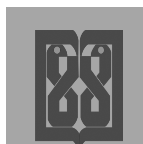

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Short Communication

Comparison of Five Simple Methods for DNA Extraction from *Echinococcus granulosus* Protoscoleces for PCR-Amplification of Ribosomal DNA

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Abstract

Background: Cystic hydatid disease is an important zoonosis, affecting humans and animals and is a significant public health and economic problem throughout the world and Iran. Since extraction of DNA from the parasite is a primary and crucial step which has a principal effect on PCR results, in the current study five simple methods for DNA extraction from protoscoleces of *Echinococcus granulosus* were applied and compared with each other.
Methods: After collecting hydatid cysts from an abattoir, DNA samples were extracted from two cyst isolates from sheep, two from goats and two from camels using five different methods involving the use of glass beads, mechanical grinder, freeze-thaw, boiling and crushing. For all DNA samples extracted, one PCR assay based on amplifying rDNA-ITS1 region was performed and amplicons resolved on 1.5% agarose gels.
Results: The methods were compared regarding to DNA and PCR bands, time and cost effectiveness and laborious amount. The target DNA was successfully amplified from all samples using all methods produced an expected band size. All methods showed some advantages and disadvantages in PCR gels. The boiling method, which was the most time and cost effectiveness method, achieved the thickest bands in the PCR following grinder, crushing, freeze-thaw and glass beads.
Conclusion: Boiling and crushing methods were the most suitable methods regarding their amplicon quality, easiness, quickness and cost effectiveness.

Keywords: *DNA extraction,* Echinococcus granulosus*, rDNA-ITS1*

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Introduction

Cystic hydatid, caused by parasite *Echinococcus granulosus*, is one of the most important zoonosis diseases in many parts of the world. The parasite has mainly domestic cycle, which involves livestock and dogs as intermediate and definitive host, respectively. Sylvatic cycle mostly is transmitted between cervid or moose and wolf, and is reported in limited regions of the world such as North America and Eurasia (1). High prevalence rates of *E. granulosus* have been described in sheep, goats, cattle, camels and dogs in different parts of Iran. Human cases of hydatid cyst are routinely reported from different parts of the country (2). The extensive variation in *E. granulosus* may affect life cycle, host specificity, development rate, sensitivity to chemotherapeutic agents plus pathology and consequently for design and development of vaccines against *E. granulosus* (3). Therefore characterizing the exact etiological agent in different areas is necessary in order to determining transmission patterns and control programs. DNA technology has had a major role in many aspects of Parasitology, including diagnosis, epidemiology, analysis of population genetic structures and vaccine development. In particular, the polymerase chain reaction (PCR) has found broad applicability, because its sensitivity allows the genes amplification from minute amounts of parasite material. This is of particular importance as it is frequently not possible to isolate adequate amounts of material from some parasites and their different life-cycle stages. Performing any DNA-based technique requires extracting DNA as the first and crucial step that can have a significant effect on the PCR result. Recently application of molecular tools has helped to resolve many of the taxonomic questions about the status of species and strains in the genus *Echinococcus* and realize causing agent of hydatid disease in every country (4-7).

In most molecular studies of *E. granulosus*, commercial kits are used for the extraction of total genomic DNA (4-6). However, such kits can be expensive and are not always readily available in some countries.

In the current study, we conducted a comparative assessment of five methods for extracting genomic DNA from protoscoleces of *E. granulosus* and achieving effective PCR-amplification using a set of primers on first internal transcribed spacer (ITS1) of ribosomal DNA. The aim was to develop an inexpensive approach for use in our laboratory.

Material and Methods

Parasite specimens

Several fresh fertile hydatid cysts of sheep, goats and camels were collected from Najafabad abattoir, central of Iran. Protoscoleces were aspirated from the cysts, washed several times with normal saline until the supernatant looked clear. Then, the sediment preserved in 70% ethanol until required.

DNA extraction

Protoscoleces from six hydatid cyst isolates (two each from sheep, goats and camels) were selected. For each method, equal volumes of packed protoscoleces (about 30 µl) were washed twice with sterile distilled water to remove ethanol. Then 300 µl lysis buffer (NaCl 0.1M, EDTA 0.01M, Tris-HCl 0.1M, SDS 1%) added to the sediment of each tube.

The subsequent DNA extraction process performed in two steps:

-Step one, which was used for disruption of cells and DNA release, was different for each method as follows:

Glass beads method: About 300 µl of 0.5 mm diameter glass beads (Glasperlen, It-

aly), was added to each tube and shaken vigorously for 5 min.

Mechanical grinder method: The protoscoleces of each tube were grinded for 3 min using mechanical grinder (Micro Multi Mixer, Ieda Trading Corporation, Tokyo, Japan)

Freeze-thaw method: Each tube was frozen and thawed three times in liquid nitrogen and boiling water interval, each for 1.5 min.

Boiling method: The protoscoleces of each tube were incubated at 100 º C for 10 min.

Crushing method: Protoscoleces of each tube inserted on the glass slide by sampler, were crushed with another glass slide for about 1 min and then product transported to the tube.

-Step two was similar for all methods:30 µg of proteinase K (Roche, Germany) was added to each tube containing samples plus 300 µl lysis buffer and incubated at 56º C for one hour. Then, 300 µl phenol-chloroform-iso amylalcohol was added and centrifuged at 5000 rpm (2000g) for 5 min. After removing the supernatant to a new tube, chloroform was added prior to shaking and spanning in 5000 rpm for 5 min. Subsequently equal volume of iso-Propanol (Merck, Germany) and 0.1 volume sodium acetate (Merck, Germany) (3M, pH=5.2) were added to the supernatant, and kept at -20 ºC for 20 min. Next, it was spun 12 min in 12000 rpm and the sediment was rinsed by 300 µl 70% ethanol. After spinning 5 min in 5000 rpm (2000g) and removing ethanol, pellet was dissolved in 50 µl deionized water, and stored at -20 º C for PCR process.

PCR process

The PCR was performed for amplification of the ITS1 from all DNA samples. The forward (EgF: 5´ GTC GTA ACA AGG TTT CCG TAG G 3´) and reverse (EgR: 5´ TAG ATG CGT TCG AAG TGT CG 3´) primers were used, which had been designed in the other study (8): PCR reaction carried out in 20 µl, with 1 µl DNA extraction product, 25 pmol/µl of each primer and premix (2x Master Mix RED, Taq DNA pol, 0.5 µM of dNTPs and 1.5 mM MgCl2, Ampliqon, Denmark). The temperature profile was : One cycle of 95ºC for 5 min (primary denaturation), followed by 30 cycles of 94 ºC for 30 s (denaturation), 66 ºC for 45s (annealing) , and 72 ºC for 45 s (extension), and a final extension 72 ºC for 5 min. The extracted DNAs and PCR products of each method were loaded on separate 1 and 1.5% TBE (Tris 0.09M-Borate 0.09M-EDTA 0.02M) agarose gel (Bio life, Italina S.r.l, Italy), respectively. The gels contained 0.5 µg/ml ethidium bromide (Roche, Germany) for staining. Electrophoresis carried out 1 hour at 80 V. The bands visualized in UV Transilluminator (UVItec, EEC) and digitally photographed.

Results

DNA extraction

Overall 30 DNA samples, extracted from 6 hydatid cyst isolates and every which by five methods, were prepared for PCR process. Fig. 1 shows the agarose gel electrophoresis of extracted DNA from samples. As it is seen all samples, except boiling have a distinct band for each sample. However, a visible smear was observed in DNA electrophoresis for all methods. The size of DNA was apparently equal in every extraction method. According to the images, it seems that grinder and freeze-thaw have quantitatively better yields of the DNA.

PCR

A PCR process to amplify rDNA-ITS1 as the target gene was set up and performed on all 30 DNA samples and negative controls, simultaneously and in the same conditions. Fig. 2 shows the agarose gel electrophoresis of PCR amplicon of DNA ex-

tracted by different methods. As seen, the target DNA was successfully amplified from all samples employing each five methods, producing an expected band size (~ 1 kb). Not any PCR amplification was seen in negative controls. In all methods, a pale band was seen below the expected band almost in all samples, that comparing with sharpness of original band is pardonable. In spit of poor quality of DNA extraction, boiling method has the thickest PCR bands for all 6 samples followed by grinder, crushing, freeze and thaw and glass beads.

Time requiring for each method

Regarding the time required for each method, step one for six samples was performed during 15, 25, 20, 10 and 15 min in glass beads, grinder, freeze-thaw, boiling and crushing methods, respectively, among which boiling was the fastest method contrary with grinder as the most time-consuming and labor intensive method. Step 2 that was similar for all five methods, took averagely about two and half hours for six samples.

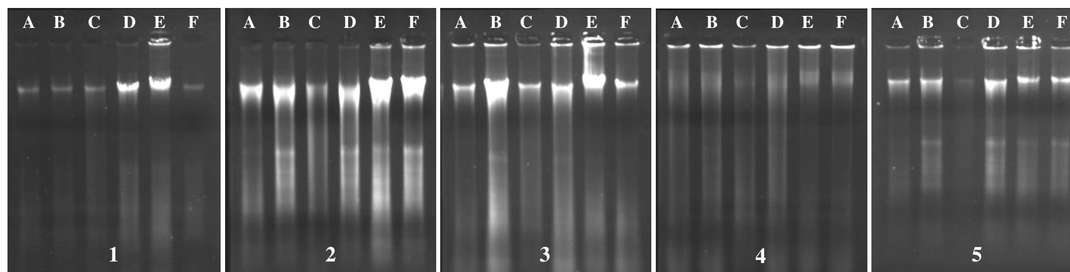

Fig. 1: Agarose gel electrophoresis of extracted DNA from 6 hydatid cysts isolates (A & B: sheep isolates, C & D: goat isolates, E & F: camel isolates) with five methods (1-5): 1. Glass beads, 2. Grinder, 3. Freeze-thaw, 4. Boiling and 5. Crushing

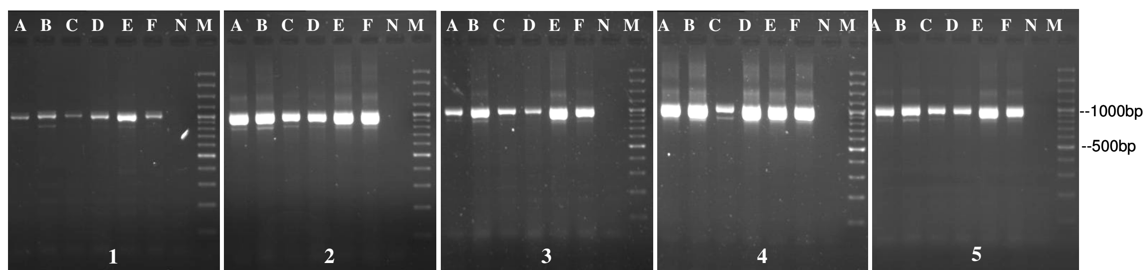

Fig. 2: Agarose gel electrophoresis of PCR on extracted DNA from 6 isolates (A & B: sheep isolates, C & D: goat isolates, E & F: camel isolates) with five methods (1-5): 1. Glass beads, 2. Grinder, 3. Freeze-thaw, 4. Boiling 5. Crushing. N and M are negative control and 100 bp DNA ladder (Fermentas, Ukraine), respectively

Discussion

PCR was used as a suitable tool for the characterization of *E. granulosus* strains in different studies. The success in PCR depends on the quantity and quality of the extracted DNA. Various sample preparation methods have been developed for PCR assays (9). In most of molecular studies of *E. granulosus*, commercial DNA extraction kits, such as High Pure PCR Preparation Kit (Roche, Germany) (4, 5, 10), QIAamp DNA mini Kit (Qiagen, Germany) (6), Wizard Genomic DNA Purification Kit (11), Q-bio Gene Kit (USA) (12) have been used for extracting DNA. A few studies have used liquid nitrogen for the crushing of protoscoleces (7, 13). Commercial kits have a proper application when a large numbers of samples containing low numbers of organisms are processed. Although the application of kits is quick, and easy for obtaining nucleic acids, they can be expensive and not readily available in some countries. Application of liquid nitrogen seems simple but laborious. Moreover, liquid nitrogen can be expensive to purchase and keeping in suitable condition.

There are some comparative studies of different DNA extraction methods for organisms, such as fungi, bacteria, viruses and protozoa (14-18). Although there are many different studies of *E. granulosus*, there were no comparative studies on methods for the extraction of DNA from protoscoleces and the present study is the first for comparison of DNA extraction methods in *E. granulosus* which five simple methods including glass beads, grinder, freeze-thaw, boiling and crushing were compared and evaluated. Our aim was to find a quick, easy to perform and cost effective DNA extraction method for application in any lab that commercial DNA extraction kits are not easily available. In all methods, DNA were extracted from cells and then purified by phenol chloroform extraction followed by alcohol precipitation. The major advantage of the "glass beads method" is that there is no need for reusable equipment, and so is a low risk for accidental contamination of DNA among samples. In this study, according to pale PCR bands of glass beads, it does not seem a proper method for extracting DNA from protoscoleces although in some comparative studies on fungi, it has been found as one of the best method (15). Grinder is relatively a new method was used on *E. granulosus* previously (8), created sharp PCR bands in our study. However, a mechanical grinder is not available in all molecular laboratories, such that the use of this method has limitations. Additionally, since each sample should be grinded separately, this method takes more time and is laborious. Moreover, as that part of the grinder, which is in contact with, organism is disposable; the cost can be high, making the method relatively expensive to perform. Thus, the "grinder method" does not appear to be the most convenient method when a large numbers of samples are to be analyzed. "Freeze-thaw method" extracted sufficient DNA and showed sharp bands in electrophoresis of PCR-products. However, application of liquid nitrogen is problematic, especially when there are a large number of samples to be examined. Other disadvantages of this method are difficulties in its handlings and safety hazards in use. Although the "boiling method" did not extract DNA efficiently from all six samples (Fig.1), it achieved the thick bands in the PCR (Fig. 2) that is probably a result of a reduction in the inhibitory factors in the sample during the boiling, but this method showed the most DNA smear in the PCR gel. Moreover, the thickness of bands can be for more amplification of the additional band that more or less observed in all methods. It seems that employing boiling

method by using less amount of extracted DNA in PCR reaction or decreasing PCR cycle numbers will attain a high quality of PCR bands. The "boiling method" took just 10 min, without any additional effort. This technique needs minimal equipments, performed at 95ºC boiling water, and is available in almost all laboratories. The "crushing method" showed the high quality PCR bands and yielded the least amount of DNA smear compare with other methods. Although glass beads methods also did not yielded DNA smear, like crushing method, but the PCR bands quality in crushing is much better than glass beads in all samples. "Crushing method" does not need to any equipment other than two glass slides.

Regarding the cost of each method, freeze-thaw was the most expensive one, followed by grinder and glass beads, whereas crushing followed by boiling methods were the cheapest. The freeze-thaw needed the manual work, in contrast to the "boiling method" followed by crushing which required the least. Glass beads and grinder methods were similar in this respect. All methods were repeatedly in our lab and the results were stable.

Overall, in PCR gels of all methods were seen some advantages and disadvantages but considering with different aspects of suitability for a DNA extraction method such as PCR band quality, time consuming, cost effectiveness, labor using and simplicity, boiling and crushing were the most suitable methods considering their amplicon quality, simplicity, quickness and low cost for the DNA extraction of *E. granulosus* protoscoleces. These methods can be used for future studies based on PCR amplification of ITS1-rDNA and probably other genes of *E. granulosus* or other organisms.

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