Original Article

Optimization of Real-Time Quantitative PCR assay for detection of *Echinococcus granulosus* in fecal samples

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Abstract

Background: Echinococcosis/hydatidosis is one of the most important zoonotic diseases. The definitive hosts for *Echinococcus granulosus* (*E. granulosus*) include a wide variety of the Canidae and dogs. Early detection of Echinococcosis in dogs is the most influential factor in improving the prevention and control of hydatidosis. The primary purpose of the present study was to optimize the real-time quantitative PCR to diagnose *E. granulosus* infection in dogs before the disposal of eggs.

Materials and Methods: Three puppies were selected to be inoculated by 70000 protoscoleces. Normal saline was inoculated to the other two puppies chosen as an experimental negative control group. Ten privately owned healthy puppies were selected for the natural negative control group. Stool samples were collected on days 7, 14, 21, 28, and 35 post-infection, DNA was extracted, then a 287bp fragment of tandem repeat region gene was amplified and cloned into linearized TA vectors. Serial dilutions of recombinant plasmid DNA and a standard curve were established. The copy amount of DNA in each sample was determined based on the standard curve.

Results: The minimum time copro –DNA could be detected in the stool sample was found on the 7th day post-infection, which was equal to 9750×10^{-8} copy number or 9.75 pg of DNA. The assay was linear in 10^5 to 10^8 copies of the recombinant plasmid per microliter.

Conclusion: The real-time PCR assay diagnosed the infection eight days earlier than the copro antigen ELISA method (7 days versus 15 days, respectively).

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Keywords: Real-time quantitative PCR, *Echinococcus granulosus*, Fecal-DNA, Tandem repeat region of DNA, Dog

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Introduction

Echinococcosis is one of the most critical zoonotic infections in the world with an indirect life cycle, recognized as a significant helminthic cyclozoonosis of great public health and

economic significance. In the lifecycle, the carnivores are the reservoir hosts and a wide range of ruminants and humans consisting of the larval stage, hydatid cyst, are the intermediate hosts[1]. Contamination of dog feces with *E.granulosus has been* reported at 8.3% to 41.3%, depending on the region[2]. The prevalence of infection with *E. granulosus* in dogs was 5% to 49% in different parts of Iran[1]. Dog as a definitive host has the most important role in the life cycle of *E. granulosus* and eggs of *Echinococcus* species shed from the feces of dogs; therefore, it should be considered the first target for control and prevention of this infection[3]. Parasitological examination of eggs is unsafe and not very useful because it is challenging to distinguish E. granulosus species from other Taenia eggs morphologically.

Furthermore, the symptoms do not appear until one month after the infection[4]. Infection in the dog has no specific clinical signs, similar to other parasitic infections. Therefore *E. granulosus* has been traditionally diagnosed in dogs ante mortem or after an Arecoline hydrobromide purge detection regardless of its limited safety precautions[3]. This method has been applied to quantitative studies. Only 43% of adult worms of *E. granulosus* have been detected in the purging at the first time (90% detecting at the second time) [5]. Since eggs of most members of the *Taeniidae* family are morphologically indistinguishable, microscopic observation of fecal samples needs acceptable efficacy. Detection of *E. granulosus* copro-DNA by the enzyme-linked immunosorbent assay (ELISA) technique is more sensitive than microscopy but cross-reacts with other *Taeniidae.* Currently, DNA-based differentiation has been described and includes the use of probes, PCR with species-specific primers, and PCR followed by restriction enzyme analysis[6]. Copro DNA detection is one of the best methods for *E. granulosus* tracing in dogs because it can prove the presence of *E. granulosus* in the small intestine. The detection and identification of copro-DNA may be advantageous over copro-antigens, as it provides the possibility of post-detection analysis of the sample DNA for precise identification of the proposed species. Some techniques include restriction fragment length polymorphism and direct comparing PCR-amplified DNA sequences[6]. Two objectives have been followed based on detecting the *Echinococcus* copro-DNA molecules by PCR in animal fecal samples: differentiation between Taenia family eggs and detecting *Echinococcus* DNA in feces to diagnose the infection[7]. The primary purpose of this study is to answer the following question: How many days after infection is it possible to detect echinococcosis infection in dogs?

Methods

Protoscoleces preparation: The present study was conducted from June 2014 to September 2015. Livers of sheep prepared from a slaughterhouse were collected after being visually inspected for the presence of hydatid cysts and were transferred into the parasitology lab in sterile transfer containers on dry ice. At first, the surface of infected organs was disinfected using 70% alcohol. Cyst contents were aspirated, and content was transferred into a V-shaped sterile graded cylinder in a refrigerator for 30 min. Then, the supernatant was removed, and protoscoleces (precipitated before) were transferred into new sterile graded tubes. The viability of protoscoleces was assessed based on flame cell activity and a 1% eosin exclusion test. In the present study, 70000 live protoscoleces were observed (on average), and protoscolex (G1 genotype) with 95% viability was used for challenging the puppies based on the Eslami and Hosseini (1998) protocol. The strain of protoscolex was determined based on morphological characterizations[8].

Preparing puppies for protoscolex challenge: According to the research ethics committee recommendations for laboratory animals in Shahid Beheshti University of medical sciences, five puppies (70-90 days old) were purchased from a dog shelter running under municipality supervision. There was no chance of infection with *E. granulosus* and treated with Drontal 10mg/kg (a broad-spectrum anti-parasitic drug combination for disinfection of possible parasitic infections). In addition, they were vaccinated against rabies and six other viral diseases. Two weeks after anti-parasite treatment, three puppies were challenged using 70000 protoscolex of *E. granulosus*, sheep strain (G1 genotype). Water was immediately given to the dogs to prevent vomiting. The puppies' feces were

collected before and after the challenge. Daily collected fecal samples were tested for detecting *Taenia* eggs by Willis's method using saturated salt water. The puppies' fecal samples were prepared on days zero (before challenge), 7, 15, 21, 28 post-challenges, and day 35, and DNA was extracted using commercial kits according to manufacturer protocols (MBST, Iran). Then the DNA was stored at -30 °C. Usually, on day 35 post-infection, 20% of the sheep strain (G1 genotype) parasites developed three segments, and on day 41, up to 58% of the parasites contained mature eggs citing the results of other studies[9, 10]. All samples were labeled in terms of the sampling information and dog numbers.

Preparing puppies for the control group: Two puppies were treated with normal saline, and the fecal samples were prepared on days zero, 7, 15, 21, 28, and 35 post-challenge, and then the samples were labeled.

Collected fecal samples in the negative group: Fecal samples were prepared from 10 puppies (privately owned), fed with cooked food, and brought to a small animal hospital with no history of *E. granulosus* infection. All days had regular amplification programs. These samples were selected as the negative control.

DNA extraction from fecal samples and Cloning TA vector (PTZ57RT plasmid): DNA extraction from fecal samples was performed using the commercial kit (MBST, Iran) based on the manufacturer's procedure. Designed primers were used to amplify a 287 bp fragment of DNA repeat region gene for *E. granulosus* detection in feces. The amplified fragment was cut from agarose gel 1.5%, and the sequence of the purified primer was designated according to the known repeat region sequence (GenBank accession number DQ157697): Forward primer was 5'CACCACGCATGAGGATTAC3', and the reverse primer was 5'ACCGAGCATTTGAAATGTTG 3' (SinaClon, Iran). The amplified fraction was extracted from the gel using the commercial kit (MBST, Iran). Then it was cloned in the TA vector (PTZ57RT plasmid, Fermentas) and was transformed into *Ecoli* DH5α After multiplication, the recombinant plasmid was extracted from the plasmid extraction kit based on the manufacturer's procedure (MBST, Iran). The Bam HI enzyme cuts the extracted plasmid to yield a linear fragment (Fermentas, Lithuania). Several dilutions of plasmid were prepared, and then the plasmid concentration was determined by Nano drop in the prepared dilutions.

Determination of plasmid copy number: The number of plasmid copies was calculated based on the following formula using NEBiocalculator,

$$\text{Number of plasmid copies} = \frac{amount\ (ng)\ \times\ 6.022\times 10^{23}}{\text{Length (bp)}\ \times\ 1\times\ 10^{9}\times\ 660}$$

Amount= amount of DNA (Nano gram), 6.022×10^{23} = Avogadro's constant, Length (bp) = Length of DNA, 1×10^{9}= conversion factor, 660= Average mass of 1bp DNA.

Real-Time PCR standard curve: The standard curve of the Real-Time PCR (Rotor-gene, Switzerland) was presented by evaluation of different dilutions of the plasmid. The fragment was amplified using specific primers and the SYBR Green kit (Takara, Japan).

Fecal-DNA quantification: The partial sequence (287bp) of Extracted Fecal DNA (samples collected the day before infection and post-infection on the 7^{th}, 15^{th}, 21^{st}, 28^{th}, and 35^{th} days) were amplified using standard PCR and Real-Time PCR by SYBR Green Kit (Takara, Japan). The amount of fecal- DNA was determined using the CT number on the standard curve, constructed by plotting the log of the initial DNA concentration versus the Ct value (Figure.3). PCR reaction was performed in a 20-microliter volume set. Then, ten microliters of 2X Master mix (Amplicon, USA) and 0.5 microliters of each primer (10µ mol/L), and five microliters of the linear plasmid of each sample were added, and sterile distilled water was added to obtain a 20 µl of final volume per reaction. These reactions occurred in hygienic conditions and were performed on ice.

It should be noted that samples and standards were put in one reaction. The program was set as follows: A cycle was performed at 95°C for 1 min, followed by 45 cycles at 95° C for 10 s, at 60.5° C for 10 s, and at 72° C for 15 s. Melting point (Melting temperature) was also determined after each reaction.

Results

The partial sequence (287bp) of DNA repeated region gene isolated from fecal samples was amplified every week for up to 35 days (Figure 1). The results of the amplification plot for the 4-fold dilution series of recombinant plasmid (as standard samples) are shown in Figure 2. The standard curve was drawn, and the results were compared with this curve (Figure 3). The standard quantification curve of the 4-fold dilution series of recombinant plasmid shows 83% efficiency (Figure 3). The quantitative measurement results of the amount of this DNA in the fecal samples were presented over different days, shown in Figure 4. The Tm (melting temperature) results were introduced in Figure5, indicating the reaction's specificity and confirming the method's reliability.

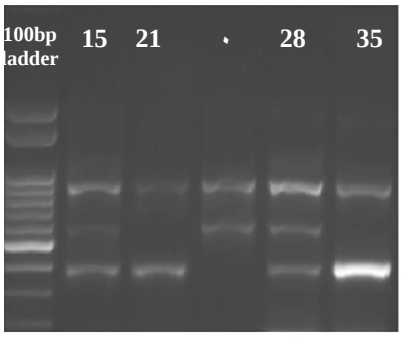

Figure 1. PCR products (287bp) of tandem repeat region DNA were obtained from fecal samples before and after the challenge with *E.granulosus*. From left to right, 100bp DNA ladder (SinaClon, Iran), day 15 after the challenge, day 21 after the challenge, day 0 before the challenge, day 28 after the challenge, day 35 after the challenge.

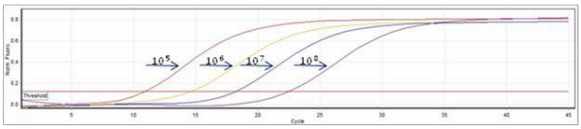

Figure 2. The amplification plot for the 4-fold dilution series (from 10 -5 to 10 -8) of Recombinant plasmid (plasmid Ptz57RT with a piece of DNA repeated region) as standard samples. The red line represents the threshold.

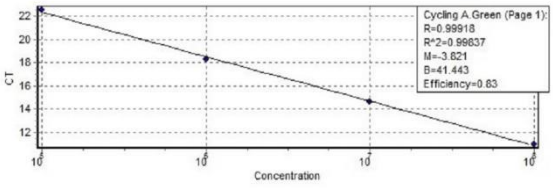

Figure 3. Standard quantification curve of *E.granulosus* tandem repeat region DNA. The standard quantification curve related to amplification of the 4-fold dilution series of Recombinant plasmid (Ptz57RT with a 287bp piece of DNA tandem repeated region) as standard samples. The standard curve shows the threshold cycle (CT) on the y-axis (12-22) and the logarithm of the dilutions of plasmid copy number is shown on the X-axis (10^5 to 10^8). The slope of the line obtained from standard curve was about 3.8 with 83% efficiency.

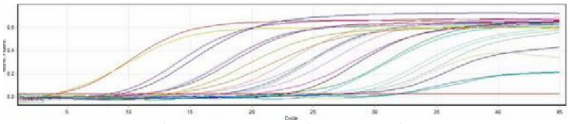

Figure 4. Amplification plots of relative fluorescence (y-axis) versus cycle numbers (x-axis) show amplified curves of fecal DNA on days 0, 7, 14, 21, 28, 35 and amplified curves of the standard samples (recombinant plasmid with serial dilutions of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5}). The standards were prepared at 9750× 10^{-5} ng to 9750× 10^{-8} ng of the recombinant plasmid.

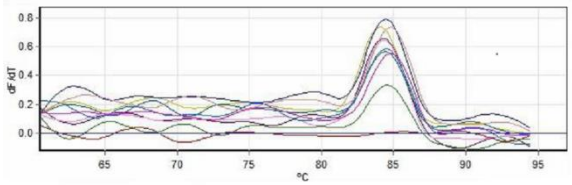

Figure 5. Melting curve analysis of copro-DNA real-time PCR products derived from -ΔF/ΔT (Y-axis) versus temperature (X-axis). The melting curve shows the proliferative pieces. 287 bp of DNA repeat region, at a denaturation temperature of 84° C, and primer-dimer is a straight line representing absence.

Discussion

E. granulosus is one of the most important and widespread of the helminth zoonoses[11]. Diagnosis of *E. granulosus* in dogs is vital since diagnosis of Echinococcosis is primarily concerned with infections in dogs. Cystic hydatid disease control programs are based on definitive host treatment to disrupt the parasite life cycle. Copro-antigen methods were used to detect infections during the patent period, but their sensitivity and specificity are low regulated by the parasite burden. The present study showed the ability of the real-time PCR method to detect *E. granulosus* infection of sheep strains (G1 genotype) from the seventh day during the patent period. Currently, diagnosis of *E. granulosus* infection in dogs involves the administration of Arecoline salts followed by collecting and analyzing purged materials[12] immunodiagnostic tests. It includes ELISA developed based on genus-specific copro-antigen detection, and serum antibodies (IgG, IgA, and IgE) were compared against Arecoline purgation to detect *Echinococcus*

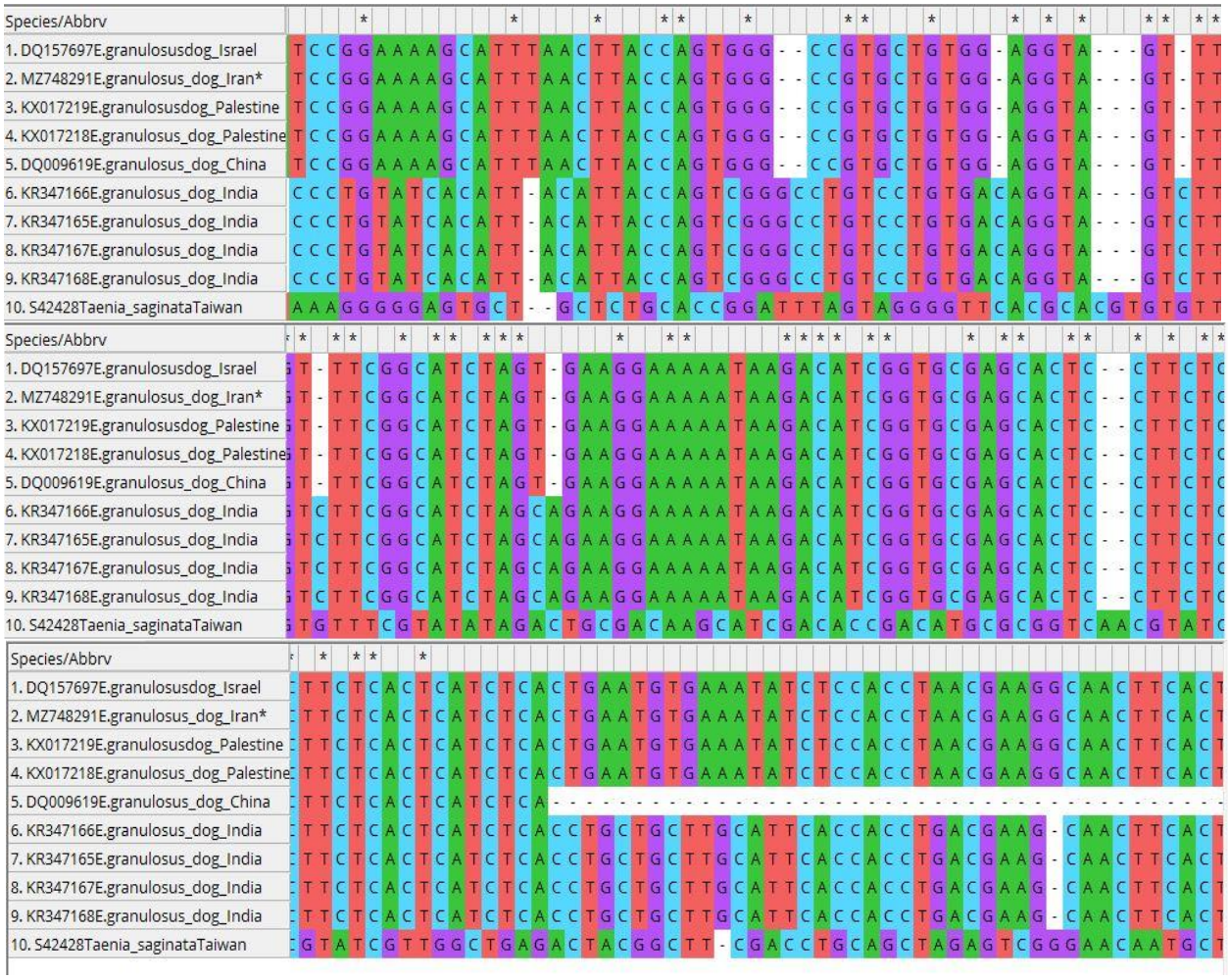

Figure 6. Alignment result of *E.granulosus* within the partial sequence of tandem repeat region in this study, accession number MZ748291, with isolates from Israel, Palestine, China, and India. The identity of the sequences was 85.7% - 100% and Polymorphic regions in aligned DNA sequences differentiate the *E.granulosus* Iran isolates from other isolates and *Taenia saginata*. Difference between aligned sequences are highlighted by different background colors of respective nucleotides. Asterisks represent nucleotides identical in all aligned sequences. The tandem of each aligned sequence consists of NCBI accession numbers followed by country name.

spp. in naturally infected dogs in Uruguay[13]. The copro-antigen in the ELISA test had a sensitivity of 76.9% compared to 34.6% obtained from the serum IgG detection in ELISA when assessed against 26 purge-positive dogs (purge worm counts ranged from1 to 4331). Copro-antigen reactivity was positively correlated to purge worm count, with a threshold of over 20 worms. There was no positive correlation between antibody levels and worm counts[13]. In the 26 matched positive dog samples, the sensitivity of serological detection increased to 69.2% when seroreactivity for IgA and IgE antibodies reached 96.2% when both copro-antigen and antibody assays were combined. The detection of current infection in individual dogs affected with *E. granulosus* by copro-antigen in ELISA test has the potential to replace with Arecoline purgation. In contrast, specific serum antibody detection should be used in assessing the exposure *to Echinococcus* in dog populations[12, 14]. Serology diagnosis of intestinal *E. granulosus* infection based on an individual dog was unreliable because an equal number of protoscoleces had not the same number of worms. It should be considered for the experimental research on *E. granulosus*[15, 16]. In Iran, Arecoline hydrobromide and autopsy are generally used to detect infection in dogs[7]. The copro-PCR method is an acceptable alternative with higher sensitivity and specificity, but inhibitor agents often influence it in fecal samples[7]. There are some limitations in using serologic methods in the

early phase of infection because in the first weeks after challenges, the antibody titer is insufficient for detection[17]. Therefore, the diagnosis should be based on detecting agents in the blood, urine, or fecal samples[18]. Sequences obtained in the present study (accession number MZ748291) were aligned and revealed 85.7%-100% identity with GenBank data (Figure6). In the present study, the 287bp gene used in repeated sequence consists of approximately 6,900 copies, arranged in groups of 2-6 repeats tandemly[20]. Tandemly repeated DNA arrangements make valuable targets for PCR diagnosis due to their high copies and specificity[21]. For instance, a highly repeated sequence of *Schistosoma haematobium (S. haematobium)* was suggested for monitoring free-living cercariae and infected snails; this sequence consists of tandemly arranged 121-bp-long units and is highly abundant and comprises 15% of the *S. haematobium* genome[22]. Real-time quantitative PCR using a standard curve was designed to detect and discriminate between different amounts of DNA: 9750×10^{-5} to 9750×10^{-8} on different days post-infection, aimed at studying *Echinococcus granulosus*. However, a larger panel of wild and domestic carnivores in field studies must be considered while dealing with helminth parasite detection and quantification[11]. This study's results showed that copro-DNA could be detected at the end of the first week or from the beginning of the second week after infection, before egg disposal (975×10^{-8}). The negative results obtained from the copro-ELISA test for diagnosis of the infection in sheepdogs, guard dogs, tracker dogs, and other dogs should be confirmed by copro- PCR. Real-time quantitative PCR for *Toxocara* spp. and *Echinococcus multilocularis* was performed in fecal samples[19]. Detecting the amount of DNA in feces could guide the researchers to estimate the burden of infection and the role of dogs in the distribution of the worm.

It becomes more critical when estimating the true prevalence of infection and analyzing the data about the role of dogs in the epidemiology of Echinococcosis. In the endemic region, the considerable importance of quantitative assays is highlighted. In Iran, early diagnosis of Echinococcosis during the patent period before eggs are excreted, particularly for sheep dogs and guard dogs, is regarded as fundamentally important and should be considered by the Iranian Ministry of Health and Medical Education and Iran Veterinary Organization.

Conclusion

In conclusion, the Real-time PCR assay improved in this study, based on the amplification of tandemly repeated DNA sequences delivers a specific and sensitive diagnostic tool for the detection of *E.granulosus* DNA in dog feces and provides alternative diagnostic method for epidemiological studies.

Acknowledgment

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