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INFECTION STATUS AND MOLECULAR IDENTIFICATION OF DIGENEAN CERCARIAE IN SNAILS IN KIM SON DISTRICT, NINH BINH PROVINCE AND BA VI DISTRICT, HA NOI

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ABSTRACT

In this study, the molecular method was used to identify digenean cercariae from freshwater snails in Kim Son District (Ninh Binh Province) and Ba Vi District (Ha Noi). A total of 9 snail species were collected and examined for cercarial infection. Three snail species (*Radix swinhoei*, *Angulyagra polyzonata*, and *Pomacea canaliculata*) were not infected, while the other 6 species (*Austropeplea viridis*, *Gyraulus convexiusculus*, *Parafossarulus striatulus*, *Bithynia fuchsiana*, *Melanoides tuberculata*, and *Tarebia granifera*) were infected with digenean cercariae at low infection rates, ranging from 2.3% to 6.3%. Seven cercarial groups were identified: Echinostome, Monostome, Parapleurophocercaria, Xiphidiocercaria, Fucocercaria, Gymnocephalous, and Megalurous. Snails *M. tuberculata* and *P. striatulus* were infected with 4 cercarial groups, *A. viridis* and *G. convexiusculus* snails were infected with 2 groups, 2 snail species *B. fuchsiana* and *T. granifera* were infected with one group of cercaria. The analyses of ITS2 sequences of the cercarial groups identified the larvae of 9 trematode species, namely *Echinostoma revolutum*, *Echinochasmus japonicus, Notocotylus intestinalis*, *Philophthalmus gralli*, *Haplorchis pumilio*, *Procerovum cheni*, *Fasciola gigantica*, *Australapatemon burti*, and *Cyathocotyle prussica*. Among them, the last three species, *P. cheni*, *A. burti* and *C. prussica*, were found for the first time in Vietnam. In addition, the ITS-2 sequence of Gymnocephalous cercariae which was previously identified as *Sphaeridiotrema monorchis*, from *P. striatulus* snail was 97% similar to that of *Sphaeridiotrema pseudoglobulus*. Likewise, ITS-2 sequence of Echinostome cercaria from *B. fuchsiana* snail was 93% similar to that of *E. japonicus* and that of Xiphidiocercaria cercaria from *M. tuberculata* snail was 93% similar to *Lecithodendrium spathulatum*.

Keywords: Digenean cercaria, freshwater snail, intermediate hosts.

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INTRODUCTION

Parasitic trematodes have complex life cycles, requiring at least two hosts. Of which, the obligatory first intermediate hosts are mollusks (usually freshwater snails), where the development of larval stages (sporocyst, redia, and cercaria) takes place. Therefore, the identification of trematode larvae in snails helps to understand life cycles of trematodes and assess the trematode infection situation of humans and animals, providing a scientific basis for controlling the intermediate hosts of trematode transmission.

Cercarial larvae of trematode are classified into 38 groups (Schell, 1970). The determination of cercarial groups is based on morphological characteristics, although it is not easy to distinguish cercariae among various species. To overcome this limitation, molecular techniques have been used for the accurate identification of trematode cercariae (Chuboon and Wongsawad, 2009; Chontananarth and Wongsawad, 2010; Anucherngchai et al., 2016).

In Vietnam, surveys on trematode larvae in snails have been carried out in some locations. However, the previous reports mainly published the prevalence of trematode cercariae or morphological identification of cercarial groups (Le et al., 1990, 1995, 2000; The, 1993; Chau et al., 1996; Kim and Vinh, 1997; Dung et al., 2010; Hung et al., 2015; Clausen et al., 2015; Dung et al., 2019). In this study, we used a molecular technique to identify trematode larvae in freshwater snails in Kim Son District, Ninh Binh province and Ba Vi District, Hanoi, where livestock and poultry are commonly raised.

MATERIALS AND METHODS

Study sites

Kim Son District, Ninh Binh Province and Ba Vi District, Ha Noi.

Methods

Freshwater snails were collected in Kim Son District, Ninh Binh province and Ba Vi District, Hanoi, and were classified according to Thanh et al. (1980).

The snails were examined for cercarial infection using the shedding and crushing methods. Each snail was kept separately in a shedding tube of 2 × 2 cm (diameter × depth). In the next morning, the shedding tubes were observed under a stereoscopic microscope to detect cercariae escaped from the snails. The cercariae were transferred to a glass slide and covered with a cover glass for morphological observation under a microscope. The cercariae were classified into a group level according to the keys described by Schell, 1970, Frandsen and Christensen, 1984. Some cercariae were used for molecular analyses.

Then, the snails were examined to observe young larvae remaining in the snails using the crushing method. For small and soft snail species, the snails were pressed between 2 glass plates. For larger species of snails, a shear was used to remove the hard shell of the back of the screw and immerse it in a small water drop on the slide glass. The slide was checked under a microscope.

Cercarial samples were molecularly analyzed to identify species using ITS2 sequences, according to the following procedure: Genomic DNA from cercariae was extracted using the QIAamp DNA Mini Kit; the ITS2 sequence was amplified by PCR technique using a primer pair: 3S (forward primer, 5'-CGC TGG ATC ACT CGG CTC GT-3') and A28 (reverse primer, 5'-CCT GGT TAG TTT CTC TTC CGC- 3') (Bowles et al. 1993); the PCR products were purified using Qiaquick PCR purification Kit (Qiagen Inc., Tokyo, Japan). The forward and reverse strands were sequenced directly using the Genetic Analyzer 3130 using Big-Dye terminator cycle-sequencing kit v3.1 (Applied Biosystems). The obtained sequences were compared with DNA sequences available from GenBank with the BLAST search program.

RESULTS

Cercarial infection in snails

We collected 9 common snail species: *Austropeplea viridis* (syn. *Lymnaea viridis*), *Radix swinhoei* (syn. *Lymnaea swinhoei*), *Parafossarulus striatulus*, *Bithynia fuchsiana*,

Melanoides tuberculata, *Tarebia granifera*, *Gyraulus convexiusculus*, *Angulyagra polyzonata*, and *Pomacea canaliculata*. The total of 1,910 snails from Ba Vi District and 2,340 snails from Kim Son District were examined. Trematode cercariae were found from 6 snail species with the prevalence ranging from 2.3% to 6.3%, but not from 3 snail species, *R. swinhoei*, *A. polyzonata* and *P. canaliculata* (table 1).

Table 1. The prevalence of cercaria infection in freshwater snails

No	Snail species	Kim Son, Ninh Binh		Ba Vi, Ha Noi	
		No. examined	No. infected (%)	No. examined	No. infected (%)
1	*A. viridis*	200	5 (2.5)	350	9 (2.6)
2	*R. swinhoei*	0		100	0
3	*G. convexiusculus*	220	5 (2.3)	260	9 (3.5)
4	*T. granifera*	0		240	8 (3.3)
5	*M. tuberculata*	250	15 (6.0)	200	12 (6.0)
6	*P. striatulus*	900	48 (5.3)	240	15 (6.3)
7	*B. fuchsiana*	350	10 (2.8)	120	6 (5.0)
8	*A. polyzonata*	220	0	200	0
9	*P. canaliculata*	200	0	200	0

Identification of cercariae

Table 2. The result of molecular identification of cercarial groups from snails

Snail species	Cercarial groups	Location	Molecular identification based on ITS2 sequence		
			Trematode species	Similarity (%)	Reference sequence in GenBank
M. tuberculata	Parapleurophocercaria 1	Ha Noi, Ninh Binh	*Haplorchis pumilio*	100.0	KX815125
	Parapleurophocercaria 2	Ninh Binh	*Procerovum cheni*	100.0	HM004164
	Monostome	Ha Noi, Ninh Binh	*Notocotylus intestinalis*	100.0	MH750029
	Xiphidiocercaria	Ha Noi, Ninh Binh	*Lecithodendrium spathulatum*	93.0	JF784192
	Megalurous	Ha Noi, Ninh Binh	*Philophthalmus gralli*	100.0	JX121231
P. striatulus	Echinostome	Ha Noi, Ninh Binh	*Echinochasmus japonicus*	99.1	KT873314
	Gymnocephalous	Ha Noi, Ninh Binh	*Sphaeridiotrema pseudoglobulus*	97.0	GQ890330
	Monostome	Ha Noi, Ninh Binh	*Notocotylus intestinalis*	100.0	MH750029
	Fucocercaria	Ha Noi, Ninh Binh	*Cyathocotyle prussica*	99.0	MH521249
A. viridis	Echinostome	Ha Noi, Ninh Binh	*Echinostoma revolutum*	100.0	KF894682
	Gymnocephalous	Ha Noi	*Fasciola gigantica*	99.9	KX198631
G. convexiusculus	Echinostome	Ha Noi	*Echinostoma revolutum*	100.0	KF894682
	Fucocercaria	Ha Noi	*Australapatemon burti*	99.4	JX977785
T. granifera	Parapleurophocercaria 1	Ha Noi	*Haplorchis pumilio*	100.0	KX815125
B. fuchsiana	Echinostome	Ha Noi, Ninh Binh	*Echinochasmus japonicus*	93.0	KT873314

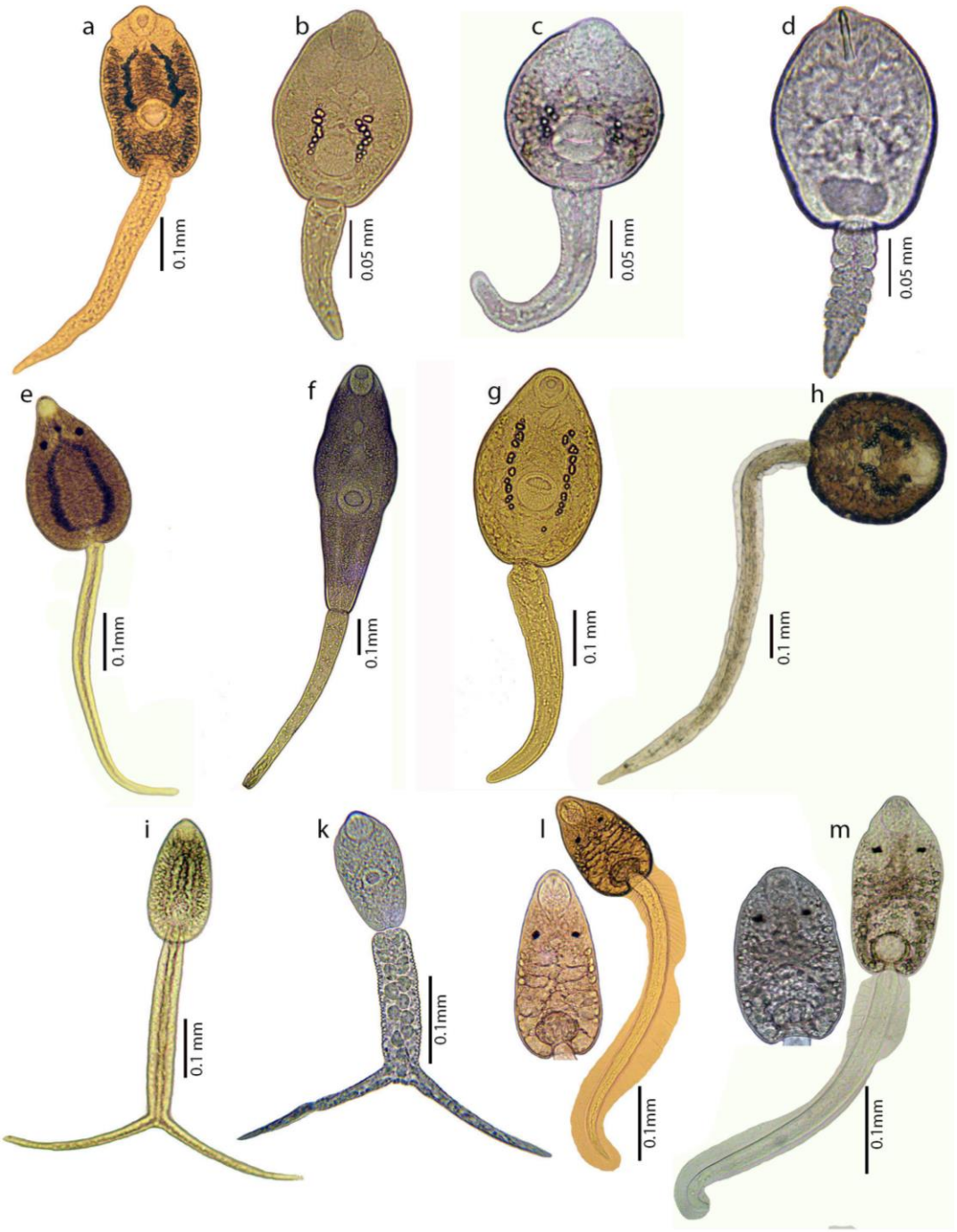

Figure 1. Cercarial groups of trematodes collected from snails

Note: a. Echinostome from snail *G. convexiusculus*; b. Echinostome from snail *P. striatulus*; c. Echinostome from snail *B. fuchsiana*; d. Xiphidiocercaria from snail *M. tuberculata*; e. Monostome from snail *M. tuberculata*; f. Megalurous from snail *M. tuberculata*; g. Gymnocephalous from snail *P. striatulus*; h. Gymnocephalous from snail *A. viridis*; i. Fucocercaria from snail *P. striatulus*; k. Fucocercaria from snail *G. convexiusculus*; l. Parapleurophocercaria 1 from snail *M. tuberculata*; m. Parapleurophocercaria 2 from snail *M. tuberculata.*

Based on morphological characteristics, we identified 7 cercarial groups (fig. 1; Echinostome, Monostome, Parapleurophocercaria, Xiphidiocercaria, Fucocercaria, Gymnocephalus and Megalurous). While snails *M. tuberculata* and *P. striatulus* were infected with 4 cercarial groups, snails *A. viridis* and *G. convexiusculus* were infected with two groups, and snails *B. fuchsiana* and *T. granifera* were infected with one cercarial group.

All cercarial groups were molecularly analyzed using ITS2 sequences as a marker. The results of the BLAST search identified the larvae of 9 trematode species with the similarities over 99% to the sequences deposited in GenBank. They are *Echinostoma revolutum* (100%), *Notocotylus intestinalis* (100%), *Philophthalmus gralli* (100%), *Haplorchis pumilio* (100%), *Procerovum cheni* (100%), *Fasciola gigantica* (99.9%), *Australapatemon burti* (99.4%), *Cyathocotyle prussica* (99.0%) and *Echinochasmus japonicus* (99.1%). In addition, Gymnocephalus group from *P. striatulus* snails was 97% similar to *Sphaeridiotrema pseudoglobulus*; Echinostome group from this snail species was 93% similar to *E. japonicus*, and Xiphidiocercaria group from *M. tuberculata* snail was 93% similar to *Lecithodendrium spathulatum*. The snail hosts, cercarial groups, trematode species identified by molecular analyses are shown in table 2.

DISCUSSION

According to previous reports, the prevalence of trematode larvae in freshwater snail species was relatively high. The (1993) reported the prevalence of *C. sinenis* cercariae in *M. tuberculata* in Nam Dinh Province up to 90%. Le et al. (1995) found trematode larvae in 30.6% of *L. swinhoei* and 26.3% of *L. viridis* snails in Ha Tay Province. The survey conducted by Kim and Vinh (1997) showed the prevalence of *Fasciola* cercariae in 62.1% of *Lymnaea* snails in Bac Ninh province. In contrast, recent surveys have revealed low prevalences of trematode larvae in freshwater snails. Dung et al. (2010) reported that 13.3% of *M. tuberculata* snails in Nghia Hung District, Nam Dinh Province was infected with trematode cercariae. A survey in Thai Binh, Nam Dinh, Ninh Binh, and Thanh Hoa Provinces revealed that the prevalence of trematode larvae in 13 snail species collected was generally low (Clausen et al. 2015). Hung et al. (2015) collected 7 snail species in Gia Vien District, Ninh Binh Province, and found a very low prevalence of cercariae in two snail species, *M. tuberculata* (7/858 = 0.8%) and *B. fuchsiana* (2/1,894 = 0.1%). Phuong et al. (2019) reported that the prevalence of cercarial infection in *M. tuberculata* snail was 14.3% in Ha Trung District, Thanh Hoa Province. Dung et al. (2019) surveyed in some suburban districts of Ha Noi, and found that among 9 snail species collected, 5 snails (*M. tuberculata*, *B. fuchsiana*, *A. viridis*, *G. convexiusculus*, *Stenothyra messageri*) were infected with cercarial larvae at low infection rates ranging from 0.3 to 2.6%. The results of the present study also indicated that the infection rates of trematode larvae in freshwater snails were relatively low (2.3% - 6.3%). These reflect a significant reduction of trematode infection in humans and animals in comparison to those in the past.

Previously published studies focused only on infection status of snails or identification of the cercarial groups. Le et al. (1995) described 3 cercarial groups (Echinostome, Fucorcercaria, and Xiphidiocercaria) from *L. swinhoei* and *L. viridis* snails in Ha Tay Province. Dung et al. (2010) identified 7 cercarial groups (Parapleurophocercous, Pleurophocercous, Echinostome, Xiphidiocercaria, Furcocercaria, Monostome, and Gymnocephalous) from snails in Nghia Hung, Nam Dinh Province. Clausen et al. (2012) mainly found Parapleurophocercous and Echinostome groups in Thai Binh, Nam Dinh, Ninh Binh, and Thanh Hoa Provinces. The survey by Hung et al. (2015) in Gia Vien District, Ninh Binh Province, showed that *B. fuchsiana* snails were infected with an Echinostome group, while *M. tuberculata* snails were infected with 4 groups: Echinostome, Xiphidiocercaria,

Parapleurophocercous, and Fucorcercaria. Phuong et al. (2019) illustrated 5 cercarial groups, namely Amphistome, Echinostome, Megaluralous, Monostome, and Parapleurophocercous cercariae, in *M. tuberculata* snails in Ha Trung District, Thanh Hoa Province. Similarly, Dung et al. (2019) detected 6 cercarial groups; Parapleurophocercaria, Echinostome, Xiphidiocercaria, Monostome, Gymnocephalous, and Fucorcercaria from snails in some suburban districts of Ha Noi. Besprozvannykh et al. (2013) identified trematode larvae in 3 snail species, *B. fuchsiana*, *P. striatulus*, and *M. tuberculata*, collected in Nam Dinh Province. They obtained the larvae of 12 trematode species belonging to 8 families, Cyathocotylidae, Pleurogenidae, Lecithodendriidae, Paramphistomidae, Heterophyidae, Notocotylidae, Psilostomidae, and Echinostomatidae. Among them, only 3 species (*E. japonicus*, *N. intestinalis* and *S. monorchis*) were identified to the species level using experimental infection and molecular analysis based on the 28S rDNA sequence.

In the present study, using ITS2 sequence analysis, we accurately identified the larvae of nine trematode species (*E. revolutum*, *E. japonicus*, *N. intestinalis*, *P. gralli*, *H. pumilio*, , *F. gigantica*, *A. burti*, *C. prussica*, and *P. cheni*). Among them, three species, *A. burti*, *C. prussica*, and *P. cheni*, were first detected in Vietnam. It should be noted that *M. tuberculata* snail has been reported as the first intermediate host of small liver fluke *Clonorchis sinensis* (rev. by Doanh and Nawa, 2016), of which cercariae belong to the Parapleurophocercaria group. In this study, Parapleurophocercaria group cercariae from *M. tuberculata* snail were identified as the larvae of small intestinal flukes, *H. pumilio* and *P. cheni*. Since cercariae of *C. sinensis* and small intestinal flukes, *H. pumilio* and *P. cheni*, all belong to the Parapleurophocercaria group, it is possible that mis-identification might occur in the previous publications, in which the classification of cercariae from freshwater snails was solely based on the morphological characteristics (Doanh and Nawa, 2016).

In this study, ITS-2 sequence of Gymnocephalous group cercariae from *P. striatulus* snail was 97% similar to that of *Sphaeridiotrema pseudoglobulus*. This cercarial group found in Nam Dinh Province was classified as *S. monorchis* through the experimental infection in ducks (Besprozvannykh et al. 2013). The present results showed that the ITS2 sequence of *S. pseudoglobulus* from *P. striatula* snail was 97% similar to that of *S. monorchis*.

The Echinostome groups from *B. fuchsiana* and *P. striatulus* snails were morphologically similar to each other, but the cercariae from *P. striatulus* was identified as *E. japonicus* with the similarity over 99%. On the contrary, the cercariae from *B. fuchsiana* snails showed a lower level of ITS-2 sequence similarity (93%) to *E. japonicus*. Thus, the Echinostoma groups from *B. fuchsiana* snails is possibly cercariae of another species, *E. beleocephalus*, which was reported in Vietnam (Le, 1995).

The Xiphidiocercaria group from *M. tuberculata* snail has the highest similarity (93%) with *Lecithodendrium spathulatum*. The trematodes of the genus *Lecithodendrium* are typical trematodes of bats. In Vietnam, three species of the genus *Lecithodendrium* (*L. daovantieni*, *L. rohdei*, and *Lecithodendrium* sp.; Le, 1995) were reported in bats. However, molecular data of these species are not available in GenBank database for comparison.

CONCLUSION

Nine snail species were collected in Ba Vi District, Ha Noi and 7 species were collected in Kim Son District, Ninh Binh Province. While three snail species, *R. swinhoei*, *A. polyzonata*, and *P. canaliculata*, were negative for cercariae, the other 6 snail species were infected with cercariae with low infection rates, ranging from 2.3% to 6.3%.

By morphology, 7 cercarial groups, Echinostome, Monostome, Parapleurophocercaria, Xiphidiocercaria, Fucocercaria, Gymnocephalous, and Megalurous, were identified. The snail hosts *M. tuberculata* and *P. striatulus* were infected with 4 cercarial groups, snails *A. viridis* and *G. convexiusculus* were infected with 2 groups, snails *B. fuchsiana* and *T. granifera* were infected with one group.

By molecular identification, cercariae of nine trematode species, *E. revolutum*, *E. japonicus*, *N. intestinalis*, *P. gralli*, *H. pumilio*, *F. gigantica*, *A. burti*, *C. prussica*, and *P. cheni*, were identified. Among them, 3 species, *P. cheni*, *A. burti* and *C. prussica*, were recorded for the first time in Vietnam. In addition, Gymnocephalous cercaria from *P. striatulus* snail, which was classified as *S. monorchis*, showed 97% similarity of ITS-2 sequence with that of *S. pseudoglobulus*. Likewise, Echinostome group from snail *B. fuchsiana* was 93% similar to *E. japonicus*, and Xiphidiocercaria group from *M. tuberculata* snail showed 93% similarity of ITS-2 sequence with that of *Lecithodendrium spathulatum*.

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