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Development of a new PCR protocol for the detection of species and genotypes (strains) of *Echinococcus* in formalin-fixed, paraffin-embedded tissues ☆

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Abstract

The aim of our study was to establish a new PCR protocol for the detection and discrimination of *Echinococcus granulosus* complex on one hand and *Echinococcus multilocularis* in formalin-fixed, paraffin-embedded tissues (FFPTs) on the other. The target sequences for all PCRs are located on a 471 bp segment of the mitochondrial ND1 gene, the fragment sizes of the amplification products are 295 bp (for the sheep strain of *E. granulosus*), 204 bp (for the pig strain of *E. granulosus*) and 252 bp (for *E. multilocularis*), respectively. In total, 80 FFPTs from patients with histologically confirmed echinococcosis (76 with *E. granulosus* and four with *E. multilocularis*) operated on in Austrian hospitals between 1978 and 2005 were examined. In 68 (85%) samples, we were able to detect specific DNA fragments with our newly established PCR protocols. Thirty-eight (47.5%) of 80 clinical samples were identified as the G1 strain, 26 (32.5%) as the G5, 6 or 7 strains and four (5%) as *E. multilocularis*. The specificity of all three PCRs was 100%; for the discrimination between G6 and G7 strains, sequencing of an additional 234 bp PCR fragment was necessary and showed that three out of 26 G5, 6 or 7 PCR-positive patients were infected with *E. granulosus* genotype G6 (the camel strain).

Keywords: *Echinococcus granulosus*; Human hydatid disease; PCR; Formalin-fixed paraffin-embedded tissues; Genotypes; PCR; Austria

1. Introduction

Cystic echinococcosis (CE) and alveolar echinococcosis (AE), caused by metacestode stages of *Echinococcus granulosus* and *Echinococcus multilocularis,* respectively, belong to the most serious helminth zoonotic diseases of man. Both echinococcoses are prevalent in Central European countries; the incidence, however, is rather low. In Austria, 0.4 cases of CE and 0.03 cases of AE are diagnosed per 100,000 inhabitants every year (Auer and Aspöck, 2002).

Although the diagnostic procedure for CE as well as for AE includes clinical, radiological (ultrasound, computer tomography, magnetic resonance) and particularly serological examinations, i.e. ELISA and Western blot, carried out before (surgical) treatment, the definitive diagnosis is usually based on pathological–histological analysis of particularly periodic acid Schiff (PAS-) stained specimens of surgically resected tissues. However, histological examinations are hampered by two difficulties. First, *Echinococcus*

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cysts may be parasitologically sterile and contain neither protoscoleces nor rostellar hooklets, so that sometimes the pathologist is not able to differentiate between the two species *E. granulosus* and *E. multilocularis*. Second, to date, eight genotypes (numbered G1–G3, G6–9 and G10), and two species, *Echinococcus equinus* (G4) and *Echinococcus ortleppi* (G5), of different pathogenicity to humans, have been identified within the former species *E. granulosus*. However, strain differentiation is not possible using histological analysis and is only feasible by molecular methods (Bowles and McManus, 1993; Dinkel et al., 2004; Obwaller et al., 2004).

The usefulness of PCR methods for the identification of *E. granulosus* eggs in dog faeces (the definitive host) has been reported in several recent publications and might be applied in cystic hydatid disease control programs (Abbasi et al., 2003; Štefanič et al., 2004; Naidich et al., 2006).

The aim of our study was to establish a sensitive and specific PCR protocol for the detection and inter- and intraspecific identification of *Echinococcus* spp. from humans with formalin-fixed, paraffin-embedded tissues (FFPTs) as a source of DNA. We examined 80 FFPTs from patients with histologically confirmed echinococcosis to evaluate the accuracy of molecular biological tools on one hand and the validity of histological examinations on the other hand.

2. Materials and methods

2.1. Patients

FFPTs from 80 patients with histologically confirmed echinococcosis, operated on between 1978 and 2005 in Austrian hospitals, were investigated in this study. Seventy-six of the 80 patients were identified as being infected with CE and four with AE by histo-pathological examination (detection of PAS-positive laminated layers and/or of protoscoleces and/or hooklets) of the resected tissues. Sixty percent of the patients were female, 40% male and the age range was 5–78 (average 42) years. The majority of the patients (36%) were of Austrian origin, 25% of Turkish origin, 24% derived from former Yugoslavia and the rest from various other countries. Details on cyst localisation (liver, lung, spleen and heart), country of origin and year of the operation of all patients are summarised in Supplementary Table S1.

2.2. DNA extraction

For each patient, two 10 μm thick sections were prepared from tissue blocks and excess paraffin was trimmed. We used disposable razorblades and cleaned carefully all equipment that might come in contact with the samples between different tissue blocks. Sections were placed in 1.5 ml microtubes and deparaffinized with 1 ml xylene for 10 min at 37 °C. Subsequently, samples were centrifuged at 1500*g* for 5 min and the supernatant was removed. This procedure was repeated once. After deparaffinization, rehydration in 100%, 90%, 80% and 70% ethanol followed. Thereafter 70% ethanol was removed and tissue lysis solution was added (Master Pure DNA Purification Kit, Epicentre). For DNA extraction, we chose a commercially available DNA extraction kit based on a simple salting out procedure, a method that gives good results with FFPT as a source of DNA (Miller et al., 1988; Howe et al., 1997), and avoids the hazardous organic solvents used in the traditional phenol/chloroform extraction. We used the manufacturers' protocol for paraffin-embedded tissues with a modification of the proteinase K step. The modification consisted of a prolonged (up to 48 h) digestion and repeated addition of 2 μl aliquots of proteinase K (50 mg/ml) every 8 h until lysis was complete (Iudica et al., 2001). The final proteinase K concentration was approximately 500 μg/ml. The incubation was performed with constant agitation at 56 °C (Thermomixer comfort, Eppendorf) and terminated with a 10 min incubation at 95 °C to inactivate the proteinase K. After drying the DNA was resuspended in 35 μl TE buffer (Master Pure DNA Purification kit, Epicentre).

2.3. Polymerase chain reaction

2.3.1. Primers

All newly established PCR protocols amplify partial regions of the 471 bp segment of the mitochondrial ND1 gene (Bowles and McManus, 1993). Three species/strain-specific primer sets were designed to amplify *E. granulosus* G1 (shPCR), *E. granulosus* G7 (swPCR) and *E. multilocu-*

Table 1
List of PCR primers used in this study

Primer	Sequence (5′–3′)	Annealing temperature (°C)	Specificity	Size of PCR product (bp)
em PCR	EM29F GATTTGCTGATTTGTTAAAGTTAGTGATC EM281R AGAACTTAAAAACGAATATTTATTGTAACT	56	*Echinococcus multilocularis*	252
sh PCR	SH172F GTTATAGAGGCCTCTCCGTGTTGTGG SH467R CGTACGATTAGTTTCACACAATATACATAT	57	*Echinococcus granulosus* G1	295
sw PCR	SW163F ATAGAGTTAGTTATAGTATGCTTTCTG SW367R TTATACCCACAACAGCATAAAGCGC	58	*E. granulosus* G6/7	204
G6/7 PCR	G6/7F TGGGGTAGTTACAATAGTTATTC G6/7R CATAATCAAATGGAGTACGATTA	53	*E. granulosus* G6/7	234

laris (emPCR), respectively. The criteria used for primer selection were: (i) the resulting amplification products should be less than 300 bp in length; (ii) the sizes of the PCR fragments for each species/strain should differ from each other by at least 40 nucleotides to allow unambiguous separation by size on an ethidium bromide stained gel; and (iii) 100% species/strain-specific amplification was expected. The primers were designed manually and located in regions with relatively high variations between the strains mentioned above, especially at the 3́ end of the primers. For all swPCR-positive samples, we had to amplify an additional 234 bp fragment of the 471 bp segment of the ND1 gene including two specific nucleotide positions in order to discriminate between the G6 and G7 strains (Table 1).

2.3.2. Amplification

To avoid carry over contamination we substituted dUTP for dTTP (Carry over Prevention kit, Applied Biosystems) during all PCRs and treated all reactions with the enzyme uracil-*N*-glycosylase (UNG). UNG was inactivated at the heat-denaturation step in normal temperature-cycling, so that new PCR products, which contained many dUs (Carry over Prevention kit, Applied Biosystems), were unaffected. We used the commercially available Carry over Prevention Kit (Applied Biosystems). Moreover, false positive results were prevented by the use of aerosol-resistant pipette tips in all pre-PCR steps and strict separation of pre- and post-PCR work areas.

Hot-start PCR was performed in a 25 µl vol. containing 1× Ampli Tag Gold Buffer, 0.01% BSA (Roche), 200 µM deoxyadenosine 5′-triphosphate (dATP), 200 µM deoxyguanosine triphosphate (dGTP), 200 µM deoxycytidine triphosphate (dCTP) (Promega), 400 µM dUTP (Applied Biosystem), 2.5 mM $MgCl_2$, 0.2 µM of each primer (MWG Biotech AG,Germany), 0.25 U UNG (Applied Biosystem), 1.5 U Ampli Tag Gold (Applied Biosystem) and 2.5 µl of the 1:5, or 1:50, respectively, diluted sample DNA.

The PCR was performed in a PTC-200 gradient thermocycler (MJ Research Inc, Waltham, MA) with an initial denaturation step of 95 °C for 10 min, followed by 45 cycles 95 °C for 30 s, 56 °C (emPCR), 57 °C (shPCR), and 58 °C (swPCR) for 45 s and 72 °C for 60 s. The PCR was terminated with a final extension step at 72 °C for 7 min.

After amplification, 10 µl of the amplification products were resolved on a 1.5% ethidium bromide-stained agarose gel and the amplified DNA fragments of specific sizes were visualised by ultraviolet fluorescence. Their sizes were verified by a standard DNA ladder (Bio-Rad) run simultaneously. The expected sizes of the PCR products were 252 bp for *E. multilocularis*, 295 bp for *E. granulosus* G1 and 204 bp for *E. granulosus* G5/6/7. Control DNA from the reference strain and negative control were included in each reaction (Fig. 1).

Each sample was run in duplicate (one reaction with DNA diluted 1:5 in distilled water, the other diluted 1:50) simultaneously with all three PCRs. For samples that showed the specific 204 bp fragment for *E. granulosus* G5/6/7 a subsequent additional PCR with the primers G6/7F and G6/7R was performed.

The following strains were used as controls:

- *Echinococcus granulosus* genotype G1, genomic DNA from an Austrian patient's cyst (AJ508022) was the positive control for all shPCRs.
- *Echinococcus granulosus* genotype G7, genomic DNA from a metacestode isolated from a pig in the Slovak Republic provided by P. Dubinsky, Helminthological

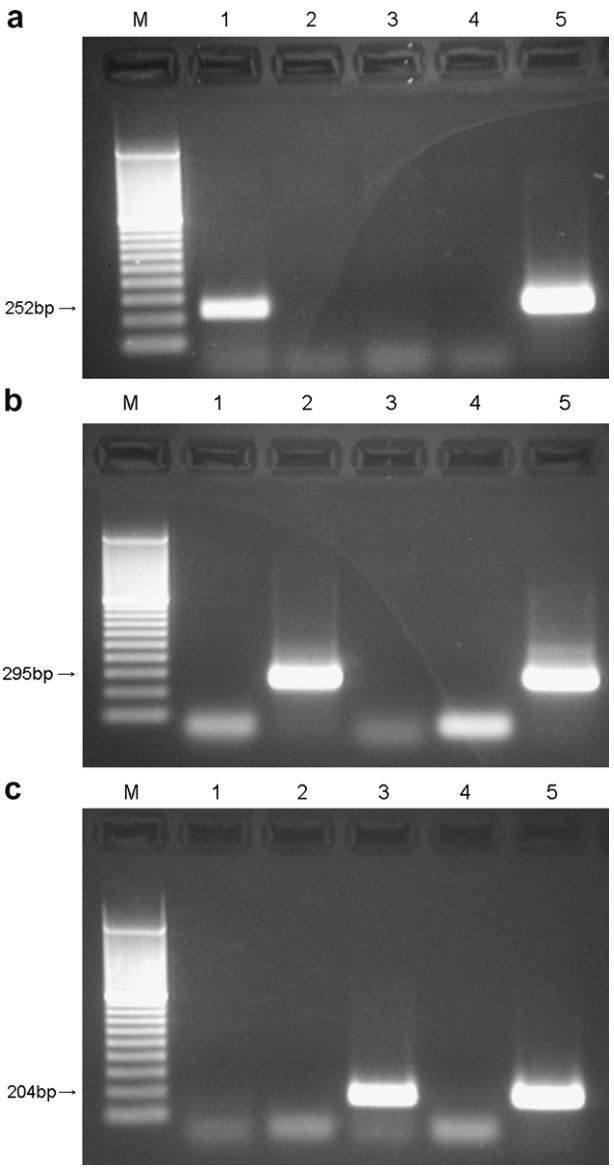

Fig. 1. Gel electrophoresis of PCR products from different strains of *Echinococcus granulosus* and *Echinococcus multilocularis*. (a) *E. multilocularis* (emPCR), (b) *E. granulosus*, G1 or sheep strain (shPCR), (c) *E. granulosus* G7 or swine strain (swPCR) run on a 1.5% agarose gel; Lane M, size marker; lane 1, sample B24; lane 2, specimen B31; lane 3, specimen A29; lane 4, negative control; lane 5, positive control (reference strain).

Institute, Slovak Academy of Science, Kosice (AJ508040) was the positive control for all swPCRs (G5/6/7) and for G6/7PCR.
- *Echinococcus multilocularis* (strainT1/85) originally isolated from a patient from The Tyrol (Austria) and propagated in laboratory mice since 1985, was extracted from infected mouse liver and used as positive control for all emPCRs.
- Human genomic DNA was used as a negative control.

2.4. PCR product sequencing

To prove the specificity of our new PCRs and to discriminate between strains G5, G6 and G7, all PCR products ($n = 94$) were purified with the QIAquick PCR Purification Kit (Quiagen) and cycle sequencing was performed.

The nucleotide sequences of the PCR products were determined by the use of Big Dye chemistry and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA) using the corresponding PCR primers (forward and reverse) as sequencing primers. Nucleotide sequence analysis was undertaken using the National Center for Biotechnology Information BLAST programs and database (www.ncbi.nlm.nhi.gov/blast/Blast.cgi).

3. Results

3.1. Sensitivity and inhibition

Sixty-eight of 80 FFPT samples (85%) of histologically confirmed echinococcosis cases gave clear positive results with one of the new PCRs, 12 (15%) showed no reaction. All samples were run in duplicate using two different DNA dilutions (1:5 and 1:50). Twenty (30%) of the positive samples were inhibited in the 1:5 DNA dilution (data not shown) but showed a specific band when DNA was diluted 1:50 or higher. Two of the remaining negative paraffin blocks showed large amounts of bile which might be a PCR inhibitor (Supplementary Table S1) and in these two samples we were unable to detect specific bands even in 1:1000 diluted template DNA. The number of successfully performed PCRs and obtained sequencing results suggest that the sensitivity of our method was high (Supplementary Table S1).

3.2. Specificity

To determine the specificity of the newly established PCRs, *E. multilocularis*, *E. granulosus* G1, *E. granulosus* G7, *E. ortleppi* (G5), *Taenia saginata*, *Taenia hydatigena* and human genomic DNA were tested with all sets of primers. Although the primers designed for swPCR contained a few mismatches, *E. ortleppi* reacted positively. Unfortunately, no samples of *E. granulosus* strains G2, G3, G4, G6 or *T. solium* DNA were available at our laboratory. Furthermore, all samples were run simultaneously with the three species/strain-specific PCRs and gave consistently clear results according to species/strain (Fig. 1). The specificity of all three PCRs was 100% but it was impossible to discriminate between *E. granulosus* strains G5, G6 and G7.

3.3. PCR

Altogether, 38 (47.5%) of the clinical samples were identified as *E. granulosus* G1 strain; 26 (32.5%) as G5/6/7 strains and four (5%) as *E. multilocularis*, whilst 12 (15%) of the FFPTs remained negative in all PCRs (Supplementary Table S1).

3.4. PCR product sequencing

Sequencing was successful with all PCR products. Nucleotide sequence identity was 100% in 65 (95.5%) of the positive patients. Two of the G1 samples and one of the G7 samples showed one nucleotide difference to the published sequence (Accession Nos. EU116938–EU116940). These samples were subjected to another round of DNA isolation, PCR and sequencing. However, identical sequencing results were obtained. The histo-pathological diagnosis of all four AE patients was confirmed by PCR and the sequencing results showed 100% identity with *E. multilocularis* M1. The sequences of the swPCR products excluded the presence of *E. ortleppi* in our samples and in order to discriminate between the G6 and G7 strains we sequenced an additional 234 bp PCR fragment. All G6 samples ($n = 3$) showed 100% identity with published G6 sequences (Bowles and McManus, 1993).

4. Discussion

FFPTs represent the most important specimens for diagnostic surgical pathology and, moreover, have proven to be an invaluable source for retrospective studies, particularly on cancer and infectious diseases. In contrast to classical histo-pathological examinations, molecular methods have the advantage of conclusive species determination as well as strain differentiation within the genus *Echinococcus,* which causes serious diseases worldwide. Numerous studies (mainly phylogenetic) have been performed on the genetic differentiation of *Echinococcus* isolates in the past; however, the materials used for these studies have been cyst fluid, membranes or protoscoleces (Dinkel et al., 2004). In general, collection of fresh human cystic material is problematic, except in hospitals located in highly endemic areas for *E. granulosus* (Scott et al., 1997). Our experience shows that in Austria and probably also in other European countries, FFPTs are the only material available for further investigation, including species and strain designation. Moreover, paraffin blocks are easy to store and to transport to the laboratory.

In the current study, in 68 out of 80 (85%) of the FFPT samples (only histologically confirmed cases were included

in the study) we amplified *Echinococcus*-specific DNA with one of the three newly established species/strain-specific PCRs and proved specificity by cycle sequencing. In 12 (15%) samples, we were unable to detect specific DNA fragments. At least two reasons might be responsible for the negative PCR results:

(i) *DNA degradation*. The paraffin blocks and slides investigated in this study were between 28 years and a few weeks old (Supplementary Table S1) when they reached our laboratory. For the past 25 years, buffered formalin has usually been used for fixation of clinical tissue samples, with duration of fixation being highly variable. However, fixation time and also the choice of fixative significantly affect the quality of the extracted DNA. For the current study we received clinical specimens from several different hospitals and therefore retrospective control for length of fixation, temperature and use of various fixatives was impossible (Greer et al., 1991; Honma et al., 1993).

From patients operated on between the years 2000 and 2005 ($n = 27$) only one sample was negative in all PCRs (Supplementary Table S1). The observation that PCR is more sensitive in FFPTs that are less than 5 years old has been also described by Imrit et al. (2006). A possible explanation for this finding is that degradation of DNA due to fixation continues even in stored blocks. The average sensitivity of PCR with FFPT as a source of DNA was 35–85% for single round PCRs and 80–100% for nested PCRs (Honma et al., 1993; Howe et al., 1997; Imrit et al., 2006).

(ii) *Inhibition*. Kamenetzky et al. (2000) were the first to describe DNA extraction from the germinal membrane of non-fertile hydatid cysts followed by strain determination using mitochondrial cytochrome *c* oxidase I gene sequencing. The polysaccharide-containing laminar layers of the non-fertile cysts were supposed to contain PCR inhibitors and the authors described a protocol to remove those. In our study, 77.5% of the samples were taken from non-fertile hydatid cysts and were therefore supposed to contain PCR inhibitors (Kamenetzky et al., 2000). However, as the protocol recommended for the removal of inhibitors is not applicable to FFPT, we tried to overcome the inhibition problem by further diluting the DNA, so that we finally obtained positive PCR results in 68 out of 80 DNA samples. We presume that in two out of the 12 PCR negative paraffin blocks, large amounts of bile acted as a PCR inhibitor.

We used the mitochondrial NADH dehydrogenase 1 gene as a target region to establish our new species/strain-specific PCRs because it has a relatively rapid rate of evolution and is therefore useful for the discrimination of closely related organisms (Eckert and Thompson, 1997; McManus, 2006). Although the resulting PCR fragments were only between 204 and 295 bp long, it was possible to detect sequence heterogeneity within the G1 strain in two cases and within the G7 strain in one case (Supplementary Table S1). Moderate sequence heterogeneity within the strains G1 and G7 has been described by others (Obwaller et al., 2004; Bart et al., 2006; Maillard et al., 2007; Roratto et al., 2006).

We assume that our shPCR would have amplified *E. granulosus* strains G2 and G3 as well, but due to lack of G2 and G3 control DNA this remained unproven. However, sequencing of the resulting 295 bp fragment would discriminate between G1 and G2/3.

The majority of our FFPT samples, ($n = 38$; 47.5%), were identified as *E. granulosus* G1, the common sheep strain or *E. granulosus* sensu stricto (Romig et al., 2006); 23 (28.75%) were identified as G7 (the swine strain), three (3.75%) as G6 (the camel strain) and the histological diagnosis of all four AE patients (5%) was confirmed by PCR (Supplementary Table S1). The patients infected with the *E. granulosus* G1 strain mainly derived from Mediterranean countries (i.e. Turkey) and those infected with the swine strain from Austria, Hungary and the former Yugoslavia. From the samples from patients of Austrian origin with CE, all except one belonged to the G7 strain. It has been suspected that *E. granulosus* G7 has low infectivity to humans (Eckert et al., 1993), but this apparently needs to be revised (Pawlowski and Stefaniak, 2003; Turcekova et al., 2003). The countries of origin of the three patients infected with the camel strain were Iran, Ghana and Afghanistan, regions in which the existence of the G6 strain has already been reported (Wachira et al., 1993; Bardonnet et al., 2002; Harandi et al., 2002; Ahmadi and Dalimi, 2006).

Thus far, most molecular studies on *E. granulosus* strains have been based on DNA isolated from fertile cysts and strain differentiation has been based on sequencing of partial mitochondrial or nuclear marker genes (Bowles et al., 1992; Šnabel et al., 2000; Turcekova et al., 2003; Bart et al., 2006; Maillard et al., 2007). Dinkel et al. (2004) established a PCR/semi-nested PCR system for rapid discrimination of the G1, G6/7 and G5 strains with reference to the epidemiological situation in Africa. Protoscoleces or cyst walls isolated from humans, camels, cattle and sheep were the source of DNA in their study and discrimination between the G6 and G7 strains required PCR product sequencing as well. In most European countries, only a small number (if any) of samples from patients with cystic echinococcosis have been examined genetically to date (Eckert, 1997; Jenkins et al., 2005; Romig et al., 2006).

Our new PCR system is sensitive and specific. It detected *E. granulosus* G1, *E. granulosus* G6/7 and *E. multilocularis*, respectively, and subsequent PCR product sequencing was only necessary for discrimination between G6 and G7 strains.

In Austria, where both AE and CE are prevalent and occur autochthonously (Auer and Aspöck, 2001), to the present no information was available on the spectrum of *Echinococcus* strains/species in clinical tissues of imported cases and from the indigenous Austrian population. The results of this study indicate that the described DNA extraction method and strain/species-specific PCRs allow

retrospective studies with FFPT as a source of DNA even from patients operated on decades before.

The described PCR protocol might also be helpful for precise diagnosis in some cases, especially with unusual cyst localisations (Georges et al., 2004; McManus, 2006) or for molecular confirmation of liver lesions in patients with possible AE lesions (Myiak et al., 2003).

With FFPT as a source of DNA for molecular biological analysis of *Echinococcus* spp., a large number of human FFPTs can be investigated and although DNA extraction is quite labour-intensive, together with clinical data and geographical information, the newly established protocols might contribute information about the epidemiological situation of echinococcosis in Europe.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2007.11.008.

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