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Research Paper

Development of a nest-PCR for detection of *Fasciola hepatica* DNA in the intermediate snail host, *Radix cucunorica*, and the prevalence in northwestern China

Si-Yang Huang[a,b,*], Jing-Zhi Gong[a], Bin Yang[a], Yi-Min Fan[a], Na Yao[a], Chun-Ren Wang[c]

[a] Institute of Comparative Medicine, College of Veterinary Medicine, Yangzhou University, Jiangsu Co-innovation Center for Prevention, Control of Important Animal Infectious Diseases and Zoonosis, Jiangsu Key Laboratory of Zoonosis, Yangzhou, Jiangsu Province 225009, PR China
[b] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, PR China
[c] College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province 163319, PR China

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ABSTRACT

Fasciolosis, a foodborne zoonotic disease, caused by *Fasciola* species which is considered an important problem for human health and livestock husbandry development. Snails are intermediate hosts of *F. hepatica*, the epidemiological surveillance of snails can evaluate the transmission risk of this disease in human and livestock. In this study, we developed a nest-polymerase chain reaction (nest-PCR) to detect the DNA of *F. hepatica* in *Radix cucunorica*, a prevalent intermediate host of this parasite in northwestern China. The nest-PCR was used to amplify a 208 bp fragment of the second internal transcribed spacer (ITS-2) of *F. hepatica* with two pairs of primers. The method was able to detect up to 0.16 fg genomic DNA in a 25 μL PCR reaction system even effected with high concentrations of snail DNA, and no cross reaction was observed from the genomic DNA of *Paramphistomum cervi*, *Clonorchis sinensis*, *Orientobilharzia turkestanicum*, *Metorchis orientalis*, *Dicrocoelium chinensis*. To evaluate the transmission risk of this disease, 409 snail samples collected from different areas of Gansu province were used to detect and analyze the transmission risk of *F. hepatica* in this area. Of 409 snail samples, the overall prevalence is 43.76%. The prevalence was 92.75% in Gannan Tibetan Autonomous Prefecture, while no snail was positive for *F. hepatica* in Linxia Hui Autonomous Prefecture. The nest-PCR was firstly used to detect the infection of *F. hepatica* in snail. It is a novel, useful and convenient method with high sensitivity and specificity. This study is the first report about the epidemiological surveillance of snail infection by *F. hepatica* in northwestern China, which will help to evaluate the transmission risk of *F. hepatica* in northwestern China.

1. Introduction

Fasciolosis, a foodborne zoonotic disease, caused by *Fasciola* species (*Fasciola hepatica* and *Fasciola gigantica*), which is a serious clinical disease for human and livestock (Mas-Coma et al., 2005; Spithill and Dalton, 1998). *F. hepatica* infects lots of different livestock, including cattle, sheep, buffalo, goats, rabbits, which causes major economic losses worldwide (Bruckner, 1999; Garcia et al., 2007; Nawrocki et al., 2019). It is estimated that about 2.4–7 million people infected by *F. hepatica* or *F. gigantica*, and over 91 million people are at risk in the world (Keiser and Utzinger, 2005). It costs conservative $3 billion financial losses every year, due to infected livestock resulting in decreased fertility and lactation (Mohammad Alizadeh et al., 2011). In China, 306 cases of human fasciolosis have been reported. Nonetheless, the prevalence of fasciolosis in China is probably underestimated because of poor sensitivity of diagnostic tests and limited epidemiological data (Ai et al., 2019). While, the prevalence of fasciolosis in animals was quite high, A study indicated that 87.35% 0f buffaloes were infected in Guangxi province, and the prevalence of fasciolosis was 28.7% in Yaks in Gansu province (Zhang et al., 2019; Zhang et al., 2017)

F. hepatica is transmitted to the definitive host (human or livestock) *via* the ingestion of the infective metacercariae which are developed inside intermediate host (snails) (Correa et al., 2010; Molloy and Anderson, 2006). Snails are important epidemiological indicators because they can reflect the environment contamination with *F. hepatica*. There are several different snails involved in the transmission of

* Corresponding author at: Institute of Comparative Medicine, College of Veterinary Medicine, Yangzhou University, Jiangsu Co-innovation Center for Prevention, Control of Important Animal Infectious Diseases and Zoonosis, Jiangsu Key Laboratory of Zoonosis, Yangzhou, Jiangsu Province 225009, PR China.
E-mail address: siyang.huang@hotmail.com (S.-Y. Huang).

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fasciolosis in China, including *Radix cucunorica*, *Galba cubensis*, and *Radix lagotis*. *R. cucunorica* was the predominant intermediate host of *F. hepatica* distributing in Gansu, Qinghai and Tibet. Investigating infected snails was able to forecast the fasciolosis prevalence in the endemic areas and then take strategies to control this disease. So, the evaluation of *F. hepatica* infection in the intermediate snail host is very important. Previous studies indicated that the internal transcribed spacers (ITSs) of ribosomal DNA (rDNA) can be used in identification of *Fasciol*a species (Ai et al., 2010; Huang et al., 2004; Lin et al., 2007; Zhang et al., 2007). Several PCR-based assays have been developed to identify *Fasciola* spp. in the intermediate host over the last several years, such as a multiplex-PCR was used to detection of *F. hepatica* DNA in *Lymnaea viatrix*, *Lymnaea columella*, *Galba cubensis* and *Galba truncatula* (Alba et al., 2015; Caron et al., 2011; Jones et al., 2018; Magalhaes et al., 2008; Magalhaes et al., 2004), Recently, a multiplex quantitative PCR assay were developed to identify *Fasciola* in *Austropeplea tomentos*a and water samples (Rathinasamy et al., 2018). Several studies indicated that *F. hepatica* was detected in snails (including *R. cucunorica*) by microscope in China. To date, no molecular detection assays were developed to identify *F. hepatica* in snails, *R. cucunorica*, the major liver fluke intermediate host in Northwestern China.

In the study, we aimed to establish a sensitive and specific nest-PCR assay to detect the *F. hepatica* in the intermediate snail host with two sets of novel primers based on ITS-2 sequences, and then investigated the prevalence of *F. hepatica* infection in the endemic areas. To our knowledge, this is the first study to demonstrate the detection method of genomic DNA from live fluke in *R. cucunorica*.

2. Materials and methods

2.1. Parasite and snails

F. hepatica were collected from livers of sheep in a slaughterhouse in the Xinjiang Uygur Autonomous Region, China, and then washed in RPMI-1640 media for 3 times at 37 °C, the clean flukes were stored at −80 °C for further studies. Adult *Paramphistomum cervi*, *Clonorchis sinensis*, *Orientobilharzia turkestanicum*, *Metorchis orientalis*, *Dicrocoelium chinensis* were stored in 70% ethanol in our lab. Snails, *R. cucunorica*, were collected from Linxia Hui Autonomous Prefecture and Gannan Tibetan Autonomous Prefecture, two typical areas of Gansu province, China. All snails were stored at −20 °C until DNA extraction.

2.2. Genomic DNA extraction

Genomic DNA from adult *F. hepatica*, *P. cervi*, *C. sinensis*, *O. turkestanicum*, *M. orientalis*, *D. chinensis* and snail were extracted using a TIANamp Genomic DNA Kit (TIANGEN, China). 20 mg snail or parasite tissue was included in each reaction, the tissue was digested by proteinase K (50 mg/mL) over night at 56 °C, and then the genomic DNA was isolated according to the manufacturer's instructions. DNA concentration and quality was measured by a Nanodrop 2000 spectrophotometer (Thermo, USA). The DNA was immediately used as template for PCR or stored at −20 °C for further study.

2.3. Primers design

The specific primers were designed on known sequences of the ITS-2 sequences of *F. hepatica* (Genebank accesion number: AB553729.1) and analyzed using Primer Premier 6.0. The primers were compared with available databases to check the specificity with ITS2 of other parasites and to verify possible cross reactions with other parasites using the BLASTn genomic database. Fh-F and Fh-R were designed to amplify the first fragment, and n-F and n-R were used to amplify the second fragment which is inside of the first fragment.

2.4. Nest-PCR amplification

Different primer concentrations were tested in each PCR assay, the PCR reaction is 25 μL with 12.5 μL 2× Premix Taq™ (TaKaRa Taq™ Version 2.0 plus dye) (TaKaRa, China), 1 μL template DNA, 2 μL primers and 9.5 μL ddH_2O. Different reaction conditions were tested, including the reaction conditions were started with a denaturation at 95 °C 5 min, followed by 35 cycles of 30 s at 95 °C, 30 s at different temperature (52 °C–60 °C) and 40 s at 72 °C, stopped by a final extension at 72 °C for 7 min. For the first run, the sample genomic DNA was used for template, Fh-F and Fh-R were used for primers, and for second run, template was changed into the PCR product of the first run, and the primers was replaced by n-F and n-R. The genomic DNA of *F. hepatica* (160 ng/μL) was positive control and the negative control was the genomic DNA of uninfected snails (100 ng/μL), positive and negative controls were included in each first run experiment. The products of the positive and negative controls in the first run were the positive and negative controls in the second run, respectively. Amplification products were observed under UV light after electrophoresis in 3% agarose gel containing GoldView™ (Solarbio, China), and confirmed by DNA sequencing.

2.5. Analytical specificity of the nest-PCR

The specificity of nest-PCR was tested under the conditions as described above, using genomic DNA of *P. cervi*, *C. sinensis*, *O. turkestanicum*, *M. orientalis*, *D. chinensis* and adult *F. hepatica* genomic DNA as a positive control. 160 ng genomic DNA was included as templet in each reaction. The products were observed under UV light after electrophoresis in 3% agarose gel containing GoldView™ (Solarbio, China).

2.6. Detection limit of the nest-PCR

The genomic DNA of *F. hepatica* (160 ng/μL) was diluted by negative snail genomic DNA solutions (100 ng/μL) by 10-flod gradient into 11 different concentrations. The sensitivity of nest-PCR was test under the conditions as described above. The products were observed under UV light after electrophoresis in 3% agarose gel containing GoldView™ (Solarbio, China).

2.7. Application of nest-PCR assays in field-collected samples

Genomic DNA of snail samples was examined for the infection of *F. hepatica* using the developed nest-PCR. The products were observed under UV light after electrophoresis in 3% agarose gel containing GoldView™ (Solarbio, China). For the suspected samples, the experiment was repeated 3 times and the products were furtherly sequenced for determination (in Sangon Biotech, China).

2.8. Statistical analysis

The variation in *F. hepatica* prevalence of intermediate snail host from different regions was analyzed by $\chi 2$ test using SAS version 9.1 (SAS Institute Inc., Cary, North Carolina, USA) (Zhang et al., 2017). The best model was judged by Fisher's scoring algorithm. All tests were two-sided, and value of $P < .05$ was considered statistically significant.

3. Results

3.1. Optimization of the nest-PCR

Primers design is the most important part of the nest-PCR optimization. The ITS-2 region of *F. hepatica* was compared with other prevalent rumen fluke in this area, such as *P. cervi*, *C. sinensis*, *O. turkestanicum*, *M. orientalis* and *D. chinensis*, and then primers were designed in the low similarity region to avoid cross reaction. The best reaction conditions were started with a denaturation at 95 °C 5 min, followed by

Table 1
Primers used in the study, annealing temperatures used in the PCR and expected sizes of the PCR products, the position of primers in ITS2(Genebank accesion number: AB553729.1).

Primer	Sequence (5′-3′)	Concentration (μM)	Annealing temperature (°C)	Product size (bp)	Position
Fh-F	ATATTGCGGCCATGGGTTAG	0.5	56	336	59–78
Fh-R	CCAATGACAAAGTGACAGCG				379–398
n-F	TATCACGACGCCCAAAAAGTC	0.5	57	208	116–136
n-R	GATCGCCAAACACACTGACA				304–323

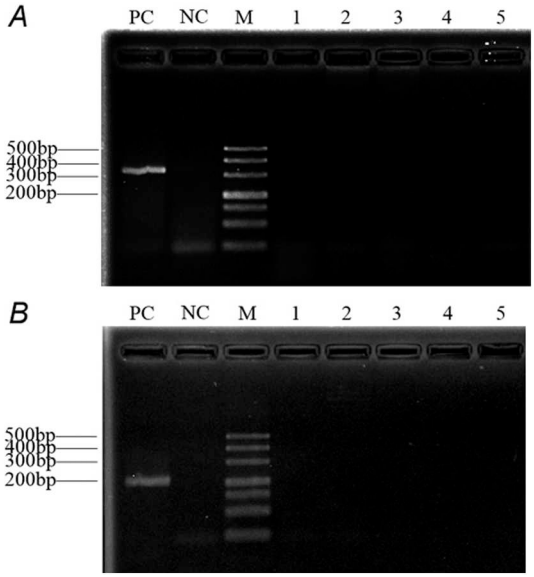

Fig. 1. Specificity of the primer sets. A) amplification with the first set of primers (Fh-F and Fh-R). B) amplification with the second set of primers (n-F and n-R). Lane PC: positive control, *F. hepatica*. Lane NC: negative control. Lane M: 500 bp DNA Marker (TaKaRa, Japan). Lane 1–5: template DNA isolated from *Paramphistomum cervi*, *Clonorchis sinensis, Orientobilharzia turkestanicum*, *Metorchis orientalis*, *Dicrocoelium chinensis*, respectively.

35 cycles of 30 s at 95 °C, 30 s at suitable temperature (56 °C or 57 °C) and 40 s at 72 °C, stopped by a final extension at 72 °C for 7 min. The primers sequence, concentrations, annealing temperature and product size were indicated in Table 1. With the first pair of primers (Fh-F and Fh-R), a clean band was amplified at the expected size (336 bp) when suitable condition was present (Fig. 1A). For the second run, the second pair of primers (n-F and n-R) were used, a clean band at the expected size (208 bp) were generated (Fig. 1B).

3.2. Analysis of cross reactions and limit of detection

The genomic DNA (160 ng) of *P. cervi, C. sinensis, O. turkestanicum, M. orientalis, D. chinensis* were used to evaluate the specificity of the nest-PCR, with the same amount DNA of *F. hepatica* as a positive control. The nest-PCR result showed expected fragment of *F. hepatica* genomic DNA at optimization condition. No PCR product was amplified with the six parasites DNA templates neither in the first or the second run PCR (Fig. 1 A and B), the results indicated that no cross reaction were observed and confirmed the specificity of the assay.

Limit of detection of the nest-PCR was analyzed using a series of 10-fold diluted adult *F. hepatica* genomic DNA. Initial concentration of template DNA was 160 ng/μL. It showed that there was a clear band of 208 bp size after 10^{-9}-fold dilution (Fig. 2B). The detection limit of the nest-PCR was at the 10^{-9}-fold dilution, the assay could detect *F. hepatica* genomic DNA up to 0.16 fg/μL. The first run PCR can only detect up to 1.6 pg/μL(Fig. 2A). These results indicated that the nest-PCR assay is 1000× more sensitive than the first run PCR.

3.3. Detection of field-collected samples

Snails were collected from Linxia Hui Autonomous Prefecture and Gannan Tibetan Autonomous Prefecture (Fig. 3). 409 snail genomic DNA samples were analyzed by the nest-PCR using *F. hepatica* genomic DNA as positive control and PBS as negative control. No sample was positive for *F. hepatica* among 216 snail samples collected from Linxia Hui Autonomous Prefecture. These results indicated that the prevalence of *F. hepatica* infection of snail is quite low in this area. While, 179 among all 193 snail samples collected from Gannan Tibetan Autonomous Prefecture were positive for *F. hepatica*, the prevalence is as high as 92.75%. Among the 193 samples, 106 were collected from small stream and 98 samples were positive, the prevalence was 92.45% (95% CI 84.12–100), 87 were collected from small ponds and 81 samples were positive, the prevalence was 93.10% (95% CI 84.02–100) (Table 2). For the field samples, we could find that two samples were negative detected in the first PCR run (Fig. 4A), while a clean band of 208 bp size was amplified from all these 10 samples in the second PCR run (Fig. 4B). These results indicated that the sensitivity of nest-PCR is much better than the conventional PCR.

4. Discussion

The high prevalence of *F. hepatica* infection along with the unfavorable impact on animal husbandry makes fasciolosis an serious problem of livestock in the world (Mas-Coma et al., 2009; Nyindo and Lukambagire, 2015; Piedrafita et al., 2010). Given no vaccine is available to prevent this disease and the drug resistance problem is serious, an alternative control method is need to reduce the transmission of fasciolosis in livestock. Metacercariae is the infective stage which develops inside intermediate host (snail). Understanding the prevalence of *F. hepatica* in snails is important to evaluate the transmission risk of this parasite. Hence, establishing specific and effective methods to detect the infection levels of snails is necessary to supervise and control development of fasciolosis.

In the present study, we developed the first nest-PCR method to identify *F. hepatica* in the snails, *R. cucunorica*, using ITS-2 as a target. The selection of the specific target sequence and nest-PCR assay resulted in a higher sensitivity with limitation up to 0.16 fg/μL, even effected by the genomic DNA of snails. Traditional fecal examinations and ELISAs used for detecting eggs, coprological antigens or serum antibodies , are not suitable for detecting the infection of snails (Becker et al., 2016; George et al., 2017; Happich and Boray, 1969; Hong et al., 2003; Mezo et al., 2007). In 1979, the prevalence of *F. hepatica* in snails was estimated through microscopy in the United Kingdom, and the prevalence was 2% detected by this method (Ollerenshaw, 1971). Another study reported a 1.7% positive rate in snails using the same detected method in France (Dreyfuss et al., 2005). Due to the limitation of microscopic technique in sensitivity and specificity, a DNA probe assay was developed, and the prevalence of *F. hepatica* in snail was 1.5% detected by this method in Florida (Kaplan et al., 1997). The sensitivity is much higher comparing to the mentioned method, including the multiplex quantitative PCR. The primers were specific to *F. hepatica* without cross-reacting with DNA from other common livestock parasites in this region (*P. cervi, C. sinensis, O. turkestanicum, M. orientalis, D.*

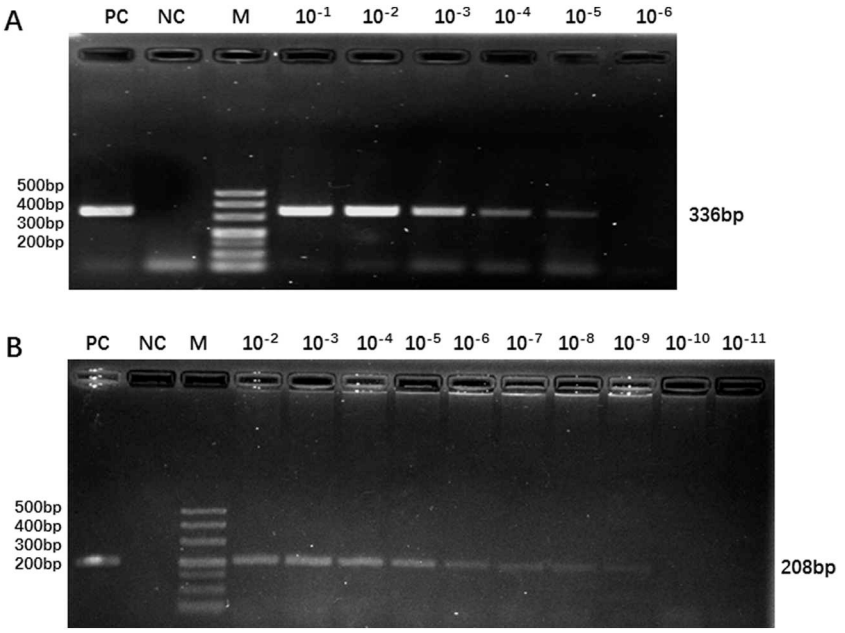

Fig. 2. Detection limit of the nest-PCR. Template was genomic DNA extracted from adult *F. hepatica* and subjected to 10-fold serial dilution with negative snail genomic DNA solutions, Template DNA was diluted by 10^{-1}(16 ng/μL), 10^{-2}(1.6 ng/μL), 10^{-3}(0.16 ng/μL), 10^{-4}(16 pg/μL), 10^{-5}(1.6 pg/μL), 10^{-6}(0.16 pg/μL), 10^{-7}(16 fg/μL), 10^{-8}(1.6 fg/μL), 10^{-9}(0.16 fg/μL), 10^{-10}(0.016 fg/μL) and 10^{-11}fold(0.0016 fg/μL), respectively. Lane PC: positive control, *F. hepatica*. Lane NC: negative control. Lane M: 500 bp DNA Marker (TaKaRa, Japan).

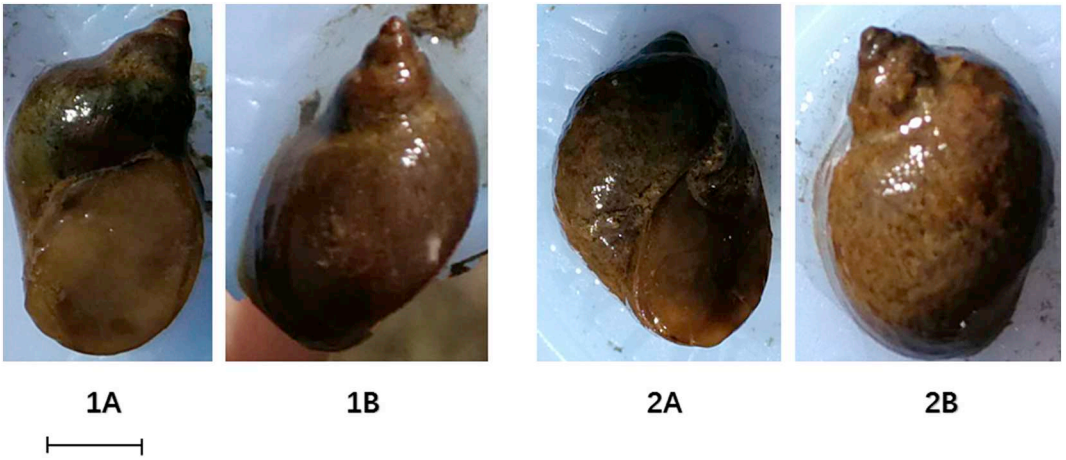

Fig. 3. Two snails collected from Linxia Hui Autonomous Prefecture (1) and Gannan Tibetan Autonomous Prefecture(2), A and B means the obverse and the reverse of snail. Scale bars: 4 mm.

chinensis). More and More new techniques were developed recently, among them, PCR was considered as a golden standard contrary to other techniques, due to its high specificity and sensitivity (Caron et al., 2008). Such as radioactive probes PCR (Schweizer et al., 2007) and conventional normal PCR were able to detect up to 10 pg of genomic *F. hepatica* DNA using primers based on the cytochrome *c* oxidase subunit 1 gene (Cucher et al., 2006). A multiplex PCR was developed to identify experimental infected *Lymnaea columella*, based on a *F. hepatica* mitochondrial DNA (mtDNA), and the limitation is up to 0.8 ng/μl (Magalhaes et al., 2004). Alba *et al* developed a multiplex PCR used to identify experimental infected *Galba cubensis*, based on a *F. hepatica* ITS2, and the detected limitation could be up to 100 pg (Alba et al., 2015). A multiplex quantitative PCR was developed to detect the infection of snails, and the detected limitation is as high as 14–50 fg, this assay was also based on ITS-2 within the rDNA gene (Rathinasamy et al., 2018). Nest-PCR has been used for diagnosing the infection of *F. hepatica* in sheep, of which specific PCR primers were designed based on a *F. hepatica* mitochondrial DNA (mtDNA) sequence in order to amplify the cytochrome C oxidase gene (Martinez-Perez et al., 2012). The specificity and sensitivity of nest-PCR is higher comparing to the normal PCR.

In the study, all of 216 snails collected from Linxia Hui Autonomous

Table 2
Prevalence of *F. hepatica* infection in the intermediate snail host in Linxia Hui Autonomous Prefecture and Gannan Tibetan Autonomous Prefecture, Gansu province, northwestern China.

Region		NO. tested	NO. positive	Prevalence(%) (95% CI)	*P* value
Linxia Hui Autonomous Prefecture	Small stream	216	0	0	–
Gannan Tibetan Autonomous Prefecture	Small stream	106	98	92.45(84.12–100)	< 0.05
	Small pond	87	81	93.10(84.02–100)	
Total		409	179	43.76	

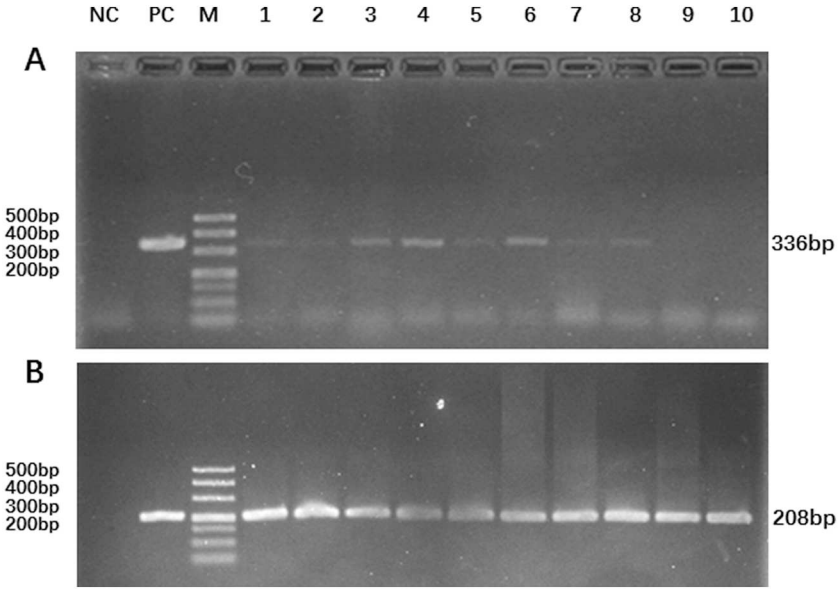

Fig. 4. Snails samples were detected by the nest-PCR. Lane PC: positive control, *F. hepatica*. Lane NC: negative control. Lane M: 500 bp DNA Marker (TaKaRa, Japan). A) Lane 1–10: The first PCR run with the template isolated from individual sample. B) Lane 1–10: The second PCR run with the template from the products of the first PCR run.

Prefecture were negative for *F. hepatica* detected by the nest-PCR method, the prevalence was extremely lower comparing to other studies , such as 2% in the United Kingdom (Ollerenshaw, 1971) and 1.7% in France (Dreyfuss et al., 2005). The main reason probably is due to the local environment without suitablities for snails living, such as the low rainfall, quite dry climate, although several rivers are crossing in this region. Another reason is that most of cattle or sheep were breed in captivity, so the rivers are not easily polluted by the feces. The prevalence of *F. hepatica* in sheep and cattle was also quite low in this area, which is consistent with the prevalence of snails (unpublished data). In the Gannan Tibetan Autonomous Prefecture, the quite different results showed the prevalence of *F. hepatica* in snails was over 90% (Table 2). Considering the plenty rainfall, distributed lots of small ponds and streams in this area, the environment is suitable for the development of snail. Livestock grazed on the pastures exacerbates water pollution by feces containing *F. hepatica* eggs, which facilitates the transmission of *F. hepatica*. It also indirectly reflected high prevalence of *F. hepatica* in Yaks in Gannan Tibetan Autonomous Prefecture (Zhang et al., 2017). From the results, we could find that the prevalence of *F. hepatica* infection varied considerably between different regions due to different grazing management and natural environment. Careful interpretation was useful to control the transmission of this disease (Takeuchi-Storm et al., 2018). The prevalence of infection of *F. hepatica* in snails can indirectly reflected the fasciolosis prevalence in domestic animals, especially for the herding animals in the endemic areas. So, the evaluation of *F. hepatica* infection in the intermediate snail host is very important to develop a policy on controlling this disease.

5. Conclusion

We established the first nest-PCR method, a rapid and highly sensitive method, to detect the *F. hepatica* infection in the intermediate snail host, *R. cucunorica*, with DNA detection levels up to 0.16 fg/μL. This method is specific for *F. hepatica* without cross reaction of *P. cervi*, *C. sinensis*, *O. turkestanicum*, *M. orientalis*, *D. chinensis*. The overall prevalence of *F. hepatica* in snails was 43.76% detected by this method in Gansu province, northwestern China.

Ethics approval and consent to participate

All experiments in this study were approved by the Science and Technology Agency of Jiangsu Province [Approval number: SYXK (SU) 2017–0044].

Competing financial interests

The authors have declared that no competing financial interests.

Declaration of Competing Interest

None.

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