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Original article

MOLECULAR CHARACTERISATION AND HISTOPATHOLOGICAL STUDY OF *EUSTRONGYLIDES EXCISUS* NEMATODE IN THE NORTHERN PIKE (*ESOX LUCIUS*)

M. R. YOUSSEFI[1], R. TABARIPOUR[2] & M. HOSSEINI[3]

[1]Department of Veterinary Parasitology, Babol Branch, Islamic Azad University, Babol, Iran; [2]Department of Cellular and Molecular Biology, Babol Branch, Islamic Azad University, Babol, Iran; [3]Department of Pathology, Babol Branch, Islamic Azad University, Babol, Iran

Summary

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Eustrongylides excisus is a parasitic nematode species whose life cycle requires two intermediate hosts, an aquatic oligochaete and a benthophagous fish. In the present study, 100 specimens of *Esox lucius* Linnaeus weighing 550–800 g were collected from Freidoonkenar, south of the Caspian Sea (Mazandaran province, Iran) and were examined for the presence of *Eustrongylides*. The bright red nematode larvae were found in the testes and encapsulated in the body musculature of 90% of *E. lucius* fish. The larvae were diagnosed as *Eustrongylides excisus*. Tissue samples were collected from the muscles and testes for histopathological examination of the lesions caused by the parasitic larvae. For molecular analysis, the nematode larvae genomic DNA was extracted and molecular characterisation of *Eustrongylides* and comparison with the corresponding sequences available in the GenBank was done. The histopathological damages caused by parasites in the muscle included external nodules, inflammation, necrosis, and granulomas. Granulomas containing multi-nucleated giant cells, epithelioid cells, lymphoid cells, macrophages and necrotic debris were observed. Microscopic examination of the testes revealed mild vacuolar degeneration in some Sertoli cells. Molecular analysis confirmed obtained larvae as *E. excisus*. Comparison of DNA sequences showed that isolated nematodes were very similar to those obtained from freshwater fish in China. The present study reported *Eustrongylides* nematodes in *Esox lucius*, and inflammatory lesions caused by *E. excisus* larvae in the muscle and testis of this species of fish for the first time. In addition, molecular characterization and phylogenic analysis of recovered larvae showed presence of microvarients.

Key words: Caspian Sea, *Esox lucius*, *Eustrongylides excisus*, pathologic lesions

INTRODUCTION

Eustrongylids are parasitic nematodes commonly long, coiled, and red in colour due to the presence of haemoglobin. Infected fish generally have several nematodes mostly in the body cavity. The nematodes can migrate out of the infected

fish into the muscles or other organs of the second fish host. After migrating into the muscle, the nematode can cause superficial injuries that look similar to a grub (Xiong *et al*., 2013). Observations on *Eustrongylides* in fish from northern Europe demonstrated large sized larvae measuring 90 mm in length which are often found in salmonids, and small larvae (length less than 40 mm) which are present mainly in percid fishes. These findings suggest that there might be an additional species present in the European region (Sosa-Medina *et al*., 2015). *Eustrongylides* sp. have complex, indirect life cycles involving a definitive host and two intermediate hosts. Definitive hosts include aquatic birds mostly from the orders Ciconiiformes, family *Ardeidae*, Anseriformes, Gaviiformes and Pelecaniformes. The eggs of all *Eustrongylides* sp. are very tough and can easily survive for a long time in fish ponds. The time required for the eggs to hatch and molt the L3 stage, which is infective to fish, is approximately 3–4 months (Ibiwoye *et al*., 2005). *Eustrongylides* sp. can be found in the muscle, "free" within the body cavity, or encapsulated on the liver and other organs of fish. Eustrongylid nematodes can affect a number of different species, including yellow perch (*Perca flavescens*), pumpkinseed (*Lepomis gibbosus*), and mummichug (*Fundulus heteroclitus*) (Spalding *et al*., 1993). Affected fish are mostly characterised by dropsy or bloated abdomens, as the nematodes often migrate into the body cavity from the gut and are quite large. In human consumers of raw or undercooked fish with *Eustrongylides* sp., gastritis and intestinal perforation have been reported (Ibiwoye *et al*., 2005).

Herein, the authors described pathological changes caused by *Eustrongylides*, especially in testes and muscles of fish. The molecular characterisation of *Eustrongylides* is also presented and compared to corresponding sequences of *Eustrongylides* sp. available in the GenBank.

MATERIALS AND METHODS

Animals

During winter 2015, 76 male and 24 female *Esox lucius* Linnaeus weighing 550–800 g were collected from Freidoonkenar (Mazandaran province, Iran) south of the Caspian Sea and transferred to the Diagnostic Necropsy Laboratories of Veterinary Medicine, Islamic Azad University, Babol Branch.

Parasites collection and identification

During the necropsy, gross lesions were recorded and parasites of the muscles and testes immediately transferred to 70% ethanol solution. Parasites were clarified using lactophenol and mounted on glass slides. The nematodes were identified as larval stage of *Eustrongylides excisus* using guidelines reported by Moravec (1994).

Tissue sample preparation

Tissue samples collected from the abdominal muscles and testes were fixed in 10% neutral buffered formalin. Cross-sections of diffent parts of the nematodes were made and stained with haematoxylin and eosin (H&E). The protocol of the study was in accordance with animal welfare law and accepted by the ethical committee of Babol branch, Islamic Azad University (No. 95314); consistent with the Principles of Laboratory Animals Care (NIH Publication no. 85–23, revised 1996).

Molecular characterisation

For molecular analysis, genomic DNA was extracted using a DNA purification kit (Roche, Mannheim, Germany) accord-

ing to the manufacturer's instructions and stored at −20 °C until further use. The sequence of rDNA ITS (18s ribosomal RNA gene, partial sequence, Internal transcribed spacer1, 5.8 ribosomal RNA gene and Internal transcribed spacer2, complete sequence, and 28s ribosomal RNA gene, partial sequence) was amplified using forward (TTGGATGATTCGGTGAG) and reverse (AACCGCTTAGTAATAT GCT) primers. PCR reaction was performed in a total volume of 50 µL containing approximately 250 µM dNTPs, 2 nM $MgCl_2$, 200 nM of each forward and reverse primers and 1.5 unit taq DNA polymerase. The amplification programme in thermocycle included initial denaturation at 94 °C for 5 minutes followed by 35 cycles of 94 °C for one minute, 58 °C for 45 seconds, 72 °C for 90 seconds and then a final extension phase at 72 °C for 10 minutes. The PCR amplification products were detected after electrophoresis on 1.5% agarose LE gel under UV transilluminator and then PCR amplicons were analysed by direct sequencing (ABI-genetic Analyzer 3730, macrogen Big-Dye). The sequence of PCR products was compared to those registered in Genbank using the Basic Local Alignment Search Tool (BLAST). The nucleotide sequence was aligned with other *Eustrongylides* sequences using the ClustalW method, Mega5 programme. Phylogenetic analysis was performed using the maximum likelihood statistical method (Mega5). Pairwise distances were corrected by the Kimura two-parameter model. For visualisation, *Pellioditis marina* (AJ867071) was defined as an out group to root the tree.

RESULTS

Our study revealed presence of larval stage of *Eustrongylides* nematodes in the muscular tissue and testes of 90% of examined *Linnaeus* fish. The number of larvae per fish ranged from 4 to 21 in every individual *Esox lucius*. Body length of larvae ranged from 21 to 30 mm, while the maximum width was 0.1 mm; buccal cavity was 0.09 mm, and the oesophagus measured 2.5–3 mm.

Results of histopathological evaluation of muscles in presence of *E. excisus* are presented on Fig. 1 and 2. Encapsulated cystic parasitic granulomas were present in the muscle tissues. In addition, there were multi-nucleated giant cells, epithelioid cells, and mononuclear inflammatory cells (lymphocyte and macrophage) around granulomas and between the muscle fibres. Hyalinisation and muscle fibre necrosis with infiltration of inflammatory cells into the necrotic area were also observed in the samples.

In the testicular tissue of *E. lucius* fish, different stages of spermatogenesis could be seen (Fig. 3). *E. excisus* caused pressure atrophy and mild necrosis in the testes' tissue. Histopathological changes including disorganisation and distortion of the seminiferous tubules were observed. Degenerative and destructive signs were also detected both in spermatogenic lineage and in Sertoli cells. Exfoliated germ cells and multinucleated giant cells accumulated in the lumen of the tubules caused by *E. excises* were observed in samples (Fig. 3).

For the molecular study, genomic DNA was extracted, and the rDNA ITS gene was amplified. The PCR product showed an expected fragment of nearly 1100 bp in length (Fig. 4).

BLAST analysis of PCR product sequences confirmed the presence of *Eustrongulides* parasitic nematodes. Multiple alignment and comparison of rDNA ITS sequence of isolated samples with other

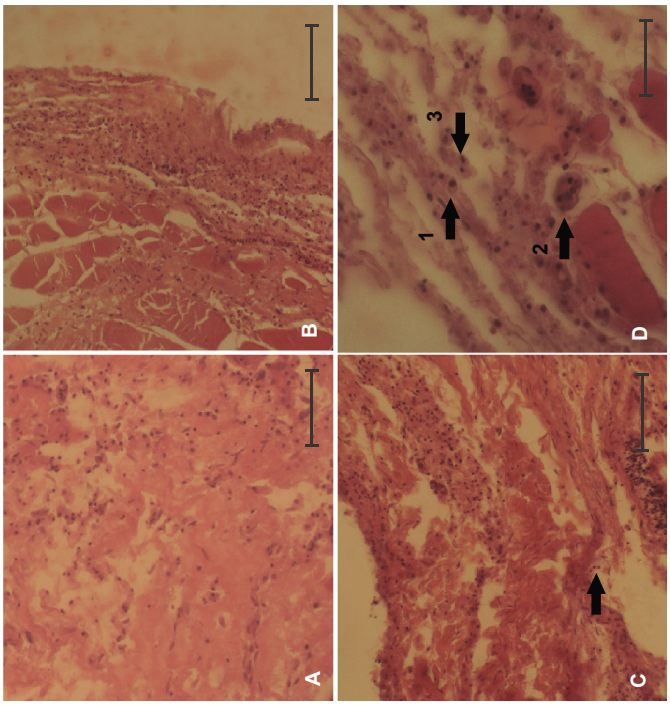

Fig. 2. Muscle tissue. **A.** Normal muscle; **B.** Hyalinisation and necrosis in muscle; **C.** Infiltration of lymphocytes (arrow); **D.** Infiltration of macrophages (1), giant cells (2) and epithelioid cells (3). H&E, bar=50 µm.

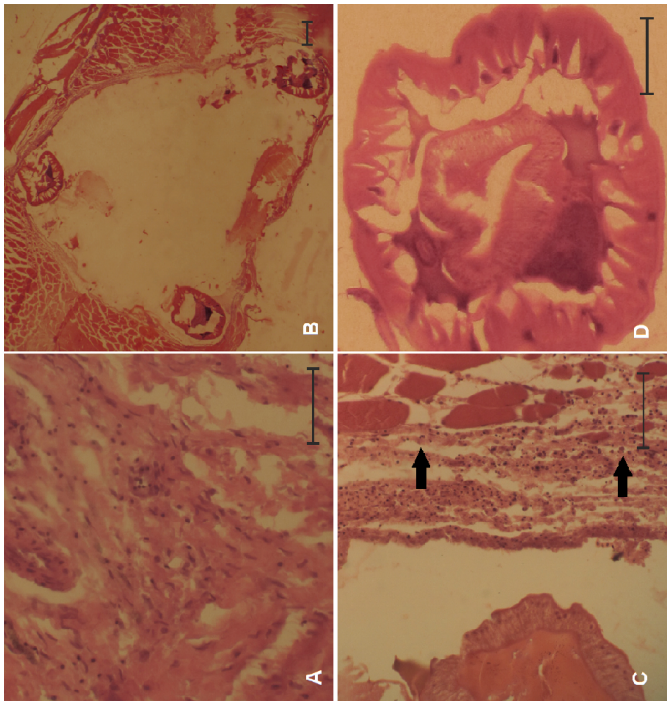

Fig. 1. Muscle tissue. **A.** Normal muscle; **B.** *E. excisus* encapsulated in the muscles; **C.** Inflammatory cells infiltration (arrows); **D.** Cross-section of *E. excisus* coelomyanian musculature, intestine, spicule in sheath and nerve cord. H&E, bar=50 µm.

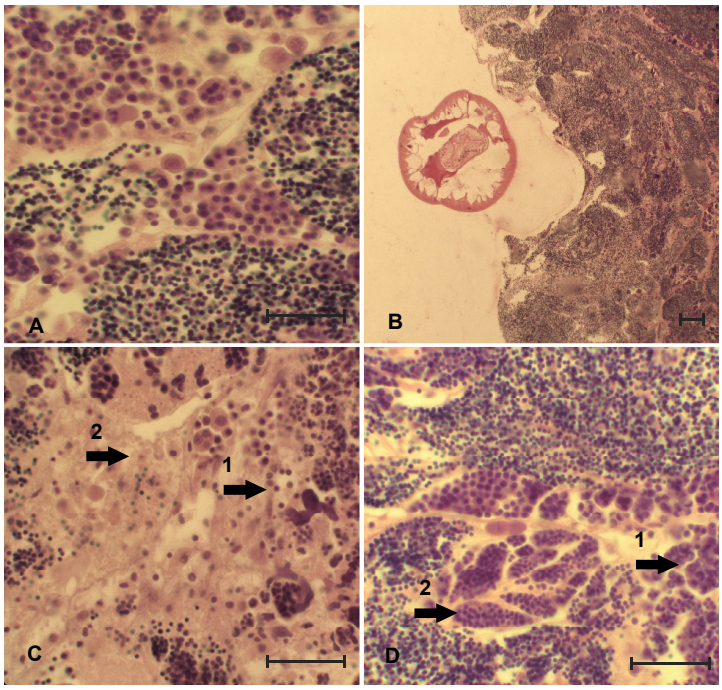

Fig. 3. Testis tissue. **A.** Normal testis; **B.** Cross-section of *E. excisus* in the testicle; **C.** vacuolar degeneration (1), necrosis (2); **D.** Cluster shape of primary spermatogonia in the lumen (1), cluster shape of secondary spermatocyte (2). H&E, bar=50 µm.

Eustrongulides sequences registered in Genbank showed high identity indicating variations among these sequences.

The phylogenetic relationship between the isolated *Eustrongylides* sequence that was registered in Genbank under accession number KU963206 for rDNA ITS and the other most identical sequences are shown on Fig. 5.

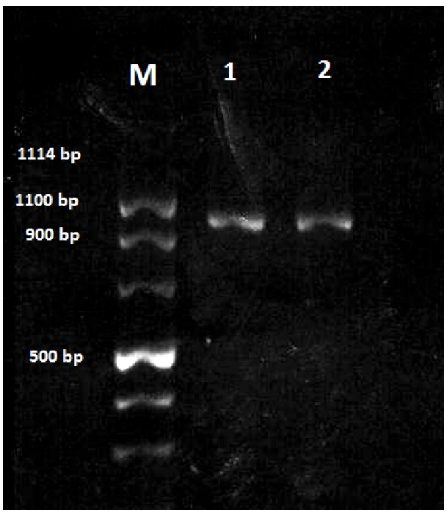

Fig. 4. Result of PCR reaction. The fragment size was confirmed by comparing to molecular weight marker (lane M). Lanes 1 and 2: PCR products.

DISCUSSION

In aquaculture systems, *E. excisus* larvae are highly pathogenic for fish and cause illness or even death (Bjelić-Čabrilo *et al.*, 2013). *Eustrongylides* species can be found in the body cavity, muscle, testes, or on the external surface of internal organs such as the liver and intestinal tract

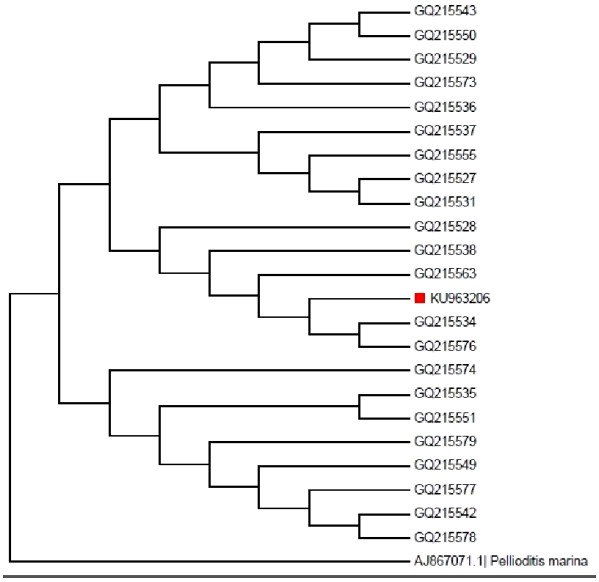

Fig. 5. Phylogenetic relation between isolated rDNA ITS *Eustrongylides* sequence and various sequences of *Eustrongulides* with high identity registered in Genbank. *Pellioditis marina* (AJ867071) is used as the out group.

of fish, and in highly infected fish may result in bloating of the hosts abdomen (Spalding *et al*., 1993). In the present study, *E. excisus* nematodes were found in muscle and testes of *Esox lucius*. Detection and identification of *E. excisus* in *E. lucius* fish in the present study is important due to histopathological lesions caused notably in the testes which may lead to decrease in fertility or even sterility, and imposes economic losses. Eustrongylid nematodes can affect various species; some aquatic birds (*Pelecanus* sp., *Phalacrocorax carbo*, *P. pigmeus*) are definitive hosts, and amphibians (*Rana ridibunda*), reptiles (*Natrix tessellate*), oligochaetes (*Lumbricus variegatus, Tubifex tubifex*, *Limnodrilus* sp.) and various fish are known to be paratenic hosts of this parasite (Spalding *et al*., 1993). *E. excisus* has been reported in various freshwater fish in Iran (Pazooki & Masoumian, 2012), Japan (Abe, 2011), Papua New Guinea (Owen, 2005), Serbia (Bjelić-Čabrilo *et al*., 2013), Bangladesh (Chandra, 2006), Slovakia (Novakov *et al.*, 2013) and Turkey (Rolbiecki, 2006). In Iran, the parasite was reported in *Acipenseridae*, *Cyprinidae*, *Percidae*, *Esocidae* and, some *Neogobiidae* fishes, and in *Rutilus frisiikutum* (Sattari *et al.*, 2008). In the present study, *E. excisus* was found in *Esox lucius* Linnaeus obtained from Freidoonkenar, south of the Caspian Sea (Mazandaran province, Iran). To our knowledge, our study is the first report of the presence of *Eustrongylides* in *E. lucius* fish.

It has been reported that *E. excius* larvae bring about heavy damage to tissues in acipenserids. In young fishes, in the sites of the localisation of larvae, large scars appeared, inflammatory lesions were sometimes found, and complete destruction of the kidney often occurred (Bjelić-Čabrilo *et al*., 2013). In the present study,

even though no lesions in the kidney were found out, *E. excius* caused external nodules, inflammation, and granulomatous necrosis in the muscle. In addition, spermatozoa clumping, necrosis, and vacuolar degeneration in some Sertoli cells were observed in the testis tissue.

Up to now, limited sequence data have been available on the genetic information of the fish parasitic *Eustrongylides* nematodes. The present study is the first sequence-based comprehensive molecular study and strain characterisation of *Eustrongylides* nematode in Iran. Comparison of sequences showed that Iranian isolated nematodes were very similar to those from freshwater fish in China (Xiong *et al*., 2013), suggesting that the origin of this parasites may be the same. Few variations between the sequence isolated from Iran and other sequences showed a probability of microvariants in *Eustrongylides* nematodes similarly to other parasites.

Overall, the present study reported *Eustrongylides* nematodes in *Esox lucius* for the first time. In addition, inflammatory lesions caused by *E. excius* larvae in the muscle and testis of this fish species have been demonstrated. Molecular characterisation and phylogenic analysis of *E. excius* was performed, showing presence of microvariants in the recovered larvae.

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Correspondence:

Mohammad Reza Youssefi
Department of Veterinary Parasitology,
Babol Branch, Islamic Azad University,
Babol, Iran
phone No: +9811324115159
e-mail: youssefi929@hotmail.com,
ORCID: 0000-0002-8606-2266