Development of real-time PCR assay for simultaneous detection and genotyping of cystic echinococcosis in humans and livestock

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ABSTRACT

Objective: To develop and evaluate a single-tube one-step real-time quantitative PCR (qPCR) assay for simultaneous diagnosis and genotyping of cystic echinococcosis (CE) in humans and livestock in the Sudan, and to compare it with conventional PCR assay.

Methods: Hydatid cysts were obtained from slaughtered animals and from humans after surgical interventions. DNA from the hydatid cysts and associated germ layers was extracted using a commercially available kit. The mitochondrial NADH dehydrogenase subunit 1 (NAD1) was used as a target for PCR amplifications. qPCR and conventional nested PCR assays were compared in this study.

Results: The qPCR assay amplified the NAD1 gene of hydatid cysts on melting temperature generated at 80 °C. Ten-folds serial dilutions of DNA with known dilution of 1 × 10⁶ to 1 × 10¹ (1 ng–1 fg) resulted in detection of as little as 1 fg of DNA with an R² value equivalent to 0.997. Similar sensitivities were encountered from both qPCR and the conventional nested PCR. The two assays did not amplify DNAs from Fasciola gigantica, Taenia saginata, Schistosoma bovis and DNA-free samples (negative controls). The PCR amplified products were purified for subsequent sequencing. The sequence data were analysed to insure the specificity of the amplified PCR products and to identify the genotype(s) of hydatid cysts. All cysts were identified as E. canadensis genotype 6 (G6).

Conclusions: The developed qPCR should be used as a rapid and reliable assay for diagnosis and genotyping of CE. The assay is highly recommended for the epidemiological surveillance in humans and livestock in endemic countries.

1. Introduction

Cystic echinococcosis (CE), due to the larval stages of Echinococcus granulosus senso lato (s.l), affects humans and a variety of domestic livestock and wild animals. The poor hygienic measures in developing countries, limited community awareness of the deleterious effect of CE on the health of affected individuals, presence of stray dogs, and home slaughtering of food animals are factors contributing towards the epidemiology of the disease in sub-Saharan Africa, including the Sudan[1-10]. Echinococcus granulosus s.l includes a number of genetically similar variants, strains or genotypes[11-13]. Ten distinct genotypes identified as G1–G10 have been described globally, based on nucleotide sequence analysis of the mitochondrial cytochrome C oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (NAH1) genes[14,15]. The genotypes have been related to the specific intermediate hosts including sheep, goats, horses, cattle, camels, pig, deer, elk, reindeer, moose and wapiti[16-26]. In humans, the disease is considered a deleterious health problem, while in animals infection may result in serious economic losses as the result of condemnations of affected organs in food animals[26,27].

Currently, infection with CE is diagnosed by radiology, microscopic demonstration of protoscolices, serology and...
molecular-based techniques. Serological reactions are useful for the identification of past infection during disease surveillance. However, cross-reactions are likely to occur with other cestode parasites. These limitations necessitate development of molecular techniques for rapid diagnosis of the parasite genome. The sensitivity of the assay is crucial in programs aiming at the prevention and control of the disease.

Previous researchers have described conventional PCR assays for the diagnosis of CE[7,28]. In addition to the conventional PCR assays used for the detection of CE, most of the researchers have also utilized the nested amplification for the purpose of increasing the sensitivity and confirming the identity of the primary amplified PCR product[9,29,30]. Moreover, the amplified PCR requires digestion by restriction enzyme using PCR-RFLPs techniques for genotyping of EG strain, which is a rather expensive, laborious and time consuming technique[6,31]. As an alternative the real-time quantitative PCR (qPCR) was developed[30,32].

Loop-mediated isothermal amplification (LAMP) assays have been developed and proved highly sensitive and specific for diagnosis of echinococcosis in the canine definitive hosts[33-35]. However, LAMP assays were described for limited applications and were not used on a wide scale. In the current investigation, a SYBR green-based qPCR assay was developed and compared to the conventional nested gel-based PCR for direct diagnosis of hydatid cysts obtained from humans and domestic animals, and the generated PCR products were sequenced and used for subsequent genotyping.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethical Approval Committee, Faculty of Veterinary Medicine, University of Khartoum, Sudan, and the Institutional Review Board of Al-Neelain University, Khartoum, Sudan. Human hydatid cysts were collected from patients subjected to surgical interventions at Khartoum Medical Teaching Hospital. The patients were informed of the objective of the sampling. Hydatid cysts from animals were collected during post-mortem examination by qualified veterinarians at the different abattoirs. The patients were informed of the objective of the sampling. Hydatid cysts from animals were collected during post-mortem examination by qualified veterinarians at the different abattoirs. The suspensions containing protoscolices and associated germinal layers were washed in nucleic acid free water to remove alcohol. Extraction of DNA from alcohol free cyst suspension was made possible using a commercially available QIAamp tissue kit (Qiagen Hilden, Germany) according to the manufacturer’s instructions. The detail for the extraction procedure was previously described[35]. A maximum yield of DNA was made possible by spinning the product at 12000 r/min for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length.

2.2. Collection of hydatid cysts

One hundred hydatid cysts were obtained from human patients and domestic animals. Ten of these hydatid cysts were recovered from humans during surgical interventions at Khartoum Medical Teaching Hospital. The remaining 90 cysts were obtained from animals during meat inspection at slaughter houses (70 cysts were collected from camels in Tamboulabattoir, 10 hydatid cysts from sheep, and 10 cysts from cattle in Omdurman slaughter house). Most of the hydatid cysts collected from sheep were rudimentary or calcified, whereas those collected from human patients and from cattle and camels were fertile. Protoscolices and associated germinal layers of the hydatid cysts were aspirated with sterile needles. The aspirates were transferred to clean sterile 50 mL bottles, to which 70% alcohol was added as preservative.

2.3. DNA extraction from intact cysts

The suspensions containing protoscolices and associated germinal layers were washed in nucleic acid free water to remove alcohol. Extraction of DNA from alcohol free cyst suspension was made possible using a commercially available QIAamp tissue kit (Qiagen Hilden, Germany) according to the manufacturer’s instructions. The detail for the extraction procedure was previously described[35]. A maximum yield of DNA was made possible by spinning the product at 12000 r/min for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length.

2.4. Selection of primers for nested and real time PCR assays

All primers were designed from the published sequences of NADH dehydrogenase 1 gene of E. granulosus genotype 6 (G6)[8]. In brief, a pair of outer primers (EGL1 and EGR2) were 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 GTA A. EGR2 included bases 447-466 of the complementary strand (5)-AAT CAA ATG GAG TAC GAT TA. Using primers EGL1 and EGR2, the primary PCR amplification will produce a 435 bp PCR product. The second pair of internal primers (EGL3 and EGR4) were designed from the same DNA sequence cited above and used for nested PCR amplification. EGL3 included bases 162-181 of the positive sense strand (5)-TTCTGTGTA. EGR4 included bases 420-437 of the complementary strand (5)-AAT CAA ATG GAG TAC GAT TA. The nested primers will result in amplification of a 276 bp PCR product, internal to the annealing cites of primers EGL1 and EGR 2. The nested pair of primers (EGL3 and EGR4) were also employed in SYBR green-based qPCR amplification assay.

All primers were synthesized on a DNA synthesizer (Milligen/ 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.

2.5. Conventional single-round PCR assay

A stock buffered solution containing 150 µL of 10× PCR buffer, 100 µL of 25 mmol/L MgCl₂, 12.5 µL of each dATP, dTTP, dGTP and dCTP at a concentration of 10 mmol/L was prepared in 1.5 mL Eppendorf tube and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. The primers were used at a concentration of 20 pg/µL. The PCR reaction mixture contained
2 μL of the primers, 5 μL of the target DNA and 42 μL of the stock solution, and 1 μL of Taq DNA polymerase at a concentration of 5 IU/μL was used. The thermal cycling profiles were as follows: a 5 min initial incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 45 s, and a final incubation at 72 °C for 10 min.

2.6. Conventional nested PCR assay

For this assay, nested primers were used at a concentration of 20 pg/μL. Then 2 μL of the 435 bp primary PCR product were used as templates in the second round of nested PCR amplification. All PCR amplification reactions were carried out in 0.5 mL PCR tubes. The thermal cycling profiles were as follows: a 5 min initial incubation at 95 °C, followed by 40 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 45 s, and a final incubation at 72 °C for 10 min. The primary and nested PCR amplification products were visualized onto ethidium bromide-stained agarose gels.

2.7. SYBR green-based real time qPCR assay

A single-tube amplification reaction was carried out using one-step QIagen kit (QIagen). In brief, a standard 25 μL reaction mixture contained in final concentration of 0.4 mmol/L dNTP was mixed with 3 mmol/L MgSO4, DNA polymerase. Then 250 mmol/ L of each nested primers (Macrogen, Seoul, Korea), 0.5 μL SYBR green 1 dye (Molecular probe, USA) diluted (1:1 000) in RNase free water were added to the PCR amplification reaction. Target genes were amplified in low-profile 0.2 mL tubes. The amplification was carried out in a LightCycler Rotor Gene machine (QIagen, Australia). No template control (NTC) was used as negative control. The cycling program consisted of initial pre-denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 15 s, and annealing at 55 °C for 45 s. Finally, a melting curve analysis was done from 55–95 °C. The fluorescence threshold limit of the Rotor Gene system was set at 0.02.

3. Results

3.1. Sensitivity of the conventional primary PCR

The conventional PCR detected DNA extracted from all hydatid cyst used in this study. The outer pair of primers EGL1 and EGR2 produced a primary 435 bp PCR product from ≥100 fg DNA. The primary PCR amplification products were visualized on ethidium bromide-stained agarose gels (Figure 1A).

3.2. Sensitivity of the nested PCR

The nested primers (EGL3 and EGR4) produced a 276 bp PCR product internal to the annealing sites of the outer primers. The nested amplification increased the sensitivity of the PCR assay and as little as 1 fg of DNA was detected by this assay as visualized on ethidium bromide-stained agarose gels (Figure 1B).

3.3. Specificity of the nested PCR and qPCR

The specificity studies of both conventional and qPCR assays indicated that the PCR product was specific and did not cross-amplify DNA of Taenia saginata, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples. The result of the specificity of the conventional nested PCR is presented in (Figure 2).

3.4. Sensitivity of the SYBR green-based real time qPCR

The one-step qPCR based on SYBR green 1 chemistry enabled rapid detection of hydatid cysts. Serially diluted E. granulosus DNA (1 × 10^6 to 1 × 10^1, i.e. 1 ng to 1 fg) yielded a detection limit of as little as 1 fg of DNA (Figure 3). The standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of the serially diluted E. granulosus DNA showed a linear range of 6 logs of dilution with a R² value equivalent to 0.99759 (Figure 4). The melting curve analysis of the amplified DNA products from hydatid cysts obtained a distinct melting peak (Tm
value) at 80 °C (Figure 5).

**Figure 3.** Sensitivity of the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA (1 × 10^6 to 1 × 10^1) as represented by numbers 1–7, respectively.

**Figure 4.** Standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA (1 × 10^7 to 1 × 10^1) as represented by numbers 1–7, respectively. The figure shows a linear range of 7 logs of dilution with a R^2^ value equivalent to 0.99759.

**Figure 5.** Melting curve analysis of the amplified DNA products from hydatid cysts, with a distinct melting peak (Tm value) at 80 °C.

3.5. **Sequencing and genotyping of hydatid cysts**

Partial sequences produced by PCR amplifications were found to align with the corresponding regions for NADH 1 gene in the GenBank confirming that the cysts contained the *E. granulosus* complex. Aligned with BioEdit, partial sequences for NADH 1 showed high similarity among all *E. granulosus* isolates recovered in this study. All cysts were identified as camel genotype (G6).

4. **Discussion**

The performance of SYBR green-based real-time qPCR assay for diagnosis of hydatid cyst was compared with the conventional gel-based nested PCR assay. Both conventional and qPCR assays showed similar sensitivity and specificity for the rapid diagnosis and differentiation of hydatid cysts from humans and animals. The developed qPCR assay showed a dynamic detection limit, which spans over a 7 log_10 concentration range. However, the nested PCR was found to be time consuming, prone to errors and complication by cross contamination resulting from multiple manipulations of the primary PCR products. Whereas, SYBR green-based qPCR required approximately 45 min from sample submission until the assay is accomplished giving final results, while the time spent for nested PCR amplification and subsequent visualization of results required at least five consecutive hours. Accordingly, the qPCR has been optimized in this study to develop an efficient qPCR assay for diagnosis and quantification of *E. granulosus* hydatid cysts because of its simplicity, high sensitivity, and specificity and cost efficiency. The additional advantage of utilizing SYBR green 1 based qPCR is that the test primer pairs are relatively easy to design and are suitable for conventional PCR analysis.

To generate a standard curve and to insure the possible detection limits of the SYBR green-based qPCR assay, 10-fold serial dilutions (1 × 10^6 to 1 × 10^1) of a known concentration of the parasite DNA were tested in the current study. The assay showed linear results for the 6 logs of the serially diluted DNA. The detection limit of the SYBR green 1-based assay was calculated to be 1 fg equivalent to DNA extracted from 10 protoscolices. Melting curve analysis was conducted to insure the existence of the specific amplicon in the reaction tube. The melting peak temperature (Tm value) was calculated to be 80 °C from the PCR products. The conventional gel-based nested PCR assay was proved to be highly specific in detecting the *E. granulosus* DNA. The first round of the conventional PCR was far less sensitive for the detection of *E. granulosus* DNA when compared to the nested PCR or SYBR green-based qPCR assay. The sensitivity was, however, significantly increased (1000 times) using a second round of nested amplification with the nested primers. Similar result was obtained with SYBR green-based qPCR and as little as 1 fg of parasite DNA was detected in both assays. The SYBR green-based qPCR is a single-tube one-step assay that does not require post amplification steps. The specificity studies of both conventional and qPCR assays indicated that the PCR products were specific and did not cross-amplify DNA of other parasites.
including cysticercus bovis, *Fasciola gigantica*, and *Schistosoma bovis* and nucleic acid free samples.

It is worth mentioning that surgical removal of hydatid cysts in hospitalized human patients requires intercostal intubation, which may result in accidental rupture of pulmonary cysts. The ruptured cyst is likely to be invaded by secondary bacteria[36]. In addition, calcification of hydatid cysts is not an uncommon finding in infected patients. Infertile or calcified hydatid cysts are common in animals, as reported in the majority of the Sudanese desert sheep and Nubian goats[6,7,28,37]. In these circumstances, the diagnosis of cystic hydatidosis by conventional techniques would be extremely difficult, if not impossible. However, in the present investigation, the detection of ruptured and calcified cysts was made possible by both the conventional and qPCR assays. The qPCR assay was preferred for its convenience and minimum sample handling, thus preventing the occurrence of cross-contamination which may decrease the quantitative reliability of the assay. It is well documented that SYBR green-based qPCR assays are less specific than the TaqMan qPCR assays. In addition, the qPCR assay can be implemented in a research laboratory setting for the purpose of rapid diagnosis and epidemiological surveillance of CE in humans and animals in developing countries, such as Sudan.

CE is widespread worldwide including the Sudan and identification of the genotype of the cyst would be advantageous for the prevention and control of the disease[37-40]. In Sudan, genotypes G5 and G6 were described in cattle and dromedary camel (*Camelus dromedarius*). The sequence analysis showed that *Echinococcus canadensis* genotype 6 (G6) is the most infectious and widespread genotype in the Sudan, which is in agreement with previous studies[28,30,41]. In conclusion, this study demonstrates that SYBR green-based qPCR can serve as a useful tool during survey of the disease among humans and susceptible animal populations. The qPCR offers advantages over the conventional gel-based nested PCR, being less time-consuming and preventing cross contaminations.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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