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RESEARCH ARTICLE

Development of a Multiplex PCR Assay for the Simultaneous Detection of *Echinococcus* spp. in Wild Canids in the Qinghai-Tibet Plateau Area of China

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Abstract

Infections of *Echinococcus granulosus sensu stricto, E. multilocularis* and *E. shiquicus* are usually prevalent or coendemic in wild canids in the Qinghai-Tibet Plateau area (QTPA) of China. Thus, an efficient method for the detection of infected hosts and the identification of *Echinococcus* species is needed. The present work aims to establish a multiplex PCR (mPCR) method that can simultaneously detect the three main *Echinococcus* species mentioned above, and provide technical support for the diagnosis, prevention and control of *Echinococcus* infection. Three pairs of specific primers were designed for this mPCR based on the *Echinococcus* mitochondrial genes in GenBank, and these primers were validated by specificity and sensitivity experiments and applied for simulated coinfection samples detection and filed samples. This mPCR method was able to successfully identify both simplex and mixed target *Echinococcus* spp. and generate expected amplicons of different sizes for each species. The sensitivity of this mPCR method was tested with serially diluted gene recombinant plasmid, and the results showed a detection threshold of less than 10^3 for both species. The specificity, which was assessed against 10 other parasites, was found to be 100%. The assay was also used on 15 simulated clinical samples, and the results confirmed the high reliability of the method, indicating that the mPCR method established in this study can be used to analyze clinical samples from the QTPA. This mPCR method has potential application in rapid detection, diagnosis and broad-scale screening and is expected to become a key technology for clinical detection and environmental monitoring.

Keywords: *Echinococcus granulosus, Echinococcus multilocularis, Echinococcus shiquicus, Multiplex PCR, Wild canids, China*

Çin'in Qinghai-Tibet Platosu Bölgesindeki Yabani Köpekgillerde *Echinococcus* spp.'nin Eşzamanlı Saptanması İçin Multipleks PCR Yönteminin Geliştirilmesi

Öz

Echinococcus granulosus sensu stricto, E. multilocularis ve *E. shiquicus* enfeksiyonları, Çin'in Qinghai-Tibet Platosu bölgesindeki (QTPA) yabani köpekgillerde genellikle yaygın ve koendemik seyretmektedir. Bu nedenle, enfekte konakçıların tespiti ve *Echinococcus* türlerinin tanımlanması için etkili bir teşhis yöntemine ihtiyaç vardır. Bu çalışmada, bahsedilen bu üç temel *Echinococcus* türünün aynı anda tespit edilmesi ve *Echinococcus* enfeksiyonlarının teşhisi, önlenmesi ve kontrolü için teknik destek sağlayan bir multipleks PCR (mPCR) yönteminin geliştirilmesi amaçlandı. mPCR için, *Echinococcus*'un GenBank'ta mevcut olan mitokondriyal genlerine bağlı olarak üç çift spesifik primer tasarlandı ve bu primerlerin özgüllük ve duyarlılıkları doğrulanarak temsili koenfeksiyon örneklerinin tespiti ve saha örnekleri için kullanıldı. mPCR yöntemi, hem bireysel hem de karma hedef *Echinococcus*'ları başarıyla tanımladı ve her tür için farklı boyutlarda beklenen amplikonlar oluşturdu. mPCR yönteminin duyarlılığı, seyreltilmiş gen rekombinant plazmidi ile test edildi ve her iki grup için 10^3'ten daha düşük bir saptama eşik değeri gösterdi. Diğer 10 parazite karşı değerlendirilerek, yöntemin spesifitesi %100 saptandı. mPCR yöntemi ayrıca temsili 15 klinik örnekte test edildi ve geliştirilen bu yönteminin QTPA'da klinik örneklerin analizinde kullanılabilecek yüksek güvenilirliğe sahip olduğu doğrulandı. Geliştirilen bu mPCR yönteminin, hızlı tespit, teşhis ve geniş ölçekli tarama çalışmalarında potansiyelinin olduğu saptanmıştır ve böylelikle klinik tespit ve çevresel izleme için kilit bir teknoloji olması beklenmektedir.

Anahtar sözcükler: *Echinococcus granulosus, Echinococcus multilocularis, Echinococcus shiquicus, Multipleks PCR, Çin*

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Introduction

Echinococcosis is a zoonotic disease caused by metacestodes (larval stage) of *Echinococcus* spp., which include one or more of nine species recognized in the most recent taxonomic revision of the genus *Echinococcus* [1,2]. Echinococcosis is currently a neglected zoonotic disease worldwide, resulting in great public health concerns and economic losses globally [3]. There are two *Echinococcus* spp. that seriously threaten human health, namely, *Echinococcus granulosus sensu stricto* (genotypes G1-G3) and *E. multilocularis*, which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. *E. canadensis* (G6) infections also result in CE, but *E. shiquicus*, which is the sister species of *E. multilocularis*, has not been reported to cause human echinococcosis thus far. To date, *E. multilocularis*, *E. granulosus s.s.*, *E. shiquicus* and *E. canadensis* (G6) have been reported in China, and *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* are mainly prevalent in the QTPA [4-6]. It is estimated that cystic and alveolar echinococcosis results in the loss of 1.000.000 and 600.000 disability-adjusted life years (DALYs) each year, respectively [7,8]. In China, there are 368 endemic counties distributed in nine provinces/autonomous regions with an estimated 166.000 cases nationally [9]. Obviously, the world's highest prevalence of echinococcosis is in China; moreover, the QTPA is located in western China, has the highest prevalence of echinococcosis in the country [9]. Echinococcosis is a very serious public health problem in the QTPA, and it causes a great threat to human health and livestock economic development.

The life cycle of *Echinococcus* parasites is complex and involves two hosts: definitive hosts and intermediate hosts [5]. *E. granulosus s.s.* is generally transmitted between canids (dogs) and livestock (yak, cattle and sheep) [10-12], while *E. multilocularis* and *E. shiquicus* are mainly transmitted between canids (dogs and foxes) and small mammals (voles and pikas) [13-15]. At present, imaging examinations, immunological methods, histological detection, and microscopic observation are the main methods widely used in clinical and surgical procedures and in survey work. However, it is difficult to morphologically distinguish the adults and eggs of some *Echinococcus* tapeworms, such as *E. multilocularis* and *E. shiquicus*. With the development of molecular biology technology, many molecular identification methods have been established using parasite total DNA as target genes [16,17]. These methods can be used to distinguish the species of the *Echinococcus* tapeworms and eggs found in the fecal samples of the definitive host. However, it is also difficult to accurately identify species in the unobvious hydatid tissue samples from different intermediate hosts. Thus PCR-based molecular detection has been used for the differential diagnosis of echinococcosis.

To date, many known PCR-related approaches targeting parasite total DNA have been designed and developed to identify different stages of the *Echinococcus* life cycle in different hosts, and some of these methods are used for the identification/discrimination of single species with singular amplification [18,19]. PCR-RFLP or LAMP for species discrimination and nested-PCR, which consists of two PCR steps with higher sensitivity, are slow, laborious and costly methods, and these methods cannot meet the needs of rapid diagnosis and large-scale detection. Several mPCR methods have been developed for identifying *Echinococcus* species [18-21].

The mPCR method can simultaneously detect different target pathogens species by using multiple specific primers in one tube, which is fast; reagent-, material- and labor-saving; and cost- effective. In addition, this method can also distinguish pathogens from mixed infections with accuracy and efficiency at the same time. The mPCR method is also very suitable for large-scale multi-sample epidemiological investigations in epidemic areas, such as studies of echinococcosis in the QTPA.

In this study, we describe the development and evaluation of a simultaneous mPCR method for the detection of single and mixed infections of three *Echinococcus* species that are prevalent in the QTPA, to quickly and accurately detect and identify the *Echinococcus* species in intermediate hosts or fecal samples of definitive hosts. This study was performed to provide technology for the epidemiological investigation of *Echinococcus* spp. and to promote the effective treatment and control of echinococcosis.

Material and Methods

Parasites

The total DNA of *E. multilocularis* (Em), *E. granulosus s.s.* (Eg), *E. shiquicus* (Es), *Taenia multiceps* (Tm), *Taenia saginata* (Ts), *Taenia asiatica* (Ta), *Dipylidium caninum* (Dc), *Taenia hydatigena* (Th), *Toxocara canis* (Tc) and *Fasciola hepatica* (Fh) are stored in the zoonotic disease laboratory of the Institute of Veterinary Medicine, Qinghai Academy of Animal Sciences and Veterinary Medicine.

Primer Design

The complete sequence of the mitochondrial genomes of *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* were compared using MEGA 5.05 software [22]. Genes with less similarity among the three species of *Echinococcus* spp. were selected as target genes, and then, the partial sequences of the selected target genes with high similarity were selected to design primers by using Primer Premier 3.0 [23]. The primers are listed in *Table 1* and synthesized by Genewiz Suzhou Biological Technology Co., Ltd. (Suzhou, China). The target genes included the NADH dehydrogenase 1 *(nad*1) gene from *E. granulosus s.s.*, the NADH dehydrogenase 5 *(nad*5) gene from *E. multilocularis*, and the NADH dehydrogenase 4 *(nad*4) gene from *E.*

Table 1. *Primer sequences used for Echinococcus spp. mPCR amplification and the expected amplicon sizes*

***Echinococcus* Species**	**Gene**	**Primer Sequence (5′→3′)**	**Product (bp)**
Echinococcus granulosus	*nad*1	*nad*1F: TTGTGTTATTAATGGCTTTGG	545
		*nad*1R: TAATAAATTAAACACTAACACCAACA	
Echinococcus multilocularis	*nad*5	*nad*5F: AGTTATTTGTCAATTTTTAGCTTT	193
		*nad*5R: ACAAAACAACGTAACCATTAAG	
Echinococcus shiquicus	*nad*4	*nad*4F: GGTTAGAGTGGGCTATAGA	354
		*nad*4R: CAACCAAAAAACTCATAAC	

shiquicus. These genes were highly conserved in these *Echinococcus* spp. *(Table 1)*.

Standard Plasmid Preparation

Three *Echinococcus* spp. recombinant plasmids were reconstructed and used as the positive controls in this study. Briefly, species-specific gene fragments were first amplified with a simplex PCR primer *(Table 1)*, and simplex PCR amplification was performed in a total of 50.0 μL, including 2.0 μL each of the forward and reverse primers (10 μmol.L), 2.0 μL of DNA template (5 ng.μL), 25.0 μL of ExTaq PCR Premix (TaKaRa, Dalian, China), and 19.0 μL of PCR grade water. The following PCR program was used: predenaturation at 98°C for 1 min, denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, for a total of 35 cycles. At end of the cycle, extension was continued at 72°C for 10 min, with a final hold at 4°C. The amplified PCR products (6.0 μL) were analyzed using agarose gel (10 g.L) containing GelStain (0.6 mg.mL) (Beijing Transgen Biotech Co. Ltd, Beijing, China) and were observed under UV light. After detection, all remaining products were purified in accordance with the EasyPure PCR Purification Kit instructions (Beijing Transgen Biotech Co. Ltd, Beijing, China). After purification, the expected PCR fragments were cloned into the pMD-19T vector (TaKaRa, Dalian, China) to obtain the recombinant plasmids. Then, the recombinant plasmids were transformed into *E. coli* DH5α competent cells (TaKaRa, Dalian, China), which were then coated onto Luria-Bertani (LB) solid plate medium containing the antibiotic ampicillin, IPTG and X-gal, and incubated at 37°C overnight. After blue-white screening, a single white colony on the transformation plate was picked, inoculated into liquid LB medium containing the antibiotic ampicillin, and cultured with constant shaking at 37°C for approximately 15 h. After PCR amplification and identification of the bacterial liquids, the positive recombinant plasmids were extracted using EasyPure Plasmid MiniPrep Kit (TransGen, Beijing China) from bacterial liquids, then the plasmids were sent to Genewiz Suzhou Biological Technology Co., Ltd. for sequencing. The identified recombinant plasmids were named pMD19-Eg, pMD19-Em and pMD19-Es. The concentration of the recombinant plasmids was determined by NanoDrop-1000 (Thermo Fisher NanoDrop,USA), and then, the plasmid copy number was calculated.

Simplex and mPCR

The mPCR primer sets, *nad*1F/R, *nad*5F/R and *nad*4F/R were individually validated to determine their specificity. Each simplex PCR condition and program was performed as described above. After completion of PCR amplification, 10 μL of PCR product was electrophoresed on a 1.5% agarose gel containing 0.6 mg.mL GelStain (TransGen, Beijing China) in Tris–acetate-EDTA (TAE) buffer at 120 V for 35 min, and the gel was visualized under UV light.

Optimized mPCR was performed in a final volume of 35 μL containing 24.6 μL ExTaq PCR Premix (TaKaRa, Dalian, China), (0.4-1.2) μL each of the forward *(nad*1/4/5F) and reverse *(nad*1/4/5R) (10 μmol/L) primers, 3.0 μL of DNA plasmids, and 2.0 μL of dNTP (10 mmol/L (each)). The reaction conditions included an initial denaturation step at 98°C for 1 min, followed by 35 cycles of denaturation at 98°C for 10 s, primer annealing at varied temperature (47.1°C-59.1°C) for 40 s, and extension at 72°C for 40 s. A final extension at 72°C for 10 min was performed. Then, 10 μL of the PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel, as described above. For multiplex primer concentration optimization, 9 sets of different primer concentrations were tested. For annealing temperature optimization, 8 gradients were used to identify the optimal reaction conditions for mPCR. The optimal primer concentrations and annealing temperatures for mPCR were determined based on obvious target bands, few primer dimers, and no nonspecific amplified bands.

Specificity of Simplex and mPCR

The specificity of simplex and mPCR was tested using other common canine parasites (including *T. multiceps*, *T. saginata*, *T. asiatica*, *D. caninum*, *T. hydatigena*, *T. canis* and *F. hepatica*). The total DNA of non targeted parasites was used as a template for mPCR amplification. The amplified products were detected by agarose gel electrophoresis to evaluate and verify the specificity of the mPCR method constructed in this study.

Sensitivity of mPCR

To determine the detection limit of the mPCR, the pMD19-Eg, pMD19-Em and pMD19-Es plasmids were used to create ten individual series of 10-fold dilutions from 1×10^9

copies/μL to 1×10^0 copies/μL. In addition, these three species of mixed plasmids were also diluted by 10-fold gradient dilutions to evaluate the sensitivity of the mPCR assays in amplifying genes from samples containing mixed plasmid DNA templates. The mPCR was performed using the optimized conditions described above.

Simulated Coinfection Samples and Field Samples Detection

Fecal samples from dogs that had been verified as positive or negative by simplex PCR were used as positive or negative samples [20]. Total DNA of *Echinococcus* spp. mixed with negative dog feces was used to create clinical samples to evaluate and verify the practicability of the mPCR method established in this study. The field samples (35 fox fecal samples) were collected in Yushu county and were tested to evaluate the capability of the mPCR method. All the positive PCR products were directly sequenced by Genewiz Suzhou Biological Technology Co., Ltd. (Suzhou, China).

Results

Primer Design and Simplex PCR Amplification

The results of comparing the sequences of the forward and reverse primers are shown in *Fig. 1*, and the primer sequences and expected amplicon sizes are shown in *Table 1*. There are multiple different sites in the primer sequences among the *Echinococcus* spp. Based on observing the gel electrophoresis results with the gel imaging system, the three pairs of specific primers for the three species of *Echinococcus* spp. could amplify the corresponding target genes, and the sizes of the bands were consistent with the expected sizes. The expected sizes of the products for *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* were 545 bp *(Fig. 2)*, 193 bp *(Fig. 2)* and 354 bp *(Fig. 2)*, respectively.

Identification of Recombinant Plasmids

The expected PCR products were purified by gel extraction and then cloned into the pMD-19T vector. The three recombinant plasmids were constructed correctly, which was confirmed by bacterial liquid PCR and sequencing identification *(Fig. 3)*, and the plasmids were named pMD19-Eg, pMD19-Em and pMD19-Es.

Establishment and Optimization of the mPCR Method

The volume of the multiple PCR primers (0.4-1.2 μL) and the annealing temperature (47.1-59.1°C) were optimized. Avoidance of the amplification of nonspecific bands was achieved, and amplification efficiency (bright or weak of bands) of the target bands and reduced primer dimer formation were observed. When the primer ratios for the three target genes were adjusted to optimize the concentration ratio to 1:1:1, the PCR amplification efficiency was high and there were no nonspecific bands produced, so 0.9 μL each of the forward primers and reverse primers of the three genes was used *(Fig. 4)*. The target gene plasmids of the three *Echinococcus* spp. were

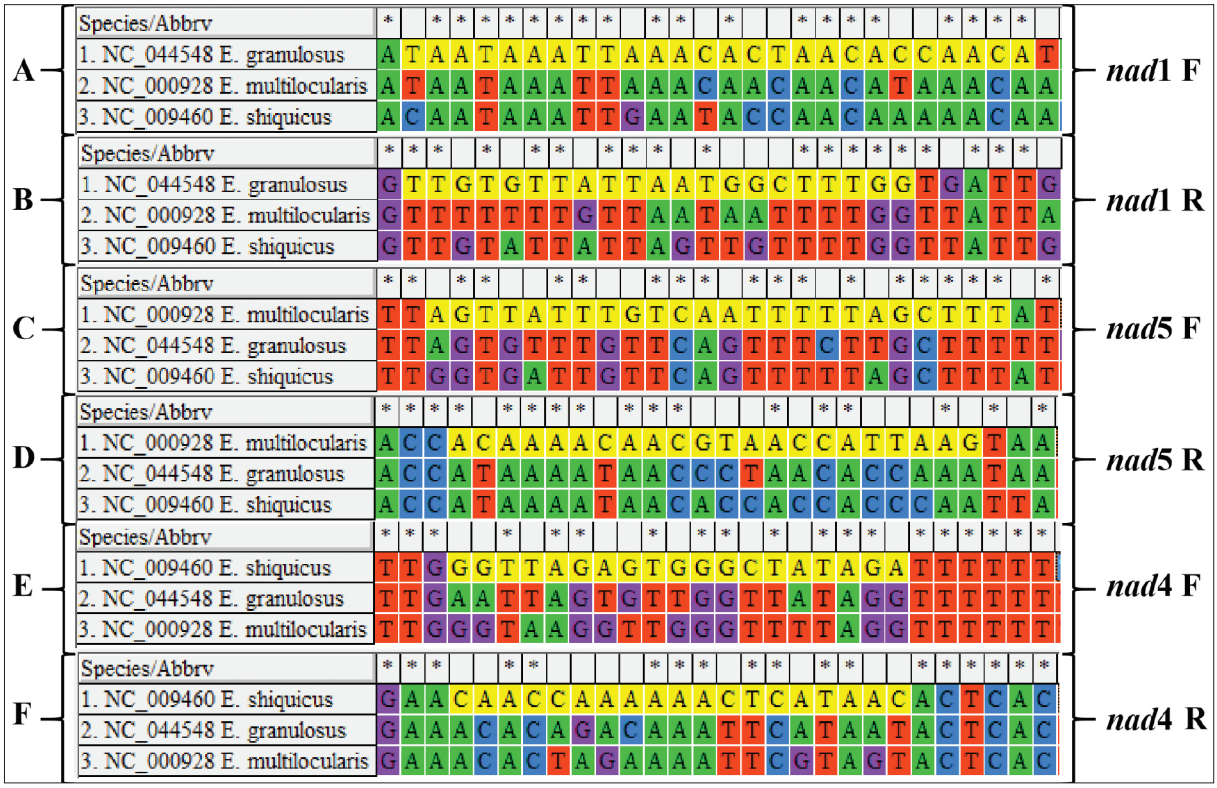

Fig 1. Sequence analysis of primers based on *Echinococcus* spp. mitochondrial target genes

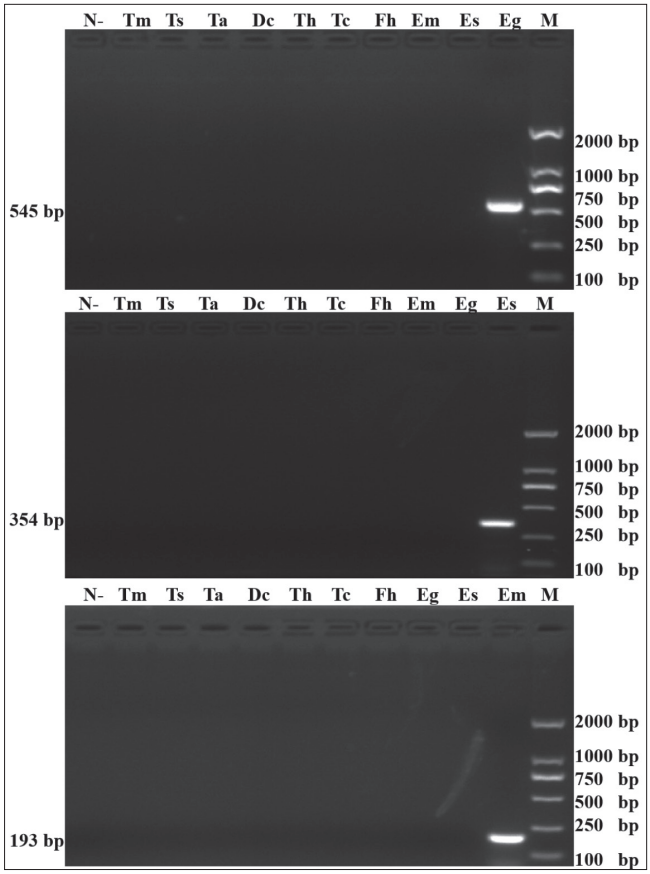

Fig 2. Simplex PCR amplification results based on *Echinococcus* spp. mitochondrial target genes. Eg: *E. granulosus s.s.*; Em: *E. multilocularis*; Es: *E. shiquicus*; Tm: *T. multiceps*; Ts: *T. saginata*; Ta: *T. asiatica*; Dc: *D. caninum*; Th: *T. hydatigena*; Tc: *T. canis*; and Fh: *F. hepatica*

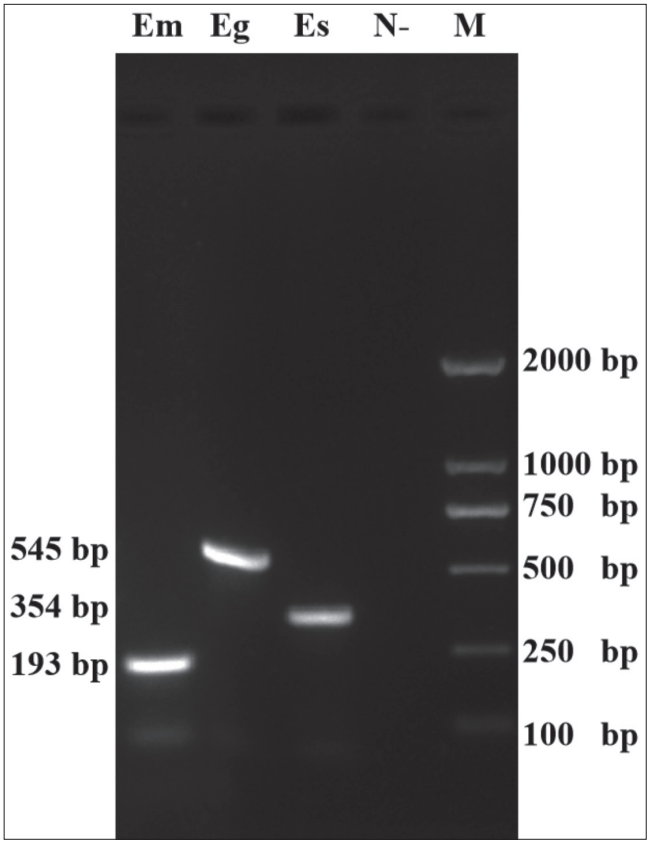

Fig 3. PCR results of the identification of the constructed plasmids of mitochondrial target genes in *Echinococcus* spp.

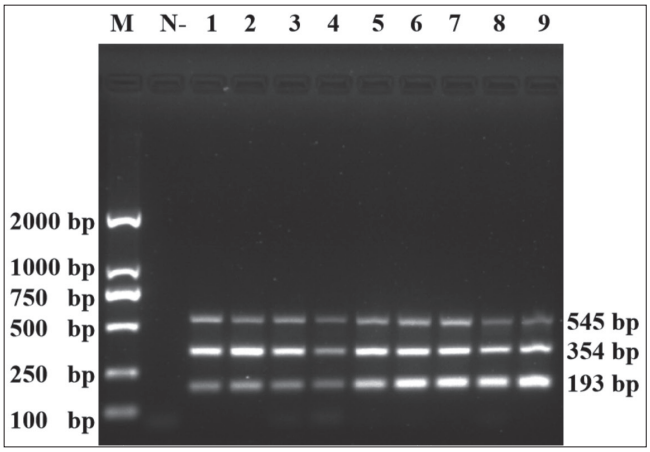

Fig 4. The results of optimization of the concentration of primers specific for *Echinococcus* spp. mitochondrial target genes. N-: Negative control, 1: 0.4 μL, 2: 0.5 μL, 3: 0.6 μL, 4: 0.7 μL, 5: 0.8 μL, 6: 0.9 μL, 7: 1.0 μL, 8: 1.1 μL, and 9: 1.2 μL; *(primer concentration: 10 μmol.L)

mixed in equal amounts as templates, and then, the annealing temperature of multiple PCR was optimized by a temperature gradient at the annealing step. The results showed that when the annealing temperature was 50°C, the amplification efficiency of the multiple PCR was the best *(Fig. 5)*. The final mPCR system included a volume of 55.0 μL. The forward and reverse primers of the three genes totalled 0.9 μL each, the templates were used at volumes of 2.0 μL each, 2×Ex Taq PCR Premix was used at a volume of 39.6 μL, and dNTP (10 mmol/L (each)) was used at a volume of 4.0 μL. The optimal reaction conditions were as follows: predenaturation at 98°C for 1 min; denaturation at 98°C for 10 s, annealing at 50°C for 50 s, and extension at 72°C for 50 s, for a total of 35 cycles; and 72°C for further extension for 10 min.

Multiple PCR Specificity Results

The total DNA of the three target parasites *(E. multilocularis*, *E. granulosus s.s.* and *E. shiquicus)* and the nontarget parasites *(T. multiceps*, *T. saginata*, *T. asiatica*, *D. caninum*, *T. hydatigena*, *T. canis* and *F. hepatica)* were used as templates. In the 55.0 μL system, only three target parasite DNA were amplified by 3 pairs of specific primers. When the three target parasite genomes were present, three corresponding target bands could be amplified *(Fig. 6*: Eg 545 bp + Em 193 bp + Es 354 bp). When only two target parasite DNA exist, only two corresponding bands could be amplified *(Fig. 6*: Eg 545 bp + Em 193 bp, Eg 545 bp+Es 354 bp, Em 193 bp+Es 354 bp). When only one target parasite DNA exist, only one corresponding target band could be amplified *(Fig. 6*: Eg 545 bp, Em 193 bp, and Es 354 bp). When nontarget parasite DNA was present, no target

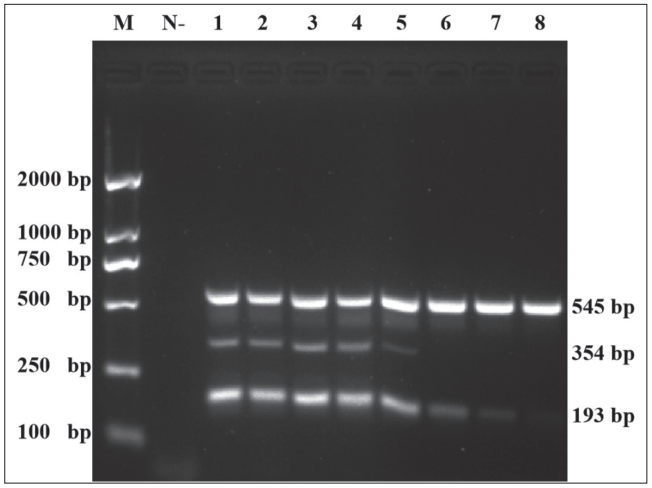

Fig 5. The results of optimization of the annealing temperature for *Echinococcus* spp. mitochondrial target genes. 1: 47.1°C, 2: 47.7°C, 3: 49.2°C, 4: 51.6°C, 5: 54.6°C, 6: 56.9°C, 7: 58.4°C, and 8: 59.1°C

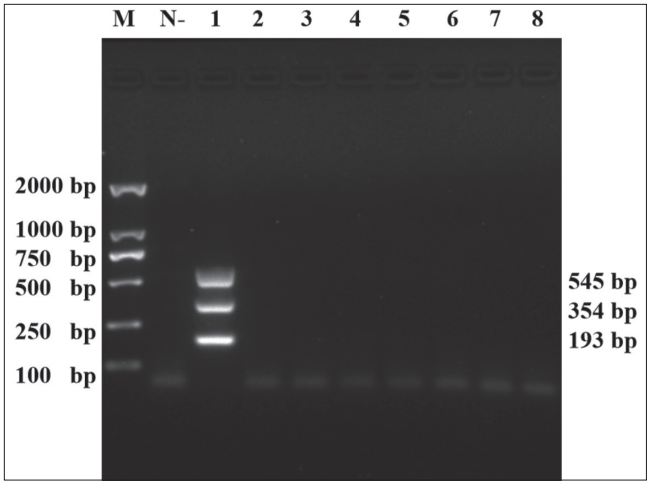

Fig 7. The results of the mPCR method for *Echinococcus* spp. species-specific detection. 1: Eg+Es+Em, 2: Tm, 3: Ts, 4: Ta, 5: Dc, 6: Th, 7: Tc, and 8: Fh

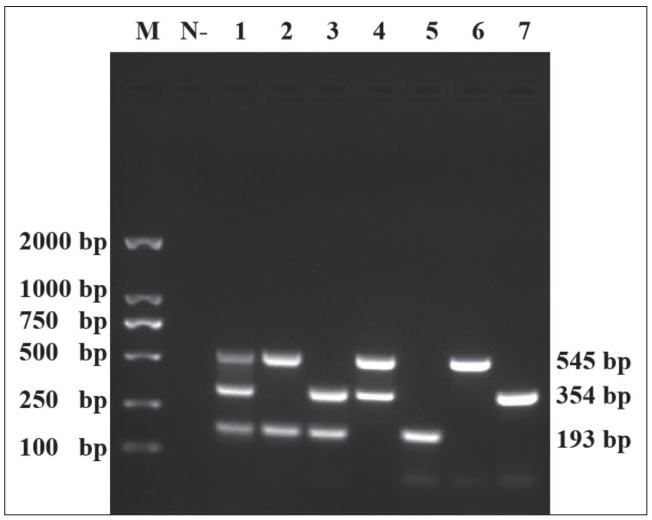

Fig 6. The results of the mPCR method for *Echinococcus* spp. intraspecies-specific detection. N-: Negative control, 1: Eg+Es+Em, 2: Eg+Em, 3: Es+Em, 4: Eg+Es, 5: Em, 6: Eg, and 7: Es

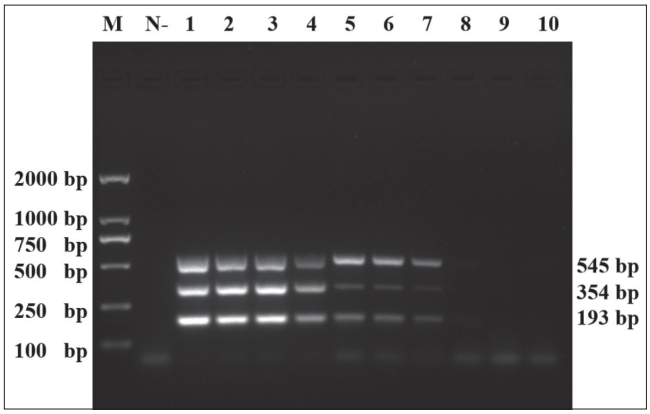

Fig 8. The sensitivity results of the mPCR method for *Echinococcus* spp. N-: Negative control, and 1-10: 1×10^9 ~ 1×10^0 copies.μL

fragments were amplified, and the result was negative *(Fig. 7)*. The same results were obtained, when the experiment was repeated 3 times, which indicated that the multiplex established a PCR method with good specificity and reproducibility.

Multiple PCR Sensitivity Results

The three *Echinococcus* spp. target gene plasmids pMD19-Eg, pMD19-Em and pMD19-Es, were diluted to produce 10 gradient dilutions between 1×10^0~1×10^9 copies/μL. Each gradient dilution of the three target gene plasmids was mixed in equal amounts to prepare the DNA templates. The sensitivity of the established multiple PCR was analyzed. As shown in *Fig. 8*, when the three DNA templates were mixed, the detection limit of each parasite was 1 $\times10^3$copies/μL.

Simulated Clinical Samples and Field Samples Detection

As shown in *Fig. 9*, we simulated triplex, duplex and simplex infections of different combinations of *Echinococcus* spp. at the same concentration. In total, 15 simulated clinical samples were examined using the developed multiple PCR method and simplex PCR with the same primers. The result showed that 11 samples were *Echinococcus* spp.-positive, including 1 triple positive sample with Eg + Em + Es, 4 double positive samples with Eg + Em (2), Eg + Es (1) and Em + Es (1), 6 simplex positive samples with Eg (2), Em (2) and Es (2), and 4 negative samples. The results of the multiple PCR method were consistent with those of the simplex PCR method. For the field samples, 35 fox fecal samples were also detected using the triplex/simplex PCR methods. Only one fox fecal samples was detected as *E. multilocularis* by both methods. All the PCR products were directly sequenced in both directions, and the sequence analysis results confirmed the species of *Echinococcus* again. It was indicated that the mPCR method established in this study can be used for clinical samples detection.

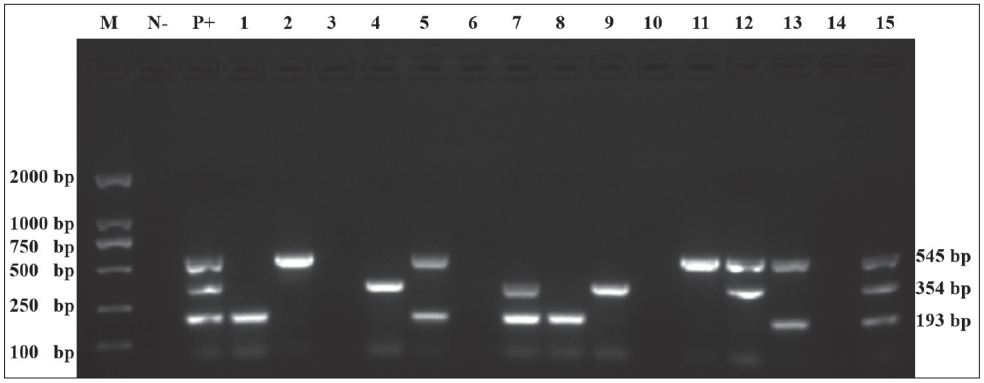

Fig 9. The results of the mPCR method for detecting simulated clinical samples. N-: Negative control, P+: Positive control, 1: Em, 2: Eg, 3: Negative, 4: Es, 5: Eg+Em, 6: Negative, 7: Es+ Em, 8: Em, 9: Eg, 10: Negative, 11: Eg, 12: Eg+Es, 13: Eg+Em, 14: Negative, and 15: Eg+Es+Em

Discussion

Echinococcosis has been one of the great public health threats in northwest China, especially, in the QTPA. This area is now the most severe pandemic region for CE in humans and livestock, which is caused mainly by *E. granulosus s.s.*, and AE in humans and small wild mammals, which is caused by *E. multilocularis* [24-26]. *E. canadensis* also causes CE in humans and livestock, but *E. shiquicus* has not been validated to infect humans and cause echinococcosis in endemic areas [27,28]. It is reported that few cases of *E. canadensis* are found in the QTPA, indicating a low prevalence of this species in this area [27]. *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* are mainly prevalent in intermediate hosts or fecal samples of definitive hosts in the QTPA [25]. The pathogenic factors causing the spread of echinococcosis in this area are quite complicated [29,30]. In fact, double infections with different species of *Echinococcus* in animal hosts have been reported [31]. Because of the close relationship between dogs and humans, humans are very susceptible to infection. In the QTPA of China, an increasingly large number of human AE and CE cases originating from infected dogs occur, causing a heavy burden on public health and veterinary services [20]. Therefore, it is necessary to develop an accurate, rapid and effective method for the simultaneous detection and identification of different life stages of the three *Echinococcus* spp. Moreover, the method will be beneficial for the monitoring, management, diagnosis and prevention of echinococcosis. It is essential to develop such a needed method for clinical and epidemiological investigations.

The mPCR is a method that can detect multiple target species by using multiple pairs of primers in a simplex reaction tube. Since this method was first described in 1988, it has been successfully applied for the identification of pathogenic bacteria, viruses, fungi and parasites [32,33]. Primer design is the key factor for the successful establishment of an mPCR method. As is known, the conventional primer design of simplex PCR is very simple. The design of mPCR primers is more complicated and difficult, because it is not a simple combination of several simplex PCR primers. When multiple pairs of primers are mixed in one reaction tube, the primers may be randomly paired in the mixture to form a new reaction system, making the process much more complicated than a simplex reaction. The design of mPCR primers needs to overcome many difficulties, including low sensitivity and specificity, different annealing temperatures of different primers, and easy formation of primer dimers [32,34]. At present, there are no software programs or methods to predict the performance of the multiple primers; thus, there are only empirical experiments and trial-and-error approaches.

Methods for recovering eggs and morphological identification are usually used in traditional tapeworm epidemiological surveys. However, it is impossible to morphologically identify the eggs of *Echinococcus*, which has prompted the development of several molecular detection methods [17,35]. When the mPCR method is used to determine the pathogens in the host, the advantages of the mPCR method become obvious.

An mPCR method for detecting and distinguishing *E. multilocularis*, *E. granulosus s.s.* and *E. shiquicus* was successfully developed in this study. This method can pair well with simplex or mixed target template DNA to amplify and produce the expected specific amplicons, which are highly consistent with their target *Echinococcus* species. There was a high degree of species specificity in testing the cross-reactivity of the assay, and there were no amplicons from any other worms or negative samples (no DNA). The multiple reactions of the primer sets (three pairs of primers) worked well on all tested templates in simplex reaction tubes, and for the three tested *Echinococcus* tapeworms, specific amplicons of the expected length were produced.

In the recent taxonomic revision, *Echinococcus* spp. was divided into nine species, including *E. felidis*, *E. equinus*, *E. oligarthra*, *E. vogeli*, *E. ortleppi*, *E. canadensis*, *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* [2]. DNA samples from the first five species of *Echinococcus* are not available because they are not found anywhere in China. Nevertheless, to minimize the possibility of nonspecific amplicons from these closely related species, the target sequences of the first six species were compared with the primer targeted sequences of the three target species, and the

results showed that there are 4-10 base pair differences between these species. Only one sequence (R) had only 2 base pair differences, and the L sequence had only 5 base pair differences (in *E. equinus)*. This result is the same as that observed in research by Liu, in which there were 6-11 base pair differences between these species, and due to such large differences, it is extremely unlikely that any amplicons would be generated from these 5 species in the mPCR detection method [20]. Therefore, due to its high species specificity, it is extremely unlikely that any amplicons would be produced from these species during mPCR.

Previous studies have shown that compared with simplex PCR, mPCR has lower sensitivity [16,36]. The designed method proved to be similarly sensitive in this study, with detection thresholds as low as 0.2 -1 pg for *E. multilocularis*, *E. granulosus s.s.* and *E. shiquicus* (plasmid conversions). The detection limit is lower than the detection limit of 0.32-1.6 pg *(E. granulosus* s. s. and *E. multilocularis)* [37] and is the same as the detection limit of mPCR reported by Liu [20] (0.1-5 ng and 10-20 pg, respectively). According to the report, each parasite egg contains approximately 8 pg of nuclear DNA [38], and it is reported that at least 15 eggs can produce a positive result [21]. Thus, when the detection method is used to detect *Echinococcus* spp. in canid feces, its sensitivity may not be good enough, especially in mixed infections. To avoid false-negative results caused by low-level infections, it is recommended to expand the detection volume to accurately detect the infection of parasitic pathogens. Due to the lack of such mixed-infection samples, none of the fecal materials showed coinfection phenomenon, but the results of the plasmid DNA fully proved that the detection method developed in this research can effectively detect double and/or triple mixed infections of the target species.

For all cyst samples previously confirmed by gene sequencing, genotyping can be successfully confirmed by mPCR. In addition, no amplicons were observed in any of the samples of fox feces and dog feces that have been identified, which indicates that host DNA does not interfere with this method. Actually, due to there were only two human echinococcosis cases infected with *E. canadensis* in China, compared with the other three *Echinococcus* tapeworms involved in the method, *E. canadensis* is almost not prevalent in the QTPA, so *E. canadensis* is not the main detection target of the detection method. If necessary, the method can be further improved by adding a pair of specific primers for *E. canadensis*, for the establishment of a four-fold PCR detection method for the ecological and biological research of *Echinococcus* species.

The established mPCR method that could simultaneously, quickly and accurately determine *E. granulosus s.s.*, *E. multilocularis* and *E. shiquicus* in this study. It has high potential for application and may greatly assist in the diagnosis of canine-derived stool samples on the QTPA. In addition, due to its reliability and accuracy, the method can be used as a useful tool for evaluation, supplementing the results of serological testing and further improving the accuracy of animal-derived stool samples. This method has potential application in rapid detection and broad-scale screening, and it is expected to become a useful technology for clinical diagnosis and environmental samples monitoring.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

X. ZHANG and Y. JIAN designed and performed experiments, analyzed results, wrote and reviewed the manuscript. Y. FU, Z. GUO and H. DUO provided advice, reviewed and edited the manuscript.

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