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Multiplex PCR assay for discrimination of *Centrocestus caninus* and *Stellantchasmus falcatus*

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ABSTRACT

Objective: To develop the multiplex PCR method based on the internal transcribed spacer 2 to discriminate the intestinal trematodes, *Centrocestus caninus* (*C. caninus*), and *Stellantchasmus falcatus* (*S. falcatus*).
Methods: Four species of heterophyid trematodes including *C. caninus*, *S. falcatus*, *Haplorchis taichui* and *Haplorchoides* sp. were amplified and the specific primer was designed based on the internal transcribed spacer 2 region. Two specific primers were used to validate the optimized PCR conditions: the specificity test and the sensitivity test.
Results: Both of these specific primers confirmed the specificity through multiplex PCR reaction which generated both PCR products (231 and 137 bp) in the mixed DNA template of *C. caninus* and *S. falcatus* with no cross-reaction with other heterophyid trematodes. The optimum annealing temperature of both primers was 54–59 °C. The sensitivity test used the two-fold serial dilution DNA template, which was concentrated between 10 and 0.3125 ng/μL. The lowest concentration of the DNA template of this multiplex PCR was 2.5 ng/μL.
Conclusions: The technique described here proved to be a species-specific technique and was found to be a rapid method for the diagnosis of *C. caninus* and *S. falcatus* in terms of the larval and adult stages in intermediate and/or definitive hosts in the endemic area.

1. Introduction

Heterophyid trematodes are major zoonotic parasites that have been known to infect humans and animals with a high rate of prevalence in Asia and Southeast Asia [1] including countries such as China, Japan, Korea, Laos, Vietnam, the Philippines, Egypt and Thailand [2–5]. Their distribution depends on the presence of the first and second intermediate hosts. Several epidemiological investigations of trematode infections in Thailand have revealed a high prevalence of heterophyid trematode infections in various species of freshwater snails and fish. For instance, recent studies on the metacercarial stage of the heterophyid trematode species (*Haplorchis pumilio* and *Centrocestus formosanus*) were found in freshwater fish collected from the Pasak Cholasid Reservoir, Thailand [6]. Particularly, both *Centrocestus caninus* (*C. caninus*) and *Stellantchasmus falcatus* (*S. falcatus*) have displayed a similar indirect life cycle and share elements of pathogenicities. Both of these species make use of freshwater snails, *Melanoides tuberculata* (*M. tuberculata*) and *Tarebia granifera*, as their first intermediate host [7,8], freshwater fish *Cyclocheilichthys repasson*, *Puntius brevis*, *Dermogenys pusillus*, *Cirrhinus mrigala* and *Anabas testudineus*, as the second intermediate host [9] and *Gallus gallus domesticus* and *Mus musculus*, as well as humans as the definitive hosts for their complete life cycle [10].

Generally, the parasitological survey was conducted by crushing the snails and the pepsin digestion technique was used to detect the metacercarial stage in the fish. The subsequent examination was performed under a light microscope. This protocol was incapable of detecting the early larval stages of infection from other parasites. The molecular diagnoses seemed

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to be more effective as an alternative method. For example, PCR-restriction fragment length polymorphism has been used to closely discriminate between various related species of parasites (*Echinococcus granulosus* [11], *Toxoplasma gondii* [12] and *Fasciola* spp. [13]).

The PCR based-method targeting internal transcribed spacer 2 (ITS2) has been applied for the molecular identification of various trematodes, such as *Stictodora tridactyla*, *S. falcatus*, *Fasciola hepatica*, *Paragonimus* sp., *Clonorchis sinensis* and trematodes in the family Paramphistomidae [14–17]. The multiplex PCR procedure has been established for its accurate and rapid features with a high sensitivity of detecting parasites in a single step. The ITS2 multiplex primer has been applied for the detection of human liver flukes, *Fasciola hepatica* in lymnaeid snails [18], *Clonorchis sinensis*, *Opisthorchis viverrini* and *Haplorchis taichui* (*H. taichui*) [19].

With regard to all of the above species, the aim of this research study was to establish and validate an accurate, sensitive and rapid analytical procedure for the detection of two closely related species of minute intestinal flukes, *C. caninus* and *S. falcatus*. This procedure can be used to detect and identify the trematodes when present in the case of a single or mixed infection in both intermediate and definitive hosts. Moreover, this multiplex PCR allows for a reliable epidemiological control model of both trematode infections in order to achieve rational control strategies, which would then be firmly established on a larger scale.

2. Materials and methods

2.1. Parasite specimens and experimental hosts

Specimens identified at three metacercarial stages of heterophyid trematode infection were collected from various freshwater fish, comprising *C. caninus* from golden fish (*Carassius auratus*), *S. falcatus* from halfbeak (*Dermogenys pusillus*), and *H. taichui* from Siamese mud carp (*Henicorhynchus siamensis*). Collection was done using the digestion technique with 1% pepsin solution [20] and specimens were then force-fed to develop as 1-day-old chicks (*Gallus gallus domesticus*). Additionally, the adult stage specimens of the *Haplorchoides* sp. were collected from bagrid catfish (*Hemibagrus filamentus*). All of the parasites were washed in phosphate buffer, at a pH value of 7.4 and stored in 95% alcohol at −20 °C.

2.2. Total genomic DNA and PCR amplification

All trematode specimens were extracted using the commercial GF-1 tissue DNA extraction kit (Vivantis, Malaysia) according to the manufacturer's instructions. Samples of the genomic DNA of each specimen were diluted to a concentration of 10 ng/μL with an elution buffer and were kept at −20 °C until being used. The PCR amplification of the ITS2 region was performed by using a pair of primers which were designed and described in the previous study [21]. This process consisted of (ITS3) 5′ GCATCGATGAAGAAC GCAGC 3′ as a forward primer and (ITS4) 5′ TCC TCCG CTTATTGATATGC 3′ as a reverse primer. The sequence of all PCR products was performed for the purposes of being checked by the BLAST program in the NCBI database in order to confirm the PCR target. Then, the specific primer of each species was designed manually from the sequence with the other nucleotide sequences acquired from the GenBank database. The designs were CC-F (5/CGG CGT GAT TTC CTT GTG CTT TGC) and CC-R (5/GCA CAC ATT AGA AAC GCG TGC AAT) for *C. caninus* (anticipated product size 231 bp); ST-F (5/CTG TTG TAG GGT GCC GGA TC) and ST-R (5/GCA CAC ATT AGA AAC GCG TGC AAT) for *C. caninus* (anticipated product size 137 bp). The PCR product of both trematodes was sequenced again to confirm identity.

2.3. Validation of multiplex PCR

Multiplex PCR was developed for a single reaction using a pair of primers. The specificity and sensitivity tests were done using different combinations of the DNA template at a concentration of 10 ng/μL in every test. The optimal temperature of both primers was tested in ranks of various annealing temperature at 54–59 °C. Furthermore, both primers were tested for the specificity period and were used to perform the molecular identification by attempting to amplify them with four heterophyid trematode species, which are wildly distributed throughout Thailand (*C. caninus, S. falcatus, H. taichui* and *Haplorchoides* sp.). For the sensitivity test, two-fold series dilution (10–0.312 5 ng/μL) of the DNA template of *C. caninus* and *S. falcatus* were amplified.

3. Results

The amplification of the ITS2 from the four species of heterophyid trematodes consistently yielded products about 400 bp fragment for *C. caninus*, *S. falcatus* and *Haplorchoides* sp, whereas, only *H. taichui* gave rise to 560 bp fragments.

The optimization of the PCR conditions for the amplified ITS2 fragment of *C. caninus* and *S. falcatus* was done at varied annealing temperatures using CC-F and CC-R primers for *C. caninus* and ST-F and ST-R primers for *S. falcatus*. The results showed that both the primer sets could be amplified at all temperatures. The same multiplex PCR conditions were used for the species-specific identification PCR with co-infection of both trematode species until two specific fragment sizes appeared together. The mix of both DNA specific primers generated strong PCR product fragments of 231 and 137 bp for the mixed DNA templates of *C. caninus* and *S. falcatus*, and no cross-reaction with the other heterophyid trematodes occurred.

Regarding the sensitivity test, sensitivity was amplified in the *C. caninus* and *S. falcatus* specimens using two-fold serial diluted genomic DNA. The initial concentration of the genomic DNA template was 10 ng/μL and was concentrated between 10 and 0.312 5 ng/μL. The specific fragments of both trematode species were generated at all concentrations, although the intensity of the band progressively decreased along with the low concentration value of the DNA templates. The lowest DNA concentration of *C. caninus* was at about 2.5 ng/μL while the lowest concentration of the DNA template of *S. falcatus* was 0.312 5 ng/μL. Consequently, this multiplex PCR could be used to detect co-infection in both trematodes at a concentration of 2.5 ng/μL.

4. Discussion

Infection of heterophyid trematodes in the intermediate hosts has generally been detected by identifying the larval stages (cercarial and metacercarial stages) under a light microscope. This

method is known to display low accuracy and sensitivity ratings and requires a higher level of experience. As a result, other methods have been developed to yield more sensitive results, such as those associated with molecular biology. These are required, principally, when various trematode species co-infect the same species of the intermediate host. For example, *M. tuberculata* serve as the first primary intermediate host of various trematode species, such as *H. taichui, Haplorchis pumilio, C. caninus, S. falcatus*, and *Haplorchoides* sp. Moreover, the heterophyid trematodes use a similar species as the second intermediate host. According to previous reports, it has been concluded that the snail family Thiaridae harbors the larvae of the intestinal and blood flukes [22]. Furthermore, *M. tuberculata* and *Tarebia granifera* have been classified as being medically important because they can serve as the first intermediate host for intestinal flukes [23,24].

The increasing numbers and prevalence of heterophyid trematodes in snails, fish and mammals place a heavy burden on veterinary services, especially in Thailand. This is particularly true in Chiang Mai, Phitsanulok, Udon Thani, Nakhon Ratchasima, and Kanchanaburi Provinces [19,22,24]. There is a potential for significant economic losses in the farming, forestry and livestock industries. Therefore, effective methods in terms of comfort, safety, timeliness and the highly accurate diagnosis for every parasitic stage in the intermediate and definitive hosts are necessary for the epidemiological study. The developed detection methods of heterophyid trematodes have so far contributed to fast and accurate identification methods. Most have been involved with coprodiagnosis and the simplex PCR technique, such as sequence characterized amplified regions marker [25], high annealing temperature random amplified polymorphic DNA profile [26] and PCR-restriction fragment length polymorphism [11,12], which are considered both time-consuming and expensive. Accordingly, a recent report that was published in 2008 concluded that multiplex PCR was the most sensitive and accurate method for specific diagnosis and could easily be applied to achieve results as a low cost technique. The ITS2 based multiplex PCR procedure, which is the first work applied to specifically amplify the *C. caninus* and *S. falcatus* DNA, has been developed in order to detect the parasitic infection in the first and second intermediate hosts. The ITS2 region proved to be a suitable target to discriminate between *C. caninus* and *S. falcatus* infections.

This method proved to be highly accurate and displayed sensitivity when being used to examine the parasitic infection. The multiplex PCR revealed that the optimal concentration of the DNA template was 2.5 ng/μL. However, the lowest concentration value of the DNA template that could be amplified by species-specific PCR reaction was around 0.3125 ng/μL, which is very low compared with other reports [27] that have revealed different fragment sizes for clear separation. The primer set in the multiplex PCR reaction yielded specific fragments of *C. caninus* and *S. falcatus*. This study performed well in all templates that were tested and no fragments were produced from the other heterophyid trematode DNA templates.

The successful development of the *C. caninus* and *S. falcatus* specific primers has provided a beneficial approach as a revelation in the epidemiological situation of both trematode species in the intermediate and definitive hosts for the purposes of monitoring parasitic diseases.

Conflict of interest statement

I declare that I have no conflict of interest.

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