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A multiplex PCR for the identification of *Echinococcus multilocularis*, *E. granulosus sensu stricto* and *E. canadensis* that infect human

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Research Article

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Abstract

Echinococcus granulosus sensu stricto (*s.s.*), *Echinococcus multilocularis* and *Echinococcus canadensis* are the common causes of human echinococcosis in China. An accurate species identification tool for human echinococcosis is needed as the treatments and prognosis are different among species. The present work demonstrates a method for the simultaneous detection of these three *Echinococcus* species based on multiplex polymerase chain reaction (mPCR). Specific primers of this mPCR were designed based on the mitochondrial genes and determined by extensive tests. The method can successfully detect either separated or mixed target species, and generate expected amplicons of distinct size for each species. Sensitivity of the method was tested by serially diluted DNA, showing a detection threshold as less as 0.32 pg for both *E. granulosus s.s.* and *E. canadensis*, and 1.6 pg for *E. multilocularis*. Specificity assessed against 18 other parasites was found to be 100% except weakly cross-react with *E. shiquicus*. The assay was additionally applied to 69 echinococcosis patients and 38 healthy persons, confirming the high reliability of the method. Thus, the mPCR described here has high application potential for clinical identification purposes, and can further provide a useful tool for evaluation of serology and imaging method.

Introduction

Echinococcosis is one of the most pathogenic helminth zoonosis worldwide caused by the larval stage of *Echinococcus*. Humans are infected by accidentally ingested *Echinococcus* eggs, which are released along with canids' feces. The parasite can reach any human organs, mainly liver and lung, causing serious life-threatening hydatid cysts (McManus *et al.*, 2003; Zhenghuan *et al.*, 2008). The infections can be asymptomatic for years and are usually found in the late stage of the disease (Piccoli *et al.*, 2013). Historically, four species have been recognized within the *Echinococcus* genus: *E. granulosus* (including 10 distinct genotypes G1–G10), *E. multilocularis*, *E. oligarthra* and *E. vogeli*. *E. shiquicus* and *E. felidis* were new species additionally discovered in 2005 and 2008, respectively (Boubaker *et al.*, 2013). Subsequently, in the newly taxonomic revision, previously *E. granulosus* species have been split into four species: *E. granulosus sensu stricto* (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–G8, G10) (Nakao *et al.*, 2013). To date, *E. granulosus s.s.*, *E. multilocularis*, *E. shiquicus* and *E. canadensis* have been identified in China (Cong-Nuan *et al.*, 2015). Both *E. granulosus s.s.* and *E. multilocularis* are widespread in western and north-western China and have long been a predominant public health and medical threat in these areas (Zhenghuan *et al.*, 2008). Approximately 90% of human echinococcosis in China are cystic echinococcosis (CE) caused by *E. granulosus s.s.*, whereas the remainder are from alveolar echinococcosis (AE) caused by *E. multilocularis*, which accounts for more than 90% of the AE burden in the world (Yang *et al.*, 2014). *Echinococcus canadensis* is also responsible for human CE (Orkhontuul *et al.*, 2018), which used to be neglected in China because the limited distribution and only two human cases of *E. canadensis* (G6) have been reported till 2005 (Bart *et al.*, 2006). However, in the past 5 years human-derived G6, G7 and G10 genotypes of *E. canadensis* have emerged in several provinces/autonomous regions of China (Zhang *et al.*, 2014; Ma *et al.*, 2015; Yang *et al.*, 2015; Cao *et al.*, 2018), which requires more research efforts.

Since different *Echinococcus* species have diverse diseases treatment and prognosis (Brunetti *et al.*, 2010; McManus *et al.*, 2012), species genotyping is essential, especially in China where co-endemic has occurred. A number of molecular approaches based on DNA detection have been designed to accurately identify different *Echinococcus* species. PCR-sequencing (Nakao *et al.*, 2000; Xiao *et al.*, 2006*a*) or PCR-RFLP (Xiao *et al.*, 2006*b*; Chaabane-Banaoues *et al.*, 2016) method can identify the genotype of *Echinococcus* species PCR amplicons by sequencing or restriction fragment length polymorphism (RFLP) in a relatively slow and costly way. Uniplex PCR (uPCR) can identify its target *Echinococcus* species based on only PCR which relatively simplify the genotyping (Boufana *et al.*, 2013). By contrast, mPCR which can simultaneously detect more than one pathogens in a single

Table 1. Information on parasite samples used in the study

Parasite	Stage	Host origin	Site	Number of individuals	Reference for PCR method
E. granulosus s.s.	Larval	Patient liver	Hospitals in Sichuan, CN	55	Nakao *et al.* (2000)
E. multilocularis	Larval	Patient liver	Hospitals in Sichuan, CN	15	Nakao *et al.* (2000)
E. Canadensis (G6)	Larval	Patient liver	Hospitals in Sichuan, CN	2	Nakao *et al.* (2000)
*E. shiquicus**	Larval	Pika lung	Shiqu, Sichuan, CN	5	Nakao *et al.* (2000)
*T. hydatigena**	Adult	Dog feces	Shiqu, Sichuan, CN	1	Boubaker *et al.* (2013)
*T. taeniaeformis**	Larval	Vole liver	Jiangyou, Sichuan, CN	4	Nakao *et al.* (2000)
*T. solium**	Adult	Human feces	Muli, Sichuan, CN	2	Nakao *et al.* (2000)
*T. saginata**	Adult	Human feces	Muli, Sichuan, CN	2	Nakao *et al.* (2000)
*T. asiatica**	Adult	Human feces	Muli, Sichuan, CN	1	Nakao *et al.* (2000)
*T. serialis**	Adult	Dog feces	Shiqu, Sichuan, CN	1	Boubaker *et al.* (2013)
*T. multiceps**	Adult	Dog feces	Shiqu, Sichuan, CN	1	Wang *et al.* (2018)
*T. leonina**	Adult	Dog feces	Shiqu, Sichuan, CN	1	Jacobs *et al.* (1997)

Note: Parasites marked with * were used to check the specificity of the mPCR assay.

PCR mixture is more rapid and have been widely applied in both clinical and laboratory research (Henegariu and Al, 1997). Some mPCR approaches have been developed for detecting certain *Echinococcus* species (Dinkel *et al.*, 2011; Boubaker *et al.*, 2013; Cong-Nuan *et al.*, 2015), but to our knowledge, no studies aiming at simultaneous identification of the three species (*E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*) that can cause human echinococcosis in China have been performed. Moreover, those methods were mostly applied on feces or animal tissues for epidemiological investigation purpose, and the effect on detection of human-derived parasite samples was seldom assessed. Therefore, in this study we aimed to set up a single-tube mPCR approach that can be applied for the accurate identification of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* (G6–G8, G10) to assist the diagnosis of human echinococcosis.

Materials and methods

Sample collection and extraction

In total, 72 parasite tissues were collected post-operatively from echinococcosis patients received surgical treatment from the West China Hospital and Sichuan Provincial People's Hospital in Sichuan Province from 2015 to 2017. Adults of *Taenia solium*, *Taenia saginata* and *Taenia asiatica* were kindly provided by Tiaoying Li (Professor of Cysticercosis Control and Research Department, Sichuan Center for Disease Control and Prevention, Sichuan, China). Other parasite individuals isolated from pikas, voles or dogs were collected by our group during routine parasite disease surveillance in Sichuan Province. In addition, blood samples were also collected from 38 healthy people ruled out parasitic infections during routine physical examinations. All the parasite samples above were listed in Table 1 and confirmed by morphological identification and PCR-based sequencing. One *E. granulosus s.s.*, one *E. multilocularis* and one *E. canadensis* (G6) isolates were selected from the 72 patient samples, respectively, as standard strains for the establishment of mPCR and the rest (54 *E. granulosus s.s.*, 14 *E. multilocularis* and one *E. canadensis*) were employed for subsequent reliability test of the mPCR.

Total genomic DNA of samples was extracted by using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, GER) according to the manufacturer's instructions. The DNA concentration was measured by using a NanoDrop One (Thermo Fisher Scientific, Wisconsin, USA), to confirm the quality of DNA extractions. Then the extracted DNA samples were stored at −20 °C until use.

Primer design

Complete mitochondria genes of all the *Echinococcus* genus, including *E. granulosus s.s.* (accession no. NC008075), *E. multilocularis* (accession no. NC000928) and *E. canadensis* (G6) (accession no. NC011121) as well as the remaining six *Echinococcus* species namely *E. shiquicus* (accession no. NC009460), *E. oligarthrus* (accession no. NC009461), *E. equinus* (accession no. NC020374), *E. ortleppi* (accession no. NC011122), *E. vogeli* (accession no. NC009462) and *E. felidis* (accession no. NC021144) were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/gene/) and aligned by MEGA7.0 (https://www.megasoftware.net/). A number of primer pairs specific for *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* were designed manually on conserved regions of *E. canadensis* (Boufana *et al.*, 2013) and then the primer pair with maximum specificity was pre-selected by aligned with sequences in the GenBank (https://www.ncbi.nlm.nih.gov/gene/). Primer sequences were subsequently synthesized by Shanghai Invitrogen Biotechnology Co Ltd.

Primer selection experiments

The designed primers were combined into various mixtures, each including three primer pairs specific for *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*, respectively, and can generate expected amplicons of distinct size for each species. These primer sets were selected by testing with single and pooled standard DNA, as well as other related species. The primer set that allows accurate discrimination of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* in both single and pooled target templates without generating any nonspecific products was retained for the mPCR approach (presented in Table 2).

Multiplex PCR conditions

The mPCR was conducted using a SmpliAmp thermal cycler (Applied Biosystems, California, USA) in a 25 μL reaction system containing 20 ng standard DNA of each *Echinococcus* species, 6 μL of double distilled water, 12.5 μL of GoTaq Hot Start Polymerase mixture (Promega, Wisconsin, USA) and 7.5 μM

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Table 2. Characteristics of primers used in mPCR

Species	Primer	Concentration (μM)	Product size (bp)	Sequence(5′–3′)	Primer length	Target gene
E. granulosus s.s.	Gd3-4F	0.3	167	TTGTTGGGTTTGAGTGGGGC	20	*nad3*
	Gd3-4R	0.3		ACCAAACAGTACCCCCTGC	19	
E. multilocularis	Mtb2F	0.3	237	TTGGCATATGGTAGGTGTAAATGT	24	*cytb*
	Mtb2R	0.3		ACCATAGAACCAACCAACGG	20	
E. canadensis	Cox1F	0.3	441	TTTTATTTACGTTTGGGGGCG	21	*cox1*
	Cox1R	0.3		CCACCAAACCAAAAGACCTG	20	

(each) of Gd3-4F, Gd3-4R, Mtb2F, Mtb2R, Cox1F and Cox1R primers. Annealing temperatures were optimized before the assay and 56 °C was confirmed to be with the best specificity and sensitivity of the method. So that the optimized thermal cycling conditions were as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min.

For all experiments, both positive and negative controls (double distilled water) were included. To avoid extraneous DNA contamination, the reaction mix was prepared with dedicated equipment, and the operation area was exposed to UV light for at least half an hour prior to the assay.

Identification of PCR products

Five microlitres of PCR products were separated by electrophoresis in a 2% agarose gel stained with Super Red (Biosharp, Hefei, CN) and visualized by using Universal Hood II UV transilluminator (Bio-Rad, California, USA) with Quantity One analyst software (Bio-Rad, California, USA).

Amplicons generated from standard DNA of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* in the mPCR were sequenced by Shanghai Invitrogen Biotechnology Co Ltd., and then compared with existing sequences in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) for further confirmation of the genotype.

Specificity evaluation of mPCR

To test the possible unspecific cross binding with other parasite species, 30 ng template DNA derived from 18 other parasite isolates including five samples of one *Echinococcus* species (*E. shiquicus*), 12 samples of seven closely related Taenia species and one non-related *Toxascaris leonina* were applied in individually performed standard mPCRs. The parasite samples for the assessment are presented in Table 1 marked with an asterisk.

Sensitivity evaluation of mPCR

Five-fold serial dilutions of three target DNA with the concentrations ranging from 1000 to 0.0128 pg were individually used to evaluate sensitivity of the mPCR approach. The lowest detection limit of each target species was determined according to the lowest amount of DNA that can yield a clearly visible band.

Reliability evaluation of mPCR

To confirm the reliability of the newly developed mPCR, a total of 69 parasite samples removed from patient livers were tested. These samples have been genotyped to be 54 *E. granulosus s.s.*, 14 *E. multilocularis* and one *E. canadensis* before according to PCR-based sequencing described by Nakao *et al.* (2000). Reliability was assessed by comparing the consistency of mPCR approaches and sequencing results. Besides, in order to exclude nonspecific reaction which may be caused by the host (human) DNA, 38 healthy human DNA extracted from blood samples was also investigated. This blood was alternative for liver tissue because healthy human liver tissue was not available in this study.

Results

Identification of PCR products

PCR products amplified with single and pooled standard DNA of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* are shown in Fig. 1. Expected amplicons of 167, 237 and 441 bp were observed for *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*, respectively. The respective diagnostic products were also detected when checked with different DNA mixtures of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* (Fig. 1, lanes 4–7). Fragment generated by each target species was highly similarity (98–99%) with respective reference sequences in GenBank: *E. granulosus s.s.* (GenBank accession no. MG672293), *E. multilocularis* (GenBank accession no. KY205670) and *E. canadensis* (GenBank accession no. MH274989), which showed 1–4 base pair differences between them.

Specificity of the mPCR

The mPCR was proved to be 100% specific when tested with other closely related Taenia species and one non-related *T. leonina* (Fig. 2, lanes 9–21), as no products were observed or their products were significantly larger than the expected bands. The only exception was *E. shiquicus* (Fig. 2, lanes 4–8), which cross react with multiple primers and generate 3–4 slightly visible bands among target bands region.

Sensitivity of the mPCR

The results showed the detection limits of mPCR assay varied among species. The lowest limit for the DNA detection of *E. granulosus s.s.* and *E. canadensis* can both reach as less as 0.32 pg, and the lowest detection limit for *E. multilocularis* was 1.6 pg (Fig. 3), which revealed the high sensitivity of the method.

Validation of the mPCR

For all 69 patient-derived parasite DNA samples, a clearly genotype-specific binding pattern was observed. Among them, 54 samples were identified as *E. granulosus s.s.*, 14 samples were *E. multilocularis* and one sample was *E. canadensis* according to the sizes of PCR products (part electrophoresis results are presented in Fig. 4A). Therefore, the results obtained by mPCR were completely in accordance with that of the sequencing. Moreover, no target band was observed from 38 normal human

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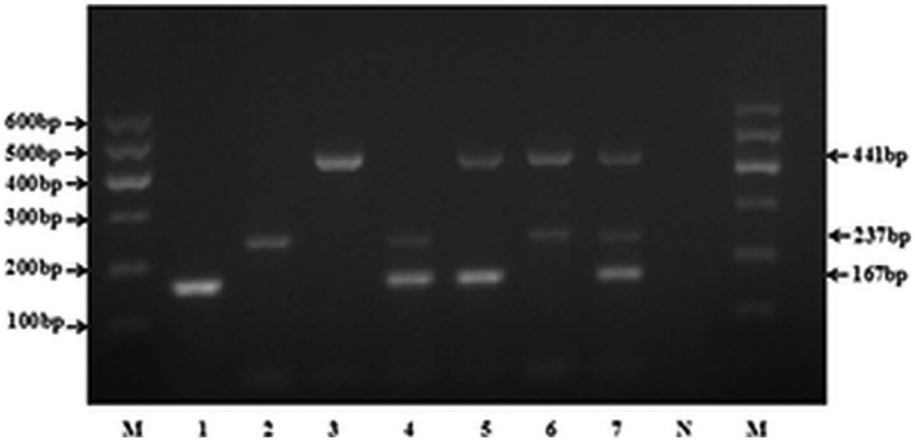

Fig. 1. PCR products of single and pooled DNA of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* in mPCR assay. Lanes 1–3, amplicons of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*, respectively; lane 4, amplicons of mixed DNA of *E. granulosus s.s.* and *E. multilocularis*; lane 5, amplicons of mixed DNA of *E. granulosus s.s.* and *E. canadensis*; lane 6, amplicons of mixed DNA of *E. canadensis* and *E. multilocularis*; lane 7, amplicons of mixed DNA of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*, N, negative control; M, DNA marker.

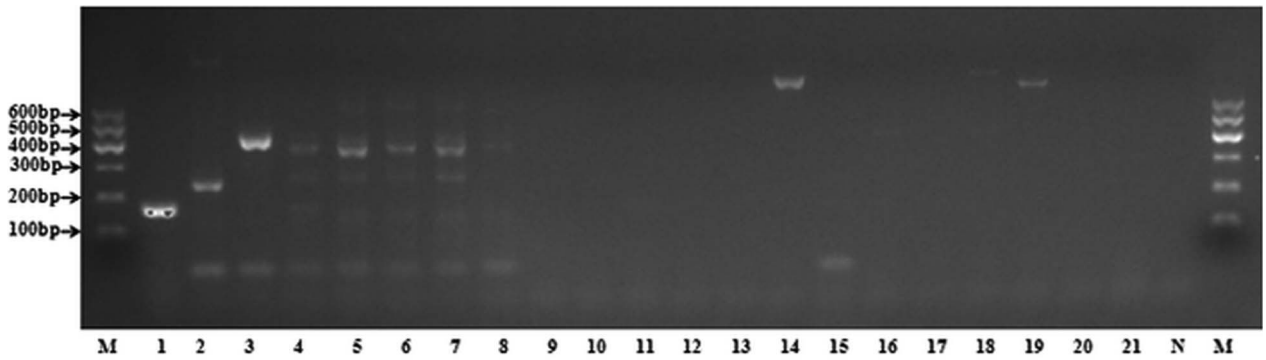

Fig. 2. Specificity of the mPCR. Lanes 1–3, positive controls (amplicons of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*, respectively); lanes 4–20, amplicons of other parasite DNA samples: *E. shiquicus* (lanes 4–8), *T. asiatica* (lane 9), *T. saginata* (lanes 10 and 11), *T. solium* (lanes 12 and 13), *T. hydatigena* (lane 14), *T. taeniaeformis* (lanes 15–18), *T. serialis* (lane 19), *T. multiceps* (lane 20) and *T. leonina* (lane 21); M, DNA marker (100–600 bp); N, negative control.

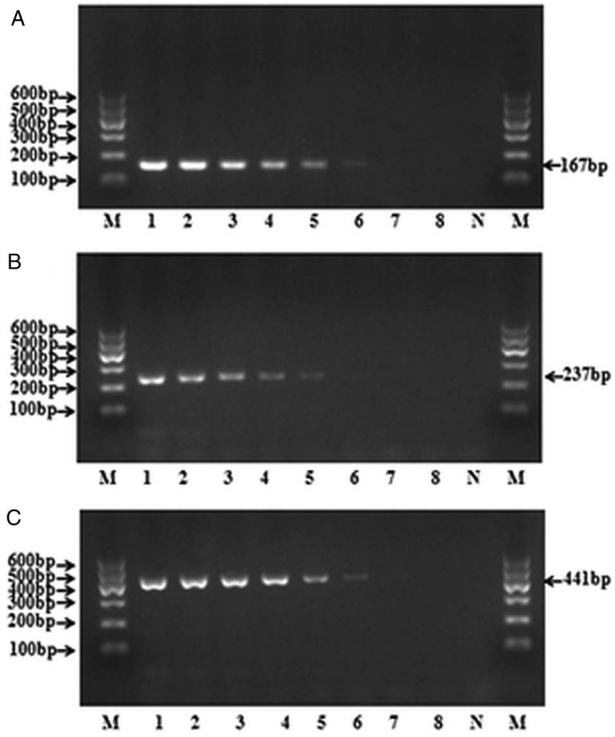

Fig. 3. Sensitivity of the mPCR for three targeted species, respectively. The DNA detection limit using a serial dilution of standard DNA of (A) *E. granulosus s.s.*, (B) *E. multilocularis* and (C) *E. canadensis*, respectively. Lanes 1–8, 5-fold serially diluted DNA templates (1000, 200, 40, 8, 1.6, 0.32, 0.064, 0.0128 pg); M, DNA marker; N, negative control.

DNA samples (part electrophoresis results are shown in Fig. 4B), which indicates that host tissue did not affect PCR outcomes.

Discussion

Multiplex polymerase chain reaction (mPCR) is a method that can detect more than one target species by using multiple primer pairs in a single reaction tube. Since its first description in 1988, this method has been successfully applied in many areas of DNA identification, such as bacteria, viruses, fungi or parasites (Henegariu and Al, 1997; Elnifro *et al.*, 2000). Primer design is undoubtedly a key factor for successfully establishing a PCR approach. In general, it is not very difficult to design primers for conventional (uniplex) PCR, but the design of mPCR primers is much more complicated because it is not a simple combination of several uPCRs. When more than one pair of primers are mixed in the same reaction tube, the primers can be randomly paired in the mixture to form a new reaction system that is far more complex than the original separated ones. At this point, the design of mPCR primers needs to overcome many difficulties, including poor sensitivity and specificity, different annealing temperatures of each primer, preferential amplification of one sequence over others (Polz and Cavanaugh,1998), easily formation of primer dimer and so on (Elnifro *et al.*, 2000). There are no other means to predict the performance characteristics of multiple primer pairs system except empirical testing and a trial-and-error approach.

The present study successfully developed an mPCR method to detect and differentiate human-derived DNA of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis* (G6–G8, G10). This method can work well with single or mixed target templates and generate specific amplicons of expected length that are highly similarity

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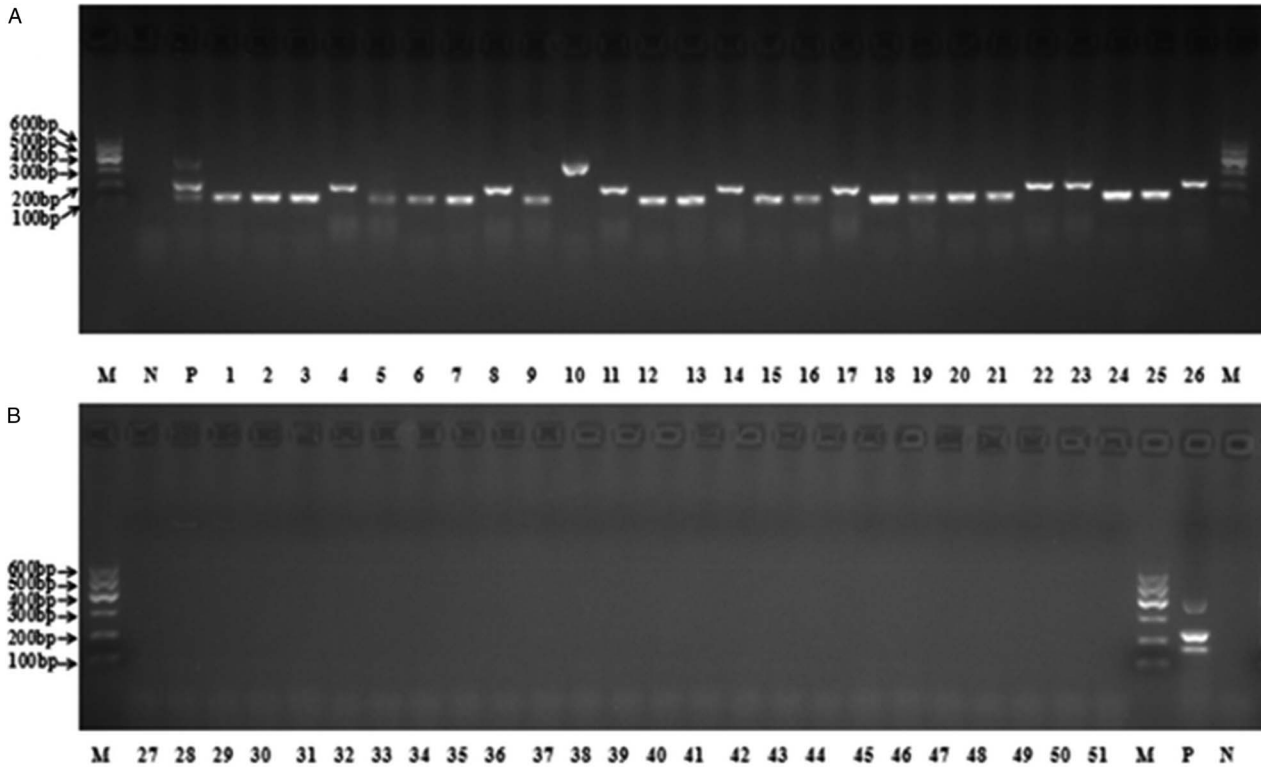

Fig. 4. Validation results of mPCR. Reliability was assessed by tested with human-derived parasite DNA that has been sequenced (A) and healthy human DNA (B). (A) Lanes 1–26, parasite DNA from humans, including one *E. canadensis* (lane 10), eight *E. multilocularis* (lanes 4, 8, 11, 14, 17, 22, 23 and 26) and the rest were *E. granulosus s.s.* (B) Lanes 27–51, healthy human DNA; M, DNA marker; P, positive control (DNA mixture of *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*); N, negative control.

with their intended target *Echinococcus* species. It achieved a high degree of species specificity because no predicted product was detected from any other tested helminths, except for the amplification of *E. shiquicus*. Although *E. shiquicus* has been found on foxes and plateau pika in Qinghai-Tibet plateau region of China (Xiao *et al.*, 2006*a*; Ma *et al.*, 2015), to date human infection of *E. shiquicus* has not been reported (McManus *et al.*, 2012; Nakao *et al.*, 2013). Thus, cross-reactivity with *E. shiquicus* is unlikely to occur in human echinococcosis detection. In addition, *E. shiquicus* can generate 3–4 weakly visible bands in the mPCR, so that it would not interfere with the detection of single or double infected samples. Confusion can only appear when encountering 3-plex infection, but this is very unlikely to happen and can be easily resolved by further sequencing the PCR products. Therefore, the effect of *E. shiquicus* on this method can be neglected. In the most recent taxonomic revision, the genus *Echinococcus* was divided into nine species, including *E. felidis*, *E. equinus*, *E. oligarthra*, *E. vogeli*, *E. ortleppi*, *E. granulosus s.s.*, *E. multilocularis*, *E. canadensis* and *E. shiquicus* (Nakao *et al.*, 2013). DNA samples of the former five *Echinococcus* species are unavailable as they have not been found in any part of China. Despite this, in order to minimize the possibility of unspecific interactions with these five closely related species, comparison of the primer target sequences for the five species with those of the three target species has also been conducted, which showed 6–11 base pair differences between them. According to Liu (Cong-Nuan *et al.*, 2015), it is highly unlikely to produce any amplicon from the five species during the mPCR assay due to such large differences, thus further illustrating the strict species specificity of the method.

Previous studies show that mPCR assay has lower sensitivity compared to uPCR assay (Dong *et al.*, 2000; Bharathi *et al.*, 2013; Boufana *et al.*, 2013). Nevertheless, the method designed in this study is proved to be highly sensitive, with a detection threshold of as less as 0.32 pg for *E. granulosus s.s.* and *E. canadensis*, and 1.6 pg for *E. multilocularis*. The detection limit is slightly lower than that was described in uPCR assays developed by Boufana *et al.* (2013) (2–10 pg), and is significantly lower than the detection limits of mPCR that were previously reported by Boubaker *et al.* (2013), and Cong-Nuan *et al.* (2015) (0.1–5 ng and 10–20 pg, respectively). With the implementation of control programmes and large-scale population echinococcosis screening in China, the majority of newly discovered patients are in the early infection stage with small lesions. Therefore, only minute amounts of tissue samples can be collected in most cases, which puts higher requirements on the sensitivity of DNA detecting methods. The mPCR method developed herein can meet its need due to its high sensitivity.

In the application test of our mPCR method, a large number of patients and healthy human samples were investigated. For all previously gene-sequencing determined patient samples, the genotypes could be successfully re-confirmed by mPCR, thus demonstrating the high accuracy and reliability of the method. Meanwhile, no amplicon was observed from any healthy human DNA samples, which indicates that the method was not interfered by contaminating DNA from the host (human). On the other side, only two human-derived *E. canadensis* (G6) samples were available for this study, because of its very low prevalence in China. Although the sample size and genotypes of *E. canadensis* were insufficient in the application test, our BLAST analyses of *E. canadensis* revealed that most *E. canadensis* (G6–G8, G10) isolates listed in GenBank can full-match with *E. canadensis* primers, which confirmed our method could successfully amplify G6–G8 and G10 genotypes. Compared to other identification methods for human echinococcosis in China, such as PCR-based sequencing, PCR-RFLP or histopathology (Bart *et al.*, 2006; Li *et al.*, 2008), the mPCR method presented herein is more advantageous: it is more rapid and less costly. Thereby, it clearly simplifies the

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identification of human echinococcosis in China and is suitable for routine tests.

The current clinical diagnosis of echinococcosis primarily relies on imaging techniques, and serological test plays a complementary role (McManus *et al.*, 2012; Sarink *et al.*, 2018). Imaging diagnosis can distinguish the two medically important CE and AE lesions (Brunetti *et al.*, 2010), but it fails to discriminate the CE lesions caused by *E. granulosus s.s.* and *E. canadensis*, respectively. In addition, misdiagnosis or uncertain cases can easily occur in imaging examination when encountering atypical pathological features, early stage or similar lesions, or inexperienced inspectors (Zhang and McManus, 2006; HołodyZaręba *et al.*, 2013; Mesut *et al.*, 2016). Although serology is a helpful tool for early diagnosis of echinococcosis, questions remain with regards to its specificity and effectiveness for clinical detection. Till now, there are no available standardized echinococcosis diagnostic kits that are generally accepted by clinical physicians (Zhang and McManus, 2006; McManus *et al.*, 2012). These all illustrate the widely used non-invasive tests could not reach accurate species identification. Invasive molecular biology technique base on the detection of parasite-specific DNA provides a more accurate approach for differential diagnosis of echinococcosis and is now considered to be a gold standard (Schweiger *et al.*, 2012). Possible due to the need for invasive examination, its development and application on clinical diagnosis is still limited. Nevertheless, treatment and prognosis of echinococcosis are diverse among species (Brunetti *et al.*, 2010; McManus *et al.*, 2012), precise differential diagnosis is fundamental for determining the optimal treatment plan with the least amount of morbidity and mortality. Therefore, an efficient molecular diagnostic method is essential and should to be employed more widely or even taken as a routine clinical diagnostic tool. The mPCR established in this study provides such a method, which can simultaneously differentiate and accurately identify *E. granulosus s.s.*, *E. multilocularis* and *E. canadensis*. It has a high application potential and may greatly contribute to the confirmatory diagnosis of human echinococcosis in China. Moreover, due to the high accuracy and reliability, this method can be a useful tool for evaluating the results of imaging and serological tests, which in turn can further develop the detection accuracy of non-invasive diagnosis.

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Conflict of interest. None.

Ethical standards. The study was approved by the Ethics Committee of Sichuan Center for Disease Control and Prevention. All the samples were collected according to the procedures and guidelines of the ethics committee.

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