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Short Communication

DNA extraction and a cost-effective detection method for *Echinococcus granulosus* protoscoleces

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ABSTRACT

Most methods of DNA purification from protoscoleces of *Echinococcus granulosus* involve the use of expensive kits and may also require a second step after extraction for an effective purification. The present work describes an optimized cost-effective method that is fast and simple. This method is based on a chemical lysis with proteinase K with a subsequent one-step PCR detection. In this study we used already available primers and newly designed primers to amplify two fragments of different size corresponding to the mitochondrial cytochrome C oxidase subunit 1 gene. By one-step PCR, both fragments were successfully amplified from even a single protoscolex. This result demonstrates that this method of extraction is efficient even with small amounts of sample and that PCR is highly sensitive. The major advantage of this lysis-PCR method is that it avoids a second step of purification resulting in a simpler and more economical method. Our research will serve as a base for future studies on *E. granulosus* genotyping, mainly with wild mammals with a low number of cysts.

Echinococcus granulosus is a cestode that infects several domestic and wild mammals. The adult form resides mainly in canids (definitive host), whereas the larval form (metacestode or hydatid cyst) is found in a wide variety of mammals, including humans (intermediate host). The metacestode, which develops in the intermediate host, is lined with an inner germinal layer that produces small immature worms named protoscoleces. These protoscoleces are capable of developing into adult worms in the gut of the definitive host.

E. granulosus comprises a number of intraspecific variants, strains or genotypes at the genetic level (Nakao et al., 2007). Up to now, 10 distinct genotypes (G1–G10) have been described worldwide based on nucleotide sequence analyses of the mitochondrial cytochrome C oxidase subunit 1 (*co1*) and NADH dehydrogenase 1 genes (*nad1*) (Bowles et al., 1992; Bowles and McManus, 1993; McManus, 2002; Moks et al., 2008). These genotypes include several strains: two from sheeps (G1 and G2), two from bovines (G3 and G5), one from horses (G4), one from camels (G6), two from pigs (G7 and G9) and two from cervids (G8 and G10). In Argentina, G2 was isolated from both sheep and humans and G7 was found in pigs; furthermore, G1 and G6 were isolated for the first time from humans (Rosenzvit et al., 1999). There are few studies, however, on the genotypes of *E. granulosus* in wild animals that could act as intermediate hosts. The studies on *E. granulosus* genotyping are carried out by collecting a large number of cysts from animals in endemic areas but unfortunately cysts in wild mammals may be scarce. Therefore, it is important to have an economical and highly sensitive detection method capable of obtaining sequences from protoscoleces removed from a low number of cysts. The DNA extraction step from protoscoleces

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is crucial for the PCR procedure. The protocols used so far involve DNA purification with a commercial purification kit (Sánchez et al., 2010). In this respect, Rostami Nejad et al. (2011) have evaluated different methods to extract DNA from *E. granulosus* protoscoleces: phenol–chloroform, modified Cinnagen extraction kit, tissue DNA extraction kit and a modified phenol–chloroform method. Their study demonstrates that the quality of the DNA extracted using the modified Cinnagen extraction kit and the modified phenol–chloroform method is high. Also, Sharbatkhori et al. (2009) performed five different methods in a first comparison study of simple DNA extraction methods for *E. granulosus* protoscoleces. In their study, they use glass beads, mechanical grinder and performed freeze-thawing, boiling and crushing. In addition, Tappeh et al. (2012) have recently compared four simple methods of DNA extraction from *E. granulosus* protoscoleces using similar protocols to those used by Sharbatkhori et al. (2009) but also using a commercial DNA extraction kit. All the methods described above require a second step of DNA purification using kit columns or performing phenol–chloroform extraction. This extra step results in processes that are more complex, expensive and time-consuming.

In this context, the aim of this study was to develop a new method performing lysis of protoscoleces and a subsequent one-step PCR procedure avoiding extra steps of purification. This will allow us to process many samples simultaneously in order to reduce costs and time and apply this method in a subsequent genotyping analysis of wild mammal cysts.

E. granulosus protoscoleces were aseptically removed from hydatid cysts of bovine livers and lungs that were obtained from infected cows at routine slaughter at the abattoir in Buenos Aires province, Argentina. Protoscoleces were decanted by gravity and washed twenty times in phosphate-buffered saline (PBS, pH 7.2) (Cumino et al., 2009). A suspension of 200 protoscoleces per μl of PBS 1× was stored at −20 °C until DNA extraction.

DNA extraction was performed in three independent experiments with three replicates each. The analyzed quantities of protoscoleces were as follows: 1000, 100, 50 or 25 protoscoleces corresponding to 20, 2, 1 and 0.5 mg of fresh weight, respectively. The different amounts of protoscoleces were obtained from the stock dilution of 200 protoscoleces/μl. In addition, several individual protoscoleces were isolated using a micropipette under a microscope (4×). Photographs (10–40×) of the protoscoleces were taken before and after lysis. The lysis of protoscoleces was performed vortexing for 10 s in 15 μl PCR compatible lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8.8, 0.4% v/v Nonidet P-40, 0.8% v/v, Tween 20 2.5 mM Cl_2Mg). The samples were digested with 267 μg/ml of Proteinase K (Biobasic) for 3 h at 56 °C and subsequently boiled for 15 min to inactivate the Proteinase K and to denaturize the proteins that may remain undigested. Finally, 35 μl of sterile water (Ultrapure) was added and the processed samples were centrifuged at 2600 × *g* for 5 min to dilute potential inhibitors that may remain in the sample. The supernatants and pellets were kept at −20 °C until use to perform PCR experiments and to verify the lysis process, respectively. An extraction control (negative control) containing only the lysis buffer was subjected to the same conditions. The DNA concentration was measured with NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) using 1 μl of each lysis supernatant.

A region of 125 base pairs (bp) of the mitochondrial *co1* gene was amplified using the following primers specifically designed for the present work: Ech1F 5′CTATAGTGTGTTTGGGTAGCAGGG3′ and Ech1R 5′CACCTTTATACCAGTAGGAACCC3′. The design of these new primers was based on sequence alignments of *E. granulosus co1* gene sequences available in the GenBank. In addition, a larger fragment was amplified using the already available primers: JB3 5′TTTTTTGGGCATCCTGAGGTTTAT3′ and JB4,5 5′TAAAGAAAGAACATAATGAAAATG3′ (Bowles et al., 1992). JB3 and JB4.5 amplify a 450 bp region of the mitochondrial *co1* gene. This primer set was designed based on evolutionarily conserved regions of the *co1* sequence published for *Fasciola hepatica* and they have been widely used to discriminate between *E. granulosus* genotypes (Bowles et al., 1992; Kamenetzky et al., 2002; Sánchez et al., 2010; among others). PCR was performed in a final volume of 12.5 μl containing 2 μl of lysis supernatant, 200 μM of each DNTP (Finnzymes), 0.4 μM of each of the primers, 2 mM of $MgCl_2$ (Fermentas) and 0.65 units of Taq DNA Polymerase (Fermentas) in 10× *Taq* Buffer with $(NH_4)_2SO_4$. PCR conditions were as follows: an initial denaturing step (94 °C for 3 min) followed by 40 cycles at 94 °C for 45 s (denaturation), 50 °C for 45 s (annealing), and 72 °C for 45 s (extension), and a final extension step (72 °C for 10 min). The negative control was included in all PCR experiments. The specificity and size of amplification products were assessed by electrophoresis in 2% (w/v) Tris–borate/EDTA (TBE) agarose gels and stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen).

To test the efficiency of the DNA extraction method, we performed classical PCR to amplify a 125 bp fragment of the *co1* gene. Lysis supernatants with decreasing amount of protoscoleces (1000, 100, 50, 25 and 1) were evaluated to determine sensitivity of PCR. The 125 bp fragment amplification was achieved in all samples; which shows that the minimal amount of protoscoleces necessary to give a positive PCR result was 1 protoscolex (Fig. 1A) In addition, three replicates of DNA extraction from 1000, 100, 50, 25 and 1 protoscolex were quantified in three independent experiments. Fig. 1A shows DNA concentration used in PCR reaction corresponding to each protoscolex amount. The 260/280 ratios measured by Nanodrop showed values of 1.5–1.7 from 1000 and even with 50 protoscoleces. From 25 protoscoleces to 1 protoscolex, DNA concentration was below detection limit of Nanodrop (values below 2 ng/μl) and the 260/280 ratio were not determined. These results allow us to demonstrate the high sensitivity of the proposed method. Further, PCR results showed that the intensity of the bands decreased with the reduction amount of protoscoleces indicating the low influence of inhibitors in lysis supernatant without a second purification step.

On the other hand, by microscopic observation of the lysates, we verified the lysis of a single protoscolex. Fig. 1B shows an intact protoscolex before lysis and free rostellar ring after lysis and PCR results proved the efficiency of this

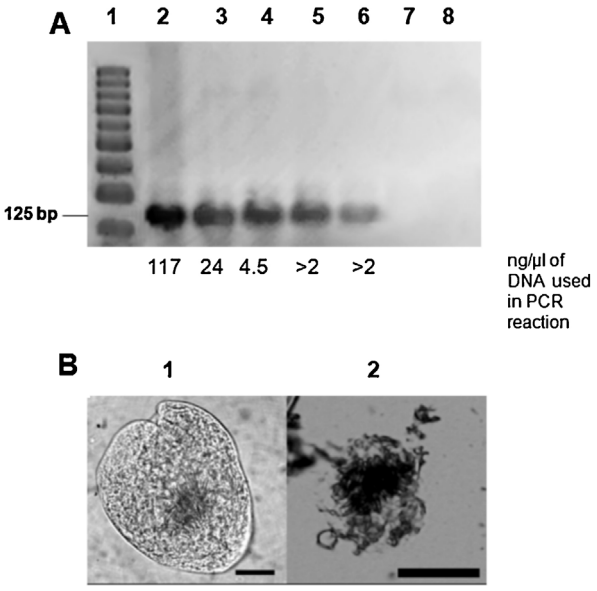

Fig. 1. (A) Lysis efficiency and DNA detection method with decreasing amounts of protoscoleces. (1) GeneRuler™ 100 bp DNA Ladder (Thermo Scientific); (2) 1000 protoscoleces (pts); (3) 100 pts; (4) 50 pts; (5) 25 pts; (6) 1 pt; (7) negative extraction control; (8) negative PCR control. In each lane: DNA concentration (ng/µl) used in PCR reaction corresponding to the different amounts of protoscoleces. (B) Lysis efficiency of the method for 1 protoscolex. (1) Protoscolex before lysis; (2) protoscolex after lysis. Free hooks in sample. (A: 40×).

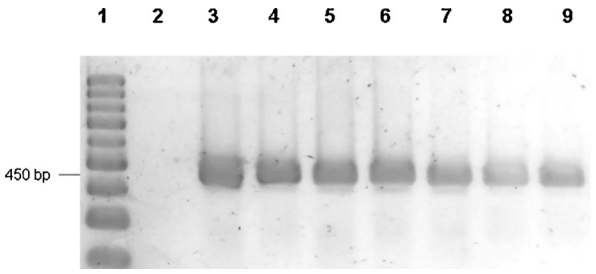

Fig. 2. Detection of the 450 bp fragment of *co1* used to genotyping from 1 protoscolex (pt). (1) GeneRuler™ 100 bp DNA Ladder (Thermo Scientific); (2) negative PCR control; (3–9) 7 replicates of 1 pt.

method to lyse and release DNA from a single protoscolex (Fig. 1A). In addition, the 125 bp fragment identity with *E. granulosus co1* gene from GenBank was confirmed by the PCR products sequencing (data not shown). Besides, no amplification was observed in extraction and PCR negative controls.

The potential application of this method in genotyping of *E. granulosus* is interesting, specially because it could be applied with a minimal amount of starting sample. For evaluating this method, the PCR amplification of 450 bp *co1* fragment was evaluated in 2 µl of DNA samples extracted from a single protoscolex coming from several different samples. The isolation of these protoscoleces was performed by using optic microscope. Although DNA concentrations measured with the Nanodrop were below the equipment detection limit, amplification products were obtained in three independent experiments. No amplification was detected in negative PCR controls (Fig. 2). For all cases the 450 bp fragment was observed.

In most molecular studies of *E. granulosus*, commercial DNA extraction kits have been used for extracting DNA. For instance, the following kits have been used: High Pure PCR Preparation Kit (Roche, Germany) (Maillard et al., 2007), QIAamp DNA mini Kit (Qiagen, Germany) (Zhang et al., 1998), Wizard Genomic DNA Purification Kit (Busi et al., 2007), Q-bio Gene Kit (USA) (Bhattacharya et al., 2007). Although they are simple and fast to apply, the use of such kits involves a higher cost, which makes it difficult to apply in laboratories with a low budget. Even though the phenol–chloroform method to extract DNA of *E. granulosus* allows extracting high quality DNA, it is time consuming and represents a risk to human health and the environment. In addition, all these methods require large amounts of starting material, at least 10 mg of biological sample (~500 protoscoleces). The method developed in this work allowed to efficiently obtain amounts of DNA detectable by PCR even from only one protoscolex. Other studies have been performed using small amounts of sample. For example, Hüttner et al. (2008) performed a genetic characterizarion of *Echinococcus felidis* from African Lion using a single egg. Although they developed a simple and fast lysis method based on an alkaline lysis, the small amounts of DNA obtained were only detected by nested-PCR.

At the present work we reported a fast, easy to perform and cost-effective DNA extraction method with a sensitive one-step DNA detection method for *E. granulosus*. Although other highly efficient methods have been reported, this extraction procedure did not require a DNA purification second step and avoid the use of expensive commercial kits. Even though, an extra purifying step was not performed with this method, no inhibition was detected in any of the PCR amplifications. Dead protoscoleces and external components that could inhibit PCR reaction were removed with washes with sterile PBS 1×. Furthermore, enzymes (including DNases) and other inhibitory proteins were denatured and inactivated by boiling the lysis supernatant. Furthermore, protoscolex proteins which could inhibit the DNA polymerase have not been described in the literature.

An advantage of this method is that it involves fewer tube manipulations when compared to traditional organic extraction methods, reducing contamination risks among samples.

Finally, the amplification of the 450 bp fragment in DNA samples from a single protoscolex suggests that the extraction method reported in the present work is an effective method to be applied in future studies to *E. granulosus* genotyping, mainly in wild mammals where starting material can be scarce.

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