Molecular genetic identification of Opisthorchiidae cercariae from Bithyniidae snails of Chany Lake (South-Western Siberia, Russia)

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Short Report

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Abstract

The studies of the opisthorchiids larval stages associated with Bithyniidae snails can provide the important and the most reliable data for opisthorchiidoses foci characterization due to the least mobility of the snails among opisthorchiids host species. Since the foci of opisthorchiosis (caused by *Opisthorchis felineus*) and metorchiosis (caused by *Metorchis bilis*) are overlapping in the basins of the Ob and Irtysh rivers the obstacles in determining the species of cercariae significantly reduce the accuracy of epidemiological conclusions regarding opisthorchiosis, which has a much higher medical significance. Moreover, the difficulties with identification are complicated by the fact that the focus of metorchiosis caused by *Metorchis xanthosomus* infecting birds of prey occur in the same territory. In this study we for the first time carried out the molecular genetic identification of West-Siberian opisthorchiid cercariae to verify morphological identification and confirmed *O. felineus* main association with *Bithynia troschelii* snail and *M. bilis* – with *B. tentaculata* snail. Thus, our study applied an integrated approach combining first the morphological identification of opisthorchiid cercariae in Bithynia snails with subsequent molecular genetic identification of the cercarial samples.

Introduction

The opisthorchiosis cased by *Opisthorchis felineus* (Rivolta, 1884) is notorious as the most frequently reported trematode-induced infection in mid-latitude countries. This food-born disease affects biliary tracts of human, domestic and many fish-eating wild animals. Natural focus of opisthorchiosis caused by *O. felineus* is located in basins of the Ob and Irtysh rivers in West Siberia. Although the main cause of opisthorchiosis there is *O. felineus*, mixed infections with other opisthorchiid flukes – *Metorchis bilis* (Braun, 1790) (syn.: *M. albidus* Braun, 1893)) were reported (Fedorov et al. 2002; Yurlova et al. 2017; Kiyan et al. 2018). Thus, the epidemiological situation with West Siberian opisthorchiidoses foci needs a more precise description at all stages of Opisthorchiidae life cycle.

The opisthorchiids liver flukes have complex life cycles which include the six stages and involve a succession of three hosts (Vogel 1934). The first intermediate hosts, Bithyniidae snails, ingest opisthorchiid eggs excreted by the definitive hosts and washed into water body. The miracidiae leave the eggs in the snails and develop into sporocysts and then rediae. The cercariae (free-living larvae) develop in the rediae leave the snails and invade the second intermediate hosts – cyprinid fishes with transformation into the metacercariae. The definitive hosts become infected when eating the fish containing the metacercariae. Human infection occurs via raw or half-raw fish consumption. The metacercariae excyst in duodenum, reach the liver, and develop into adult worms. The majority of the second intermediate and definitive hosts are shared by *O. felineus* and *M. bilis*. The only difference in their life cycles is the employment of Bithynia snails species as the first intermediate hosts (Schuster 2010).

The *O. felineus* is assumed to parasitize in the snail *Bithynia troschelii* (Paasch, 1842) (member of *inflata/leachii/troschelii* species group) (Vogel 1934; Fedorov 1979) while the *M. bilis* - in *Bithynia*
The clinical features of *M. bilis* infection are assumed to be similar at onset of disease to those of *O. felineus* infection. But the risks for carcinogenesis of bile ducts due to chronic infection with *O. felineus* are now intensively studied (Fedorova et al. 2016). Since chronic infection by *M. bilis* is not reliably reported so its carcinogenic potential is not studied yet. Given the pronounced difference in epidemiological significance between the *O. felineus* and *M. bilis* it is very important to investigate the natural factors contributing to differential reproduction and propagation of the either flukes jointly with their prospective intermediate hosts.

Noteworthy the studies of the opisthorchiids larval stages associated with Bithyniidae snails can provide the most reliable data for opisthorchiidoses foci characterization due to the least mobility of snails among host species. Recent applications of molecular genetic techniques for identification of opisthorchiids cercariae allow to update epidemiological data on the opisthorchiidoses foci (Schwelm et al. 2020). This study is aimed to identify opisthorchiid species at larval stages in first intermediate hosts using a combination of morphological and molecular methods and thereby create a methodological basis for the data updating on opisthorchiidoses epidemiological situation.

**Materials And Methods**

The study was carried out at the Chany Field Station of the ISEA of SB RAS. The materials collected between 2012 и 2013 in the estuary of the Kargat River (N 54°37.76'; E 78°13.07'), and Malye Chany Lake (54°37'21 N 78°09'21 E) were analyzed. The two species *B. troschelii* and *B. tentaculata* were morphologically identified according Starobogatov's key (Starobogatov 1977).

Family assignment of pleurolophocercous cercariae emitted out of snail was done using the routine light microscopic methods (Sudarikov et al. 2002). The neutral red and Nile blue dyes were used for vital staining. Morphometric studies included measurement of 4 variables (length and width of body and tail) in *O. felineus* 15 cercariae and *M. bilis* 15 cercariae. The MANOVA was performed using the STATISTICA software.
For DNA extraction from opisthorchiid cercariae, the Biosilica kit (Novosibirsk, Russia) was used according manufacturer’s protocol. Eluted DNA was dissolved in TE and stored at -20°C. To identify opisthorchiid species, the standard PCR was applied using the primer pairs targeting ITS2 locus (Kiyan et al. 2018). The PCR-products were Sanger-sequenced using BigDye terminator cycle sequencing kit and ABI 3130XL Genetic Analyzer (Applied Biosystems, USA), according to the manufacturer’s specifications in SB RAS Genomics Core Facility. The ITS2 sequences were deposited in NCBI GenBank: MK517653 (O. felineus isolate) and MK517652 (M. bilis isolate). The two sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) with sequences available in GenBank for Opisthorchiidae ITS2 locus: Amphimerus sp. (AB678442); Euamphimerus pancreati (KT740984); Clonorchis sinensis (JQ048600, KJ137224), M. bilis (KT740978, KT740979, KT740980); M. orientalis (HM347227); M. xanthosomus (KT740983, KT740977); O. felineus (DQ513407, DQ513403); O. noverca (KC109193); O. viverrini (AF408147, KT894940); Pseudamphistomum truncatum (EU483072). Phylogenetic analyse was conducted using MEGAX64 (Kumar et al. 2018). The phylogram with 1000 boot-strep replications was performed by the maximum likelihood (ML) algorithm.

**Results And Discussion**

The 453 Bithyniidae snails were examined and the average infection rate by trematode larvae was estimated as 9.32% (variation range: 1.6% – 24.1%). Specifically of the 232 B. tentaculata only 35 (15.09%) were found to be infected with cercariae assigned to 7 Digenea families. Of the 237 B. troschelii – 23 (9.95%) displayed infestation by cercariae of 9 Digenea families. The opisthorchiid-like cercariae were found in B. tentaculata with infection rate 0.86% and in B. troschelii – 0.45%. These opisthorchiid-like pleurolophocercous cercariae are shown in Fig. 1.

As mentioned above, the three types of opisthorchiid cercariae can be found in West Siberian Bithyniidae, therefore, for completeness of the comparative analysis, the data by Heinemann (1937) for M. xanthosomus were added. The measurements of cercarial morphological features for the three opisthorchiids are presented in Table 1.

<table>
<thead>
<tr>
<th>Host</th>
<th>Bithynia troschelii</th>
<th>Bithynia tentaculata</th>
<th>Bithynia tentaculata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opisthorchiidae species</td>
<td>O. felineus</td>
<td>M. bilis</td>
<td>M. xanthosomus</td>
</tr>
<tr>
<td></td>
<td>n = 15 (original data)</td>
<td>n = 15 (original data)</td>
<td>n = 27 (Heinemann, 1937)</td>
</tr>
<tr>
<td>Body length</td>
<td>163.29 ± 21.27</td>
<td>217.27 ± 38.79</td>
<td>197.1 ± 13.6</td>
</tr>
<tr>
<td>Body width</td>
<td>74.16 ± 11.36</td>
<td>75.85 ± 6.2</td>
<td>66.5 ± 10.01</td>
</tr>
<tr>
<td>Tail length</td>
<td>392.76 ± 73.82</td>
<td>369.77 ± 54.11</td>
<td>405 ± 21.7</td>
</tr>
<tr>
<td>Tail width</td>
<td>32.95 ± 11.32</td>
<td>35.43 ± 10.48</td>
<td>30 ± 0</td>
</tr>
</tbody>
</table>
The cercarial morphological features showed no statistically significant differences between the three species of opisthorchiids (Fig. 2). This conclusion confirmed the opinion that the opisthorchiid cercariae are poorly distinguishable (Filimonova 1998; Serbina and Yurlova 2002). The need to use the molecular genetic methods for cercariae species identification is obvious.

The few cercariae samples from either B. troschelii or B. tentaculata were analyzed by PCR followed by sequence analysis. The BLAST analysis revealed that ITS2 sequence of cercaria sample from B. troschelii (MK517653) displayed 100% similarity with sequence DQ513403 (isolate OpNk1 of Opisthorchis felineus), while the cercaria sample from B. tentaculata (MK517652) – with sequence KT740979 (voucher 3LF-1870 of Metorchis bilis). The grouping of the sequences MK517652 and MK517653 on the phylogenetic tree is shown on Fig. 3.

In this paper, a comprehensive study of opisthorchiid cercariae was carried out using classical zoological and molecular methods. The need for such an integrated approach is determined by the fact that studies of the epidemiological situation in the West Siberian foci of opisthorchiidoses are hindered due to the complexity with the species identification of opisthorchiid cercariae emitted by bithyniid snails acting as the first intermediate hosts. Since the foci of opisthorchiosis and metorchiosis are overlapping in the basins of the Ob and Irtysh rivers the obstacles in determining the species of cercariae significantly reduce the accuracy of epidemiological conclusions regarding opisthorchiosis, which has a much higher medical significance. Moreover, the identification difficulties are increasing by the fact that in the same territory the focus of metorchiosis caused by M. xanthosomus infecting birds of prey and perhaps few other focus of less studied Opisthorchiidae flukes can occur.

Attempts to determine the opisthorchiid cercariae species based on the Bithynia snail species are also unreliable, as it has been shown that host specificity is not strict (Serbina and Yurlova 2002). Therefore, the identification of opisthorchiid cercaria species continues to be important for opisthorchiidoses foci studies. The conventional method used to examine trematode infections in snails is cercarial shedding observation with microscope. However the Opisthorchiidae pleurolophocercous cercariae display few morphologic features for species differentiation: O. felineus cercariae have 20 penetration glands, wider base of tail, and fin-folds on both sides of the tail, while M. bilis cercariae – 14 penetration glands, narrower base of tail, and M. xanthosomus cercariae – 14 penetration glands, but base of tail is somewhat wider then in M. bilis. Furthermore the application of this method of cercarial release has strict seasonal limitation in West Siberia: 30–45 days (since end of June till beginning of August) (Serbina 2012). These methodical difficulties reduce the ability to obtain reliable estimates of opisthorchiid infection rate in snails for practical purposes.

Thus, our study applied an integrated approach combining the morphological identification of opisthorchiid cercariae with subsequent molecular genetic identification of the cercariae species. Thus we for the first time confirmed that O. felineus develops in B. troschelii and M. bilis – in B. tentaculata in the water bodies of West Siberia.
Declarations

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Author contribution

AVK: conceptualization, methodology, molecular genetic analysis, writing-original draft. EAS: conceptualization, methodology, morphology and ecology analysis, writing-original draft. Both authors read and approved the final manuscript.

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Data availability The sequences generated in the study have been deposited in the GenBank database under the accession number MK517653 (O. felineus isolate) and MK517652 (M. bilis isolate).

Code availability Not applicable.

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Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

References


**Figures**

**Figure 1**

Photos of *O. felineus* cercariae ex *B. troschelii* (a) and *M. bilis* cercariae ex *B. tentaculata* (b).
Figure 2

Plot of MANOVA results for cercarial morphometry. Open rhombus - *O. felineus*, black squares - *M. bilis*, grey circles - *M. xanthosomus*. 
Figure 3

The ML phylogram of ITS2 sequences for the cercarial specimens analyzed together with corresponding opisthorchiid sequences from GenBank.