensitivity; SP: specificity)	Approx. number of animals/samples investigated per person and day
Sedimentation and counting technique (SCT) (WHO, 2001)	SE and SP ≈ 100%. Application at necropsy, laborious for routine screening; polyspecific for intestinal helminths, precise quantification; Reference method	10 animals (necropsy included)
Intestinal scraping technique (IST) (Hofer *et al*. 2000)	SE 78% (compared with SCT); SP ≈ 100%. Application at necropsy; laborious; polyspecific for intestinal helminths; semiquantitative; Parasitological routine test at necropsy	20 animals (necropsy included)
Coproantigen ELISA (Deplazes *et al*. 1999)	SE ≈ 80% (compared with SCT); SP 95–99%. Allows *in vivo* and *post mortem* diagnosis and testing of field faecal samples, rapid and easy test, infection detectable in prepatent stage; Routine test for mass screening	200 samples
Coproantigen ELISA (Sakai *et al*. 1998)	SE ≈ 87% (compared with SCT), SP ≈ 70% (on genus level). Test characteristics see above	200 samples
Coproantigen ELISA (CHEKIT® Echinotest, Dr. Bommeli AG, CH-3097 Bern)	SE 60–80% (for *E. multilocularis* in foxes; Deplazes, unpublished), SP 80–95% (on genus level)*** Test characteristics see above	200 samples
Combined microscopy/PCR* (Mathis *et al*. 1996)	SE 94% (compared with SCT), SP 100%. Allows *in vivo* and *post mortem* diagnosis and testing of field faecal samples, laborious, in the first step (microscopy) polyspecific for helminth eggs, PCR detects only eggs; Confirmation test for coproantigen-positive results or for identification of taeniid eggs	15 samples
PCR* (Monnier *et al*. 1996)	SE 82% (compared with SCT), SP 96%. Allows *in vivo* and *post mortem* diagnosis and testing of field faecal samples, laborious, total DNA isolation from faeces allows to detect eggs and parasite tissue; Alternative method to necropsy and for confirmatory purposes	15 samples
PCR** (Dinkel *et al*. 1998)	SE 89% (compared with IST), SP 100%. Test characteristics as above	15 samples
PCR** (van der Giessen *et al*. 1999)	SE not evaluated, SP 100%. Test characteristics as above	15 samples

* Target gene: U1 snRNA gene (Bretagne *et al*. 1993); ** target gene: mt 12S rRNA gene (Dinkel *et al*. 1998); *** (Christofi *et al*. 2002).

collected from the environment. Coproantigen detection was used with fox faeces collected for monitoring the infection pressure in Hokkaido (Japan) (Nonaka *et al*. 1998; Morishima *et al*. 1999; Tsukada *et al*. 2000). Stieger and colleagues (Stieger *et al*. 2002) evaluated with field faecal specimens an EM-ELISA which had been validated with intestinal contents of foxes (Deplazes *et al*. 1999). The cut-off value was calculated to be slightly higher than in the original evaluation, possibly due to environmental influences on these faeces. Sensitivity of the test for patent *E. multilocularis* infections, as determined by PCR on isolated eggs (see below), was 88%. They tested a further 604 fox faecal samples from all over the City of Zurich resulting in 156 (28·8%) positive ones with a distinct increase in the proportion of positive samples from the urban to the periurban zone. Furthermore, samples collected in the border zone had significantly more coproantigen-positive results during winter. Both findings are consistent with prevalence data obtained from foxes at necropsy in an earlier study from the same area (Hofer *et al*. 2000). In France, parasite detection at necropsy in foxes, originating from two areas with significantly different prevalences of *E. multilocularis* (14·7% and 65·3%), was compared with

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coproantigen detection in faecal samples collected in the field using two types of ELISAs (Kohno *et al.* 1995; Deplazes *et al.* 1999). The results of both coproantigen tests were in the range expected from the necropsy data, and it was concluded that this new strategy can be applied in epidemiological studies and fundamental research on transmission ecology (Raoul *et al.* 2001).

In two studies, coproantigen detection in field samples was used to assess the effect of long-term distribution of praziquantel-containing baits on the prevalence of *E. multilocularis* in foxes (Tsukada *et al.* 2002; Hegglin, Ward & Deplazes, 2003). Both studies recorded significant decreases in the percentage of coproantigen-positive specimens from baited areas compared to control areas. This was paralleled in both studies by a decrease in egg count in fox faeces and the prevalence in intermediate hosts in the baited areas toward the end of the baiting period of more than 1 year.

Detection of copro-DNA

No commercially available kits exist for copro-DNA detection. Methods for the isolation of DNA from faecal samples and PCR assays that have been evaluated are described in detail below.

Sample preparation. Parasite DNA excreted with eggs, proglottids or parasite cells can be detected from faeces after amplification by PCR (virtually no cell-free DNA is present in faecal material due to the activity of the intestinal microflora). DNA isolation from faeces is either based on an alkaline lysis step (Bretagne *et al.* 1993; Dinkel *et al.* 1998) or on boiling the samples in 0·5% SDS and proteinase K digestion (van der Giessen *et al.* 1999). Due to the presence of substances that are inhibitory for DNA amplification, only a limited amount of material can be processed (0·5–4 g) with these methods, and extensive purification of the DNA is absolutely indispensable (phenol/chloroform extractions and use of DNA adsorbing matrices). Sensitivity of downstream PCR was reported to be 1 egg in 1500 μl of diluted fox faeces (Dinkel *et al.* 1998). However, several groups have reported that despite these high purification efforts some samples still exhibited very strong inhibitory effects on DNA amplification. For example, no conclusive PCR results could be obtained with 9 of 250 (3·6%) examined faecal samples due to inhibition (Dinkel *et al.* 1998). In another PCR-system, an increase in sensitivity from 24% to 82% was achieved (Monnier *et al.* 1996) after introduction of an additional DNA purification step to an already laborious protocol (Bretagne *et al.* 1993).

One approach to overcome the limitations of restricted specimen volume and PCR inhibition is to modify the original DNA isolation system (Bretagne *et al.* 1993) by including an initial step of concentrating helminth eggs by a combination of sequential sieving and an in-between step of flotation in zinc chloride solution (Mathis, Deplazes & Eckert, 1996). Hence, helminth eggs, which are highly resistant in the environment, can be concentrated from large sample volumes into a few μl of fluid and detected by means of an inverted microscope in a closed tube. As microscopic egg detection using this approach was shown to be very sensitive, only samples containing taeniid eggs need to be further investigated by PCR. DNA isolation from these eggs was achieved using a simplified protocol of the alkaline lysis method with no need for organosolvent extractions. No inhibition of the PCR was observed in 55 samples investigated as demonstrated by the amplification of a size-modified target in parallel reactions. The tests were undertaken with fresh faeces stored in 70% ethanol, but parasite detection was also possible after inactivation of eggs by deep-freezing ($-80\ ^{\circ}C$) or by incubation of the faeces at $+70\ ^{\circ}C$ for 2 h. Obviously, this approach is suitable for the diagnosis of gravid infections only with eggs being present in the faeces.

PCR. Two different genes have so far been targeted in diagnostic PCR for the detection of intestinal *E. multilocularis* infection in faecal samples of foxes, the U1 snRNA gene (Bretagne *et al.* 1993; Mathis *et al.* 1996; Monnier *et al.* 1996) and the mt 12S rRNA gene (Dinkel *et al.* 1998; van der Giessen *et al.* 1999) (Tables 1 and 2).

The specificity for *E. multilocularis* of the single primer pair targeting the U1 snRNA gene (Bretagne *et al.* 1993) was initially confirmed with other tapeworms (*T. crassiceps*, *T. taeniaeformis*, 2 isolates of *T. saginata*, 3 isolates of *E. granulosus* without identification of the strains). In other studies using these primers, no reaction products were found with Japanese isolates of *T. pisiformis* (1 isolate), *T. hydatigena* (2), *T. taeniaeformis* (3), *T. crassiceps* (1), one *E. granulosus* isolate originating from Uruguay (Yagi *et al.* 1996) and with 20 *E. granulosus* isolates of unknown origin (Monnier *et al.* 1996). However, in a recent investigation these primers were shown not to be strictly species-specific for *E. multilocularis* as a product of the expected size was also obtained with a horse strain of *E. granulosus* (van der Giessen *et al.* 1999).

When using these primers with DNA obtained from taeniid eggs which had been concentrated from faecal specimens of foxes, no false positive results were obtained (specificity 100%). In addition, 33 out of 35 samples from animals with proven infection (SCT) were also PCR positive (sensitivity 96%) (Mathis *et al.* 1996).

The original PCR protocol (Bretagne *et al.* 1993) was modified (Monnier *et al.* 1996) by introducing a second round of amplification with nested PCR using DNA isolated directly from faeces. Hence,

Table 2. Sequences of primers used for PCR-diagnosis of *E. multilocularis**

(Primer designation) Primer sequence (5′→3′)	Ref.	Diagnosis in definitive (DH) or intermediate (IH) hosts; comments
(BG1) TCATTCTGGTCACTCATTGTTCACC (BG2) GCAGTCTATTCTCCTCTCAACTGCC	1	IH; lack of diagnostic sensitivity when applied with faecal specimens (Gottstein, personal communication); confirmation by Southern hybridisation
GTGAGGCGATGTGTGGTGATGGAGA GAAGGCAAGTGGTCAGGGGCAGTAG	2	DH; may yield non-specific products when used with meta-cestode material containing host DNA (unpublished observation)
(PF9) CAAAGACGGCAATCCAA (PF18) CTACATCGACTCAAACTGTT	3	IH; used in RT-PCR with biopsy material
Outer primers (P60.for) TTAAGATATATGTGGTACAGGATTAGATACCC (P375.rev) AACCGAGGGTGACGGGCGGTGTGTACC Inner primers (Pnest.for) ACAATACCATATTACAACAATATTCCTATC (Pnest.rev) ATATTTTGTAAGGTTGTTCTA	4	DH, IH; used in two-tube nested PCR
Outer primers (Em-1) TAAGATATATGTGGTACAGGATTAGATACCC (Em-2) GGTGACGGGCGGTGTTGTA** Inner primers (Em-3) ATATTACAACAATATTCCTATC (Em-4) ATATTTTGTAAGGTTGTTCTA	5	DH; primers modified from (Dinkel *et al*. 1998) for use in one-tube nested PCR
(EM-H15) CCATATTACAACAATATTCCTATC (EM-H17) GTGAGTGATTCTTGTTAGGGGAAG	6	IH; primers modified from (Dinkel *et al*. 1998) for use in single PCR

* Diagnostic parameters see Table 1; ** additional T (four nucleotides from 3′ end) as compared to sequence of primer P375.rev and GenBank entries (AB018440, AB031351).
[1] (Gottstein & Mowatt, 1991); [2] (Bretagne *et al*. 1993); [3] (Kern *et al*. 1995); [4] (Dinkel *et al*. 1998); [5] (van der Giessen *et al*. 1999); [6] Mathis (unpublished).

application of this method resulted in an increase of sensitivity from 62% of the single primer pair PCR to 82% as determined with 17 proven positive samples. The specificity for *E. multilocularis* of the nested PCR amplifying part of the mt12S rRNA gene (Dinkel *et al*. 1998) was confirmed with *E. granulosus* (3 isolates, without strain identification), *T. crassiceps* (1), *T. hydatigena* (3), *T. martis* (2), *T. mustela* (1), *T. ovis* (1), *T. pisiformis* (1), *T. polyacantha* (2), *T. serialis* (1), *T. taeniaformis* (2), *Mesocestoides leptothylacus* (1) and several isolates of nematodes of fox origin (*Toxocara* sp., *Uncinaria* sp.). In this study, the specificity of the nested PCR for *E. multilocularis* was additionally confirmed by hybridisation of the PCR products from positive faecal samples with an internal probe. This test was extensively evaluated with total DNA extracted from rectal samples of 250 wild foxes. A specificity of 100% and an overall sensitivity of 89% as compared with the intestinal scraping technique (ICS) were obtained. Most interestingly, this test allowed the detection of prepatent infections with an overall sensitivity of 78% in 63 foxes. The sensitivity was dependent on the worm burden, reaching 100% with foxes harbouring more than 1000 immature worms and 70% with animals with less than 10 parasites.

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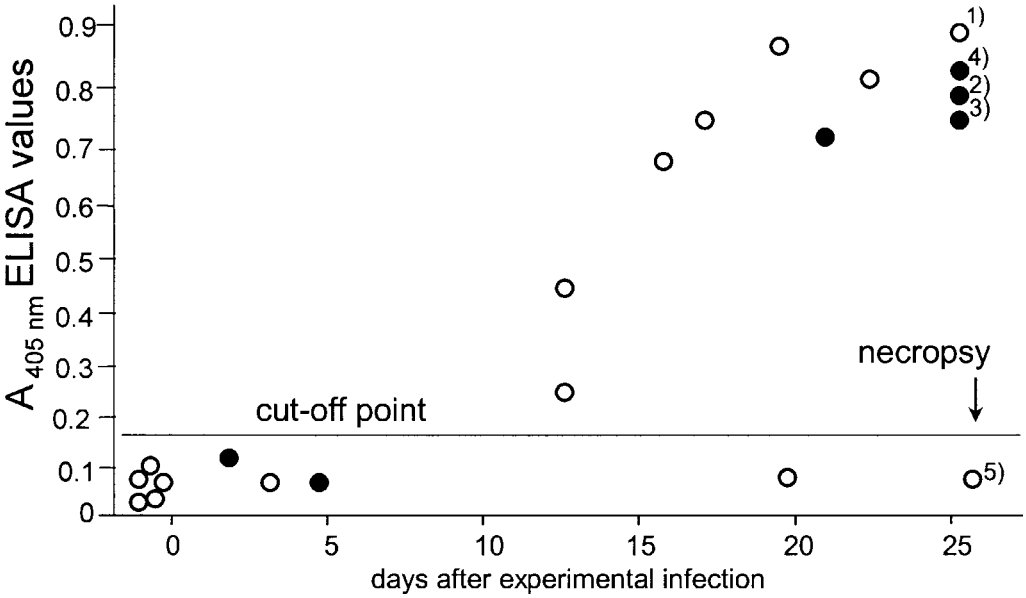

Fig. 1. Follow-up of coproantigen detection in ELISA (Deplazes *et al*. 1999) and amplification of copro-DNA by PCR (Dinkel *et al*. 1998) during experimental infections of 5 cats with *Echinococcus multilocularis* (Eckert *et al*. 2001*b*). Symbols represent individual faecal samples of the cats without animal identification; numbers identify individual samples of cats at necropsy. Worm burdens (mature, non-gravid worms) of the cats were: cat 1: 5720 worms; cat 2: 1475 worms; cat 3: 282 worms; cat 4: 6833 worms; cat 5: 20 worms. Open symbols represent PCR negative samples, solid symbols PCR positive samples (Deplazes and Dinkel, unpublished).

However, coproantigen and copro-DNA detection do not seem to correlate during the prepatent infection. As shown in Fig. 1, specific coproantigens were not detected by ELISA during the first days of infection in experimentally infected cats but PCR was positive in 2 of 3 samples. During the second third of the prepatent period 5 of 6 samples were coproantigen positive whereas only one turned out to be positive by PCR. At the end of the prepatent period (day 20–27), 3 of 7 highly coproantigen-positive samples were negative by PCR. At the beginning of the infection PCR probably detected protoscolices which did not establish and were excreted. The failure of coproantigen detection in these samples can be explained with the low concentration of coproantigens in protoscolex extracts (Deplazes *et al*. 1999). During the fast growing phase of the worms during the prepatent period high metabolic activities might be responsible for the high concentrations of coproantigens, but it seems that only little cellular parasite tissue was excreted during this time resulting in negative PCR results. Towards the end of the prepatent period, PCR-positive results indicate the excretion of worms or single proglottids.

In *E. multilocularis*, the intrinsic additional value of diagnostic PCR, the downstream identification of strains or genotypes, cannot be exploited as only a very small range of genetic heterogeneity has been discovered within numerous isolates by analysing various loci (both coding and non-coding regions) (Bowles, Blair & McManus, 1992; Bowles & McManus, 1993; Gasser & Chilton, 1995; Bretagne *et al*. 1996; Haag *et al*. 1997; Rinder *et al*. 1997; von Nickisch-Rosenegk, Lucius & Loos-Frank, 1999; van Herwerden, Gasser & Blair, 2000). No biological differences have so far been attributed to a distinct genotype. Furthermore, results on the relation of genotypes and geographic distribution are conflicting. Whereas 11 isolates investigated could be grouped into the traditional subspecies *E. multilocularis multilocularis* and *E. multilocularis sibiricensis* according to their origin and genotype (two genotypes differing at one polymorphic site in 1·3 kb of the rDNA locus) (Rinder *et al*. 1997), the geographical distribution of genotypes A and B, as determined by sequencing several loci (Haag *et al*. 1997), did not follow the pattern of the conventionally accepted North American and European strains. Similarly, only one single polymorphic site in part of the mt 12S rRNA gene was identified amongst 22 isolates of *E. multilocularis*, which reflected neither the geographic origin nor the intermediate host species from which the metacestodes were isolated (von Nickisch-Rosenegk *et al*. 1999). For epidemiological and transmission studies, microsatellites might be valuable genetic markers. Bretagne and colleagues (Bretagne *et al*. 1996) have identified microsatellite polymorphisms in the region upstream of the highly repetitive U1 snRNA gene yielding three different patterns among parasite isolates, but *E. multilocularis* microsatellites of single-copy loci need to be known for practical studies.

PCR not only allows us to distinguish *E. multilocularis* eggs from the morphologically identical eggs of other taeniids, but it also offers the possibility to

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determine egg numbers. However, due to the intermittent shedding of eggs, such a quantitative PCR, which to our knowledge has yet to be established, is of limited use for investigating single faecal samples from few animals. However, such an approach might contribute to transmission/epidemiological studies when employed on a larger scale in populations of definitive hosts over a prolonged period of time.

Diagnostic strategy

For large-scale screenings of definitive hosts the choice of the diagnostic methods has to consider economics, methodology and logistics (e.g. storage of material, stability of material and transport). Screening tests should be highly sensitive, fast and cheap. The coproantigen detection by ELISA fulfils these requirements and has been shown to be useful for large-scale investigations. In animal populations with a low prevalence of *E. multilocularis*, such as dogs and cats, ELISA results have a very high negative predictive value but low positive predictive value. Therefore, positive ELISA results need further confirmation with the more laborious PCR. Such a PCR can be done with total DNA isolated from all coproantigen-positive samples (with the inherent risk of co-isolating PCR-inhibitory substances) or with DNA from only those samples from which taeniid eggs could be obtained after concentration by a sieving/flotation method (with the risk to obtain false-negative PCR results for prepatent infections). The latter strategy has been successfully applied in dog and cat populations (Deplazes *et al*. 1999; Gottstein *et al*. 2001). As an alternative to this ELISA-based approach, the microscopical detection of taeniid eggs concentrated by conventional methods or by using the sieving/flotation system (Mathis *et al*. 1996) could be used followed by investigating samples containing taeniid eggs by PCR. This strategy can also be applied for the investigation of environmental (dust, earth, sand, food and water) samples.

MOLECULAR DIAGNOSIS OF *E. MULTILOCULARIS* IN INTERMEDIATE HOSTS

Monitoring the prevalence in intermediate hosts has been performed in recent studies aimed at determining spatial and temporal transmission of the parasite (Stieger *et al*. 2002) or at studying the effects of long-term anthelmintic baiting of foxes with praziquantel (Tsukada *et al*. 2002; Hegglin *et al*. 2003). The diagnosis of *E. multilocularis* metacestode infections in necropsied rodents is based on pathognomonic macroscopic and histological (HE- and PAS-stain) findings. However, very small, atypical or calcified liver lesions are recalcitrant to these methods. Specific metacestode antigen (Em2G11) can be detected in such cases by visualisation of fragments of the laminated layer using the EmG11 monoclonal antibody on squashed metacestode material (Hofer *et al*. 2000) or by a sandwich-ELISA (Deplazes & Gottstein, 1991). The method of choice for identifying *E. multilocularis* from such lesions, however, is PCR using proteinase K digested lesion material. Gottstein and colleagues (Gottstein *et al*. 2001) used primers derived from a specific DNA probe (Gottstein & Mowatt, 1991) in their survey in intermediate hosts from an area with high prevalence. In other studies, a nested PCR (Dinkel *et al*. 1998) or a single PCR with the slightly modified inner primer pair EM-H15 and 17 (Table 2) were used. Stieger and colleagues (Stieger *et al*. 2002) investigated 161 unidentifiable liver lesions from *Arvicola terrestris* resulting in 55 *E. multilocularis* positive results in addition to the classically diagnosed 26 infected animals. PCR examination of DNA isolated from 373 unidentifiable liver lesions originating from several intermediate host species trapped in an area of low endemicity yielded 11% *E. multilocularis* positive results (Dinkel, 1998). A study carried out in an area of high endemicity examining 386 *Arvicola terrestris* resulted in 35 positive animals (9·1%), of which 27 showed immature or non-fertile lesions only diagnosable by PCR (Dinkel *et al*. 1996; Merli *et al*. 1996). In an experimental study on developmental aspects of *E. multilocularis* metacestodes in the common vole *Microtus arvalis* (Merli *et al*. 2001) it was shown that species diagnosis for lesions <2 weeks old could only be achieved by PCR.

The primers described by Bretagne and colleagues (Bretagne *et al*. 1993) for PCR-detection in faecal samples yielded non-specific products when used on rodent liver specimens.

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