MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF ZOONOTIC TREMATODE METACERCARIAE (*Haplorchis taitui*) IN FRESHWATER FISH IN IRAN

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ABSTRACT

The intestinal trematode, *Haplorchis taichui* (Nishigori, 1924), is an important in public health that causes infection in humans and animals especially in Asia and in parts of Africa and the Americas**.** *Haplorchis taitui* metacercariae were found in the gills of *Cyprinion macrostomus* (Heckel) and *Capoeta barroisi persica* (Karaman) collected from the Shapour River. Morphological excysted metacercariae were identified as wet mounts under a stereomicroscope. Then, the samples were subjected to molecular analysis. The result showed that 69% of examined fish (n = 30) were diagnosed infected with encysted metacercariae in gills. The mean intensity was 8.3 ± 16.9 parasites per fish. The morphometrical values agree with the findings of other studies with the small differences and polymerase chain reaction product length and nucleotide sequence analysis of 18S ribosomal deoxyribonucleic acid gene showed a similarity of over 99% between the specimens and the *Haplorchis taichui* (Nishigori, 1924) recorded in GenBank.

Keywords: trematoda, Heterophyidae, Shapour River**,** PCR

IDENTIFICAÇÃO MORFOLÓGICA E MOLECULAR DE METACERCÁRIAS DE TREMATÓDEOS ZOONÓTICOS (*Haplorchis taichui*) EM PEIXES DE ÁGUA DOCE NO IRÃ

RESUMO

O trematódeo intestinal, *Haplorchis taichui* (Nishigori, 1924), é importante na saúde pública que causa infecção em humanos e animais, especialmente na Ásia e em partes da África e das Américas. Haplorchis taitui metacercariae foram encontradas nas brânquias de Cyprinion macrostomus (Heckel) e Capoeta barroisi persica (Karaman) coletadas no rio Shapour. Metacercárias foram identificadas microscopicamente sob um estereomicroscópio. Em seguida, as amostras foram submetidas à análise molecular. O resultado mostrou que 69% dos peixes examinados (n = 30) foram diagnosticados infectados com metacercárias encistadas em brânquias. A intensidade média foi de 8,3 ± 16,9 parasitas por peixe. Os valores morfométricos concordam com os achados de outros estudos com as pequenas diferenças e o comprimento do produto da reação em cadeia da polimerase e a análise da sequência nucleotídica do gene do ácido desoxirribonucléico ribossômico 18S mostraram uma similaridade de mais de 99% entre as amostras e o Haplorchis taichui (Nishigori, 1924) registrado no GenBank.

Palavras-chave: trematoda, Heterophyidae, Shapour River, PCR

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IDENTIFICACIÓN MORFOLÓGICA Y MOLECULAR DE METACERCARIAS DEL TREMATODO ZOONÓTICO (Haplorchis taitui) EN PECES DE AGUA DULCE EN IRÁN

RESUMEN

El trematodo intestinal, *Haplorchis taichui* (Nishigori, 1924), es importante en la salud pública que causa infección en humanos y animales, especialmente en Asia y en partes de África y América. Se encontraron *Haplorchis taitui* metacercariae en las branquias de Cyprinion macrostomus (Heckel) y Capoeta barroisi persica (Karaman) colectadas del río Shapour. Las metacercarias se identificaron bajo un microscopio estereoscópico. Luego, las muestras fueron sometidas a análisis molecular. El resultado mostró que el 69% de los peces examinados (n = 30) fueron diagnosticados infectados con metacercarias enquistadas en las branquias. La intensidad media fue de 8.3 ± 16.9 parásitos por pece. Los valores morfométricos concuerdan con los hallazgos de otros estudios con las pequeñas diferencias y la longitud del producto de reacción en cadena de la polimerasa y el análisis de la secuencia de nucleótidos del gen del ácido desoxirribonucleico ribosómico 18S mostró una similitud de más del 99% entre las muestras y el Haplorchis taichui (Nishigori, 1924) registrado en GenBank.

Palabras clave: trematoda, Heterophyidae, Río Shapour, PCR.

INTRODUCTION

Trematodes of the Family *Heterophyidae* are important both in terms of fish disease and as agents causing zoonotic infections in people who eat raw or uncooked fish, but they are potentially expanding through the transmission of fish or infected snails to non-endemic areas (1). The intestinal trematode, *Haplorchis taichui* (Nishigori 1924), is an important in public health that causes infection in humans and animals especially in Asia and in parts of Africa and the Americas (2,3,4). *Haplorchis taichui* is the most common species among the intestinal flukes in eastern Asia (5). Currently, this parasite has widely spread in Taiwan, the Philippines, Bangladesh, India, Sri Lanka, Palestine, Iraq, Egypt, Malaysia, Thailand, Laos, Vietnam, and South China (3,6,7,8,9,10).

Different molecular methods have been performed to identify *Haplorchis taichui* in intermediate and definitive hosts. Larvae and adult stages of *Haplorchis taichui* and *Haplorchis pumilio* (Looss 1896) were detected by using sequencing of ITS-2 gene technique (11). Wongsawad et al. (5) developed the specific primer of *Haplorchis taichui* by generating the 256 bp amplicon which had shown a positive result. In this paper, we present the development of molecular approach for *Haplorchis* metacercariae detection in natural infected *Cyprinion macrostomus* (Heckel) and *Capoeta barroisi persica* (Karaman) freshwater fishes using PCR (polymerase chain reaction) of 18S rRNA (Ribosomal ribonucleic acid) and nucleotide sequence analysis.

In this paper, we present the development of molecular approach for Haplorchis metacercariae detection in natural infected *Cyprinion macrostomus* and *Capoeta barroisi persica* freshwater fish using morphplogical identification and PCR of 18S rRNA and nucleotide sequence analysis.

MATERIALS AND METHODS

Sampling and Morphplogical Identification

A total of 30 fish were collected between September 2017 and March 2018 from the Shapour River (51° 31' 771" S and 30° 7' 542'' W), located in the Kazerun, Fars, Iran. The specimens were caught and transported alive to the laboratory, killed by decapitation and then examined for parasites under a stereomicroscope. The gills were removed, transferred to glass slides containing saline solution (NaCl 0.85%) and analyzed by means of optical microscopy to determine whether metacercariae were present and to count them. For the morphological study, subsample of excysted metacercariae (n = 30) were examined and images captured at magnifications of 40X and 100X oil immersion, using a digital microscope camera and the diagnostic variables were measured using the Axiovision software (Carl Zeiss Vision AxioVision LE Rel.) on the images captured. The drawings of the taxonomic features were made from the captured images. The results were compared with taxonomical keys (12) and morphological description metacercariae of the genus Haplorchis (13,14). Data analyses of the parasite and host were done using SPSS Package, version18. An analysis of variance (ANOVA) was performed in order to test for the relationship between the body weight and size of the studied fish and parasite intensity.

Molecular Identification

DNA (deoxyribonucleic acid) extraction of 20 samples performed by using High Pure PCR Template Preparation Kit (Roche, Diagnostics GmbH Sandhoferstrasse 116 DE-68305 Mannheim, Germany) according to instruction protocol of the company. PCR amplification was performed in a Biometra T3 thermocycler (Fisher Scientific, Hampton, New Hampshire, United States) using 60 µl reaction volumes. The reaction mixtures consisted of 20ng DNA template, 1 unit of BioTaq DNA polymerase (DNA-Technology, Varshavskoe shosse (high-way), 125Zh, Bld. 6, fl. 5, Moscow, 117587, Russia), 1 mM dNTP (deoxynucleotide triphosphate), 1.5 mM MgCl2 and 1 µM of the 2 primers. In order to amplify the 18S ribosomal DNA (rDNA) gene, the primers 5'-GCC AAG GAT GTT TTC ATT GAT CT-3'as a forward primer and 5'-GAA ACC GTC ATT GTA GCG CA-3'as a reverse primer were used (15). The PCR procedure consisted of a pre-denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 15 s and elongation at 72°C for 15 s, and finally post-elongation at 72°^{C} for 1 minute. The products were analyzed by 1.5% ethidium bromide-stained agarose gel electrophoresis. Identification of metacercariae was based on sequencing a part of the 18S rDNA gene. The amplified genome fragment was extracted from an agarose gel by illustra TM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, Illinois, United States), then nucleotide sequencing were performed at Macrogen Inc. (10F, 254 Beotkkot-ro, Geumcheon-gu, Seoul, South Korea).

RESULTS

In examination of 30 fish samples using morphological characteristics 44.4% were infected with *Haplorchis taitui* metacercariae encysted in the gills, which were found adjacent to the cartilage of the gill filaments. The mean intensity of encysted metacercariae (EMC) was 3.58± 23.99 (range 1–15) cysts per fish. A total number of 63 EMC were isolated. Result showed that nucleotide sequence was searched by Blast in the gene bank, which most identity

resembled more than 99% of the *Haplorchis taichui* (accession number: AY245705). Obtained morphological characteristics as follows (all parameters in µm).

the examined cysts were oval-shaped, measuring 250 (246 – 258) x 184 (181 –184) possessing an excretory vesicle containing dark excretory granules. Excysted metacercariae were elongate, measuring 278 (250– 318) x 96 (84 – 135) and completely covered with tegumental spines. A scattering of dark cells was apparent in the whole region of the worm, with the most prominent being the group that will develop into several organs. The oral sucker was located at the anterior end, 44 (35 – 46) long, 40 (30 – 42) wide. The pharynx was well developed, measuring 27 (24 – 28) x 26 (22 – 29). The oesophagus was short, anterior to the ventral sucker, the intestine bifurcated into two caeca extending towards the posterior end to reach the anterior level of the excretory vesicle. The ventral sucker was somewhat oval, measuring 32 (25 – 36) x 26 (21 – 34), and posterior to the middle of the body. Gonotyl was present nearby ventral sucker. Testis and ovary were somewhat oval in shape, in the posterior third of the body. The excretory vesicle was o shaped and larger than other internal organs with black pigments (Figure1).

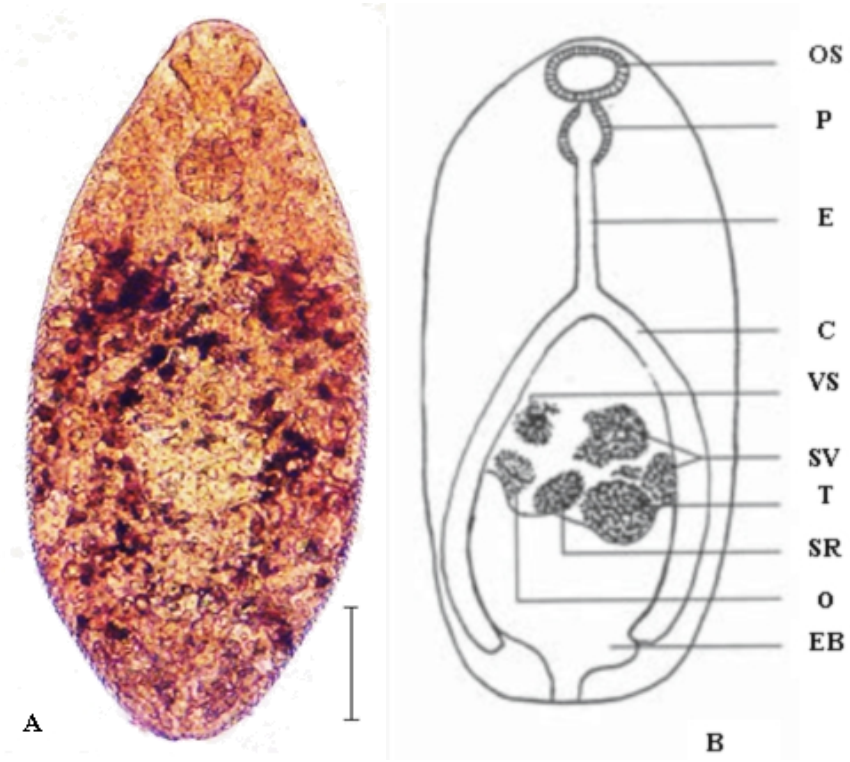

Figure 1. Newly excysted juvenile Haplorchis taichui. A: Light micrograph of a worm stained with carmine. B: Diagrammatic of internal organs of a worm showing the oral sucker (OS), ventrogenital sac (VS), pharynx (P), esophagus (E), cecum (C), testis (T), ovary (O), seminal receptacle (SR), seminal vesicles (SV) and excretory bladder (EB); 2000, scale bar 200 µm (24)

The amplified gene fragment of the 18S rRNA with a pair of universal primer has a length of 603 base pairs. The size of the DNA band obtained from PCR was equal to the expected size, which was obtained by analyzing the *H. pumilio* and *H. taichui* samples recorded in the GenBank (accession numbers 328751350 and 328751367, http://www.ncbi.nih.gov). The DNA band is very bright and strong, indicating a high amplification of the DNA of the sample. By comparing the density of the DNA band of the sample with the ladder, it is determined that the amount of DNA in the band of sample is

much higher than the value in the ladder. From the above, we can say that the PCR method used in this study was successful. The nucleotide sequence of the PCR product was completely and without a gap. This nucleotide sequence was searched by Blast in the gene bank, which most identity resembled more than 99% of the *H. taichui* (accession number: AY245705). The nucleotide alignment result shows that there are 4 nucleotide differences between the nucleotide sequences obtained in this study with the identical sequences (accession number: AY245705) registered in the gene bank which include the substitution T/C, T/A, A/C and T/C at bp 19, 26, 30 and 365 respectively. There was no nucleotide deletion. Also the Phylogenetic tree analysis by 18S rDNA was shown in Figure 2.

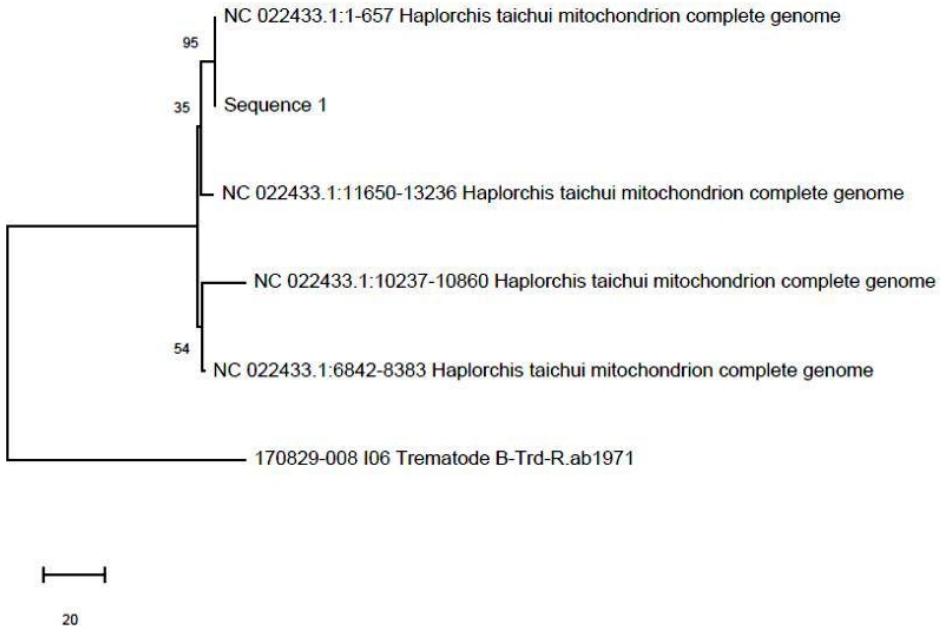

Figure 2. Phylogenetic tree analysis by 18S rDNA (ribosomal deoxyribonucleic acid) of sample recorded in the gene bank (accession number: AY245705).

DISCUSSION

The present examination illustrated, surprisingly, the identification of *Haplorchis taitui* in freshwater fish from Southwest of Iran, Shapour River. In our investigation, we identified metacercariae by using morphological and molecular techniques at the genus level. Due to the identification key, the metacercariae of genus Haplorchis was diagnosed. There were some detailed description of the morphological characteristics of the *Haplorchis taitui* metacercariae (16,17,18).

The morphometrical values found by these authors confirmed each other. In this study, the morphometrical values agree with the findings of mentioned studies but they are in line with the findings of Sukontason et al. (19) with the small differences. However, the use of morphological characteristics to detect specific parasites, and especially other developmental stages of the parasite, such as cercaria and egg, is difficult and obscure. (19,20,21,22,23,24). Therefore, we started using molecular approaches based on the PCR, because these techniques are more precise for the detection of parasites than morphological diagnostic techniques. So far, no study has been done on the molecular analysis of heterophid trematoda substances in Iran. The analysis of 18S and ITS nucleus genes is one of the most commonly used targets for genetic diagnostic tests for parasites. Among the nuclear genome, the ITS rRNA region has been used by various researchers to distinguish specific species of helminthes (20,22,23).

However, the observation of the genetic diversity in the ITS rRNA region in some of the echinostomatids and *Paragonimus westermani* has caused the use of this genetic element to be suspected in the diagnosis of parasitic species (11,25). Conserved regions of 18S ribosomal rRNA that contains polymorphic regions is broadly used to design general primers for the detection of new species of parasites (15,26,27). In the present study PCR of 18S ribosomal rRNA and DNA sequencing techniques have been successfully developed for identification of *Haplorchis taitui* taken from fresh water fish. Selected primers were universal primers capable of amplified the DNA of trematoda in a wide range of helminthes. Amplified 18S rDNA genes contain a variable nucleotide sequences that enables specific species of parasites to be identified by using species specific primers or PCR-RFLP techniques. The technique used in this study, in addition to fish, the parasite can be detected in the first intermediate hosts (snails) and the final hosts as well as in the soil (15). The impact of infections on snails, fish and birds could be significant, and humans ingesting raw or undercooked infected fish may be at risk. It is important to emphasize that *Melanoides tuberculata* naturally infected by the parasite (14) which recently reported from Shapour River (28), so it has become an environment suitable for maintenance of the life cycle of heterophid trematoes such as *Haplorchis taitui*. Generally, prevention and transmission control of these parasites is difficult and requires good management and efficient approaches.

Control strategies should include training of traders and farmers in order to monitor the health condition of imported fish. However, the main concern of veterinarians and traders should be biosecurity measures, e.g. border inspection and fish quarantine measures. Infected fish should be discarded (euthanized) and subsequent disinfecting of equipment in quarantine facilities must be performed. Appropriate legislation regarding requirement of health certificate showing the health status of imported batches of fish is also needed (2,29).

CONCLUSION

The important issue of *Haplorchis taitui* is the ability to cause a disease in humans and the importance of public health for this parasite, which is worth the recognition of it. Therefore, in order to detect different aspects of this parasite in Iran, extensive studies are needed. Among the most important issues that require extensive research are the species specific detection, the identification of the intermediate and definitive hosts of the parasite, life cycle, the abundances and geographical distribution of the parasite, the pathogenicity, the control and prevention of the parasite. Fortunately, in Iran, due to not eating raw or half cooked fish, there are still no reports of human infection, however, since one of the most important ways of parasite is through the import of contaminated fish, Therefore, the present report underlines the need to respect control and quarantine procedures with regard to imported ornamental fishes in Iran.

ACKNOWLEDGEMENTS

This study has been financially supported by research deputy of Islamic Azad University, Kazerun branch. The authors would like to thank for the financial support which made it possible to attend the study.

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Recebido em: 20/02/2020
Aceito em: 28/02/2021