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Detection and Differentiation of *Echinococcus granulosus*-Complex Using A Simple PCR-Based Assay

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Abstract: A nested Polymerase Chain Reaction (PCR) assay, for detection of intact and calcified hydatid cysts of *Echinococcus granulosus* (EG)-complex, was developed and evaluated. The NADH dehydrogenase 1 gene was used as a target DNA for PCR amplification. Two pairs of primers, (EGL 1 and EGR 2) and (EGL 3 and EGR 4), were designed and used in 2 amplification steps. First, the outer pair of primers (EGL 1 and EGR 2), derived from a highly conserve region of NADH 1 gene produced a primary 435 base pair (bp) PCR product from different stains of EG-complex including, sheep strain (genotype) designated (G 1), Cattle strain (G 5), camel strain (G 6) and pig strain (G 7). Second, a pair of internal (nested) primers (EGL 3 and EGR 4), designed internal to the annealing sites of primers (EGL 1 and EGR 2), produced a 276 bp PCR product. The primary 435 bp and the nested 276 bp EG specific PCR products were easily identified following visualization onto an ethidium bromide-stained agarose gel. However, the primary or the nested PCR products were not amplified from DNA extracted from *Cysticercus tenuicollis* and *Coenurus cerebralis*, the larval stages of the adult dog cestodes *Taenia hydatigena* and *T. multiceps*, respectively. The described nested PCR assay could be used to detect sheep, cattle and camel strains of EG-complex circulating in Sudan. This PCR assay could also, be used for rapid detection and differentiation of EG-complex from other related cestodes. This assay should be considered during an epidemiological survey of the disease in areas of endemicity.

Key words: Hydatid disease, cystic echinococcosis, *Echinococcus granulosus*, complex, genotyping, molecular diagnosis, PCR

INTRODUCTION

Hydatidosis, caused by larval stage of *Echinococcus granulosus*, affects livestock and humans in the Sudan, hence the disease is of major public health importance (Abdel Malek, 1959; Eisa *et al*., 1962, 1977; El-Khawat *et al*., 1979; El-Husein and Ali, 1990; El-Mahdi *et al*., 2004; Omer *et al*., 2004; Osman *et al*., 2007). Ten genotypes of *Echinococcus granulosus* (EG) designated (G 1- G 10) are recognized worldwide (Bowles and McManus, 1993a; McManus, 2002; McManus and Thompson, 2003; McManus *et al*., 2003; Lavikainen *et al*., 2003; Eckert and Deplazes, 2004). Sheep strain (G 1), cattle strain (G 5) and Camel strain (G 6) are enzootic in the Sudan (Omer *et al*., 2004; Elmahdi *et al*., 2004; Osman *et al*., 2007). Recent epidemiological studies indicated that the camel strain (G 6) was reported to be the most prevalent strain in Sudan (Omer *et al*., 2004; Osman, 2007; Osman *et al*., 2007). However, it is well documented that Sudanese breeds of sheep and goats are known to harbour calcified cysts of this cestode parasite (Saad and Magzoub, 1989b). Therefore, the development of a rapid, reliable, reproducible, sensitive, specific and inexpensive diagnostic assay for detection and differentiation of EG-complex would be advantageous for a variety of circumstances including, epidemiological investigations and control programs. In previous reports, single PCR amplification assays for detection of EG genotypes were described based on different target genes (Bowles *et al*., 1992; Bowles and McManus, 1993a; Gasser and Chilton, 1995; Scott and McManus, 1994). A semi-nested PCR, based on rRNA gene sequence analysis

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of EG, was also, developed and evaluated (Dinkel *et al*., 2004). Previous studies showed that NADH 1 gene is the most highly conserved among cognates of different genotypes of EG-complex. Although, these PCR-based detection assays proved highly sensitive and specific, their sensitivity for detection of calcified or degenerated cyst was not investigated sufficiently. To address this problem, we describe a simple, rapid, reproducible, sensitive and specific method for rapid detection of intact, degenerated or calcified hydatid cysts using a nested PCR-based detection assay.

MATERIALS AND METHODS

Infective materials: A total of seventy nine hydatid cysts were used in this study. Of these, fifteen calcified cysts were obtained from sheep and goat origin. Three human samples were recovered from surgically operated patients admitted to Khartoum Medical Teaching Hospital, Khartoum Sudan. Sixty one cysts of camel origin were collected from different slaughter houses in Central Sudan (Khartoum, Kharoum North and Tamboul). The cysts were collected from liver, spleen, muscles, heart and lungs of the infected patients. Hydatid fluid containing protoscolices was aspirated with sterile needles. The protoscolices were then kept in clean sterile 50 mL bottles and 70% Alcohol was added as preservative.

DNA extraction from intact samples: Protoscolices were washed in nucleic acid free water to remove alcohol. Extraction of DNA from hydatid cysts was made possible using a commercially available QIAamp tissue kit (QIAGEN Inc. Chatsworth, CA) according to the manufacturer's instructions. Briefly, 200 μL of protoscolices suspension, 20 μL of proteinase K stock solution and 200 μL of lysing buffer were pipetted into 1.5 mL eppendorf tube and the mixture was incubated at 37°C for 1 h and then at 70°C for 30 min. Total two hundred micro liter of absolute ethanol was then added to the sample and mixed by vortexing. The mixture was then transferred to the QIAamp spin column and placed in a clean 2 mL collection tube and centrifuged at 8000 RPM for 1 min at room temperature. The QIAspin column was washed twice using 500 μL of washing buffers by spinning for 1 min. The QIAamp spin column was placed in a clean 1.5 mL eppendorf tube and the DNA was eluted with 200 μL of double distilled water preheated at 70°C. A maximum DNA yield was obtained by spinning at 12,000 RPM for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length. Five Micro liter of the suspended nucleic acid was used in the PCR amplification.

DNA extraction from calcified samples: One gram of calcified tissues resected from hydatid cyst was dropped in 500 μL double distilled water. Calcified tissues were then homogenized and treated by repeated freezing and thawing. The homogenate was then incubated at 7°C in water bath. DNA extraction procedure was then performed as described for the fresh samples.

Positive control DNAs: Positive control DNAs for sheep strain (G 1), cattle strain (G 5), camel strain (G 6) and pig strain (G 7) were obtained from the Department of Parasitology, Institute of Zoology, University of Hohenheim, Stuttgart, Germany.

Primers selection and PCR amplification: For the first amplification step, a pair of outer primers (EGL 1 and EGR 2) was selected from the published sequences of NADH dehydrogenase 1 gene of *E. granulosus* genotype 6 and used in the PCR assay (Bowles and McManus, 1993b). This pair of outer primers was designed for the synthesis of the primary *E. granulosus*-specific PCR product. Primer EGL1 included bases 32-53 of the positive sense strand (5)- TGA AGT TAG TAA TTA AGT TTA A. EGR 2 included bases 447-466 of the complementary strand (5)-AAT CAA ATG GAG TAC GAT TA. Using primers EGL 1 and EGR 2, the primary PCR amplification will produce a 435 bp PCR product.

For nested amplification, a pair of internal primers (EGL 3 and EGR 4) was designed from the same DNA sequence cited above. EGL 3 included bases 162-181 of the positive sense strand (5)-TTA TAG TAT GCT TTC TGT GT. EGR4 included bases 420-437 of the complementary strand (5)-AAC ACA CAC ACC AAG AAT. The nested primers will result in amplification of a 276 bp PCR product, internal to the annealing cites of primers EGL 1 and EGR 2.

The primers were synthesized on a DNA synthesizer (Milliigen/Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA) as per manufacturer's instructions.

Polymerase chain reaction: A stock buffered solution containing 250 μL 10 X PCR buffer, 100 μL of $MgCl_2$, 12.5 μL of each dATP, dTTP, dGTP and dCTP was prepared in 1.5 mL eppendorf tube. The primers were used at a concentration of 20 pg μL^{-1} and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. Two micro liter of the primers, 5.0 μL of the target DNA and 42 μL of the stock solution were added onto 0.5 mL PCR tubes and mixed by vortexing. One micro liter of Taq DNA polymerase (Perkin Elmer) at

a concentration of 5.0 U μL^{-1} were used. All PCR amplification reactions were carried out in a final volume of 50 µL. The thermal cycling profiles were as follows: a 2 min initial incubation at 95°C, followed by 40 cycles of 95 °C for 1 min, 55°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ). Following amplification, 15 µL from each PCR containing amplified products were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the PCR products were easily identified following visualization under UV light.

Nested Polymerase Chain Reaction (nPCR): For the nested PCR amplification, 2.0 µL of the primary PCR product produced by EGL 1 and EGR 2 were transferred to 0.5 mL PCR tube containing (2 µL of nested primers; 45 µL of stock PCR buffer and 1 µL Taq DNA polymerase at a concentration of 5.0 U μL^{-1}). The nested pair of primers (EGL 3 and EGR 4) was expected to amplify a 276 bp PCR ampilicons, internal to the annealing sites of primers EGL 1 and EGR 2. All PCR amplifications were carried out in a final volume of 50 µL. The thermal cycling profiles were as follows: a 2 min incubation at 95°C, followed by 30 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 15 µL from each PCR product were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the nested PCR products were visualization under UV light.

RESULTS AND DISCUSSION

The described PCR-based assay afforded sensitive and specific detection of intact and calcified hydatid cysts of *Echinococcus granulosus*-complex obtained from naturally infected animals and humans. The outer pair of primers EGL 1 and EGR 2 produced a primary 435 bp PCR product from hydatid cysts of humans, sheep, cattle and camel origin. The primary 435 bp PCR product was visualized on ethidium bromide-stained gel from >10 pg DNA extracted from hydatid cyst. The primary 435 bp PCR products were detected directly from a variety of DNA extracted from 79 hydatid cysts samples used in this study (Fig. 1). Using the nested primers (EGL 3 and EGR 4), the PCR assay resulted in amplification of a 276 bp PCR product from as little as 1.0 pg DNA. The nested amplification increased the sensitivity of the PCR assay

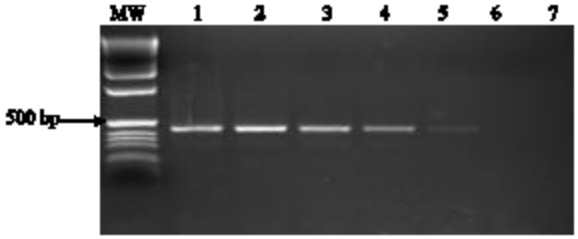

Fig. 1: Sensitivity of the PCR assay for detection of *E. granulosus*-complex using the outer pair of primers (EGL 1 and EGR 2), MW: Molecular Weight marker; Lane (1-6): DNA extracted from hydatid cyst of camel origin at concentrations of 100, 10 and 1 ng, 100, 10 and 1.0 pg, respectively; Lane 7: negative control sample

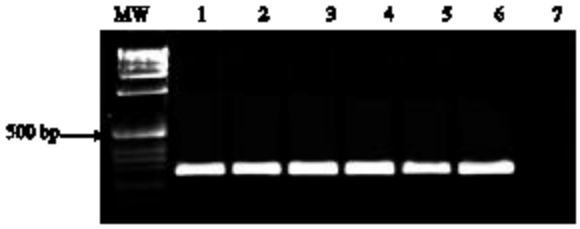

Fig. 2: Detection of *E. granulosus*-complex using the internal (nested) pair of primers (EGL 3 and EGR 4) of the above gel. The sensitivity was increased and as little as 1.0 pg of DNA was detected. MW: Molecular weight marker; Lane (1-6): DNA extracted from hydatid cyst camel strain (G 6) at concentrations of 100, 10 and 1 ng, 100 10 and 1.0 pg, respectively; Lane 7: Negative control sample

and confirmed the identity of the nucleotide sequences of EG complex (Fig. 2). However, the primary specific 435 bp PCR product and the nested 276 bp PCR products were not amplified from DNA extracted from larval stages of closely related cestodes, *Cysticercus tenuicollis and Coenurus cerebralis* (Fig. 3).

Very little information is currently available in regard to the influence of the genotypes (strains) of the parasite on the pathogenicity of the disease in man and animals. In Sudan, it has also been reported that sheep and goats seem to harbor calcified or infertile cysts of EG complex (Saad and Magzoub, 1989b; Omer *et al*., 2004; Osman, 2007). Thus, sheep and goats are considered unimportant host for transmission cycle of the parasite and maintenance of the disease. It was suggested that Sudanese breeds of sheep and goats have natural resistance to infection against the disease. However, this assumption requires further investigation. In Sudan,

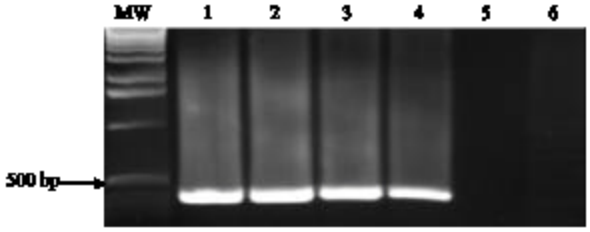

Fig. 3: Visualization of the specific 435 bp PCR products from 100 ng DNA extracted from different genotypes of *E. granulosus* hydatid cysts using primers (EGL 1 and EGR 2). MW: Molecular Weight marker; Lane 1: calcified cyst from liver of sheep; Lane 2: Camel hydatid cyst; Lane 3-4: Cattle hydatid cysts; Lane 5 and 6: DNA extracted from *Cysticercus tenuicollis* and DNA extracted from *Coenurus cerebralis*, respectively (negative controls)

camels are owned by migratory pastoralists as a source of milk, meat as well as riding animals. Extensive research has been conducted to evaluate the role played by Sudanese camels for transmission of parasitic infections with special emphasis on cystic echinococosis (Saad and Magzoub, 1986; Omer *et al*., 2004). Several studies were also conducted to evaluate the prevalence of cystic echinococcosis by Enzyme Linked Immunosorbent Assay (ELISA) in humans and animals (Craig, 1986; Benito and Carmena, 2005). The prevalence rate of the disease in camel was found to be as high as 48.69% (Saad and Magzoub, 1989a), 56.6% (Omer *et al*., 2004). The camel strain (G 6) was reported to be the most prevalent genotype of *E. granulosus* in Sudan and that camels seemed to play an important role in the transmission cycle of the parasite and the epidemiology of the disease. To advance beyond the current knowledge of the epidemiology of the disease, we proposed that attempts should be made to collect representative samples of adult worms from naturally infected dogs at different parts of the Sudan. These experiments should provide additional information to understand the biology, ecology and molecular epidemiology of this cestode parasite. In previous study, we validate the application of a nested PCR for detection of EG-complex and specific identification of the camel genotype (G 6) circulating in Sudan (Osman *et al*., 2007). Strikingly, all strains of hydatid cysts used in this study were identified as the camel strain (G 6 strain) irrespective of their origin. It is well documented that, PCR assays have been successfully applied for detection of EG complex and for identification of different genotypes of this cestode parasite. So far, there is no comprehensive data on the use of PCR for detection of calcified or degenerated hydatid cysts. The described PCR assay provides simple, rapid, sensitive and specific method for detection of EG complex and can be recommended for inclusion in surveys and control programmes. In the present study, we validated the nested PCR for detection of Sudanese genotypes of EG complex with special emphasis on sheep (G 1), cattle (G 5) and camel (G 6). The PCR, described in this study will probably detect the remaining genotypes of EG-complex. However, further research work would be necessary to confirm this assumption. Selection of these universal primers was based on the observation that the NADH dehydrogenase 1 gene has a highly conserved nucleotide sequence (Bowles and McManus, 1993b). The nested PCR assay was a simple procedure that efficiently detected all genotypes under the same stringency condition used in this study. The laboratory detection limit indicated that the nested PCR protocol was capable of detecting the amount of 1.0 pg equivalent to a single copy of total EG genomic DNA.

The specificity studies indicated that the 435 bp primary or the nested 276 bp PCR products were not amplified from DNAs extracted from other related cestodes under the same stringency condition described in this study. Based on these sensitivity and specificity results of this study, the described PCR should be considered as the most highly sensitive and specific assay compared to the previously described PCR-based detection assays. The second amplification step using the nested primers EGL 3 and EGR 4 confirmed the specificity of EG complex primary PCR product and increased the sensitivity of the PCR based assay by at least 100 folds. The use of nested amplification for confirmatory diagnosis renders this PCR assay a rapid and an inexpensive assay. The nested PCR does not require hybridization assay and this removes the hazardous and cumbersome radioactive laboratory procedures of working with P^{32} or P^{33} in hybridization assays or the cumbersome enzyme labeling procedure of the DNA probe. In addition, confirmation of the nucleic acid sequence of the PCR product with DNA sequencing machines is rather tedious and expensive. Sample preparation and DNA extraction using QIAamp extraction kit was a simple procedure, which takes one hour. The thermal cycling profiles for production of the primary and the nPCR products were consistently 4 h. Running of the agarose gel and electrophoresis usually takes an hour. Thus, confirmatory diagnosis using the described nested PCR assay could be made within the same working day. Application of this nested PCR as a coprodiagnostic procedure for detection of the parasite eggs in feces of infected canines is in progress.

CONCLUSION

The described PCR assay, using well characterized primers (EGL 1 and EGR 2), provides a simple, rapid, sensitive and specific method for detection of different genotypes of EG-complex including G 1, G 5 and G 6 but not other related cestodes. In addition, this PCR assay should be used as a valuable tool to identify calcified or degenerated hydatid cysts.

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