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## Occurrence of larval anisakid in imported mackerel (*Scomber scombrus*) in Astana fish markets

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**Abstract**

Background and Aim: This study aimed to investigate the presence of *Anisakis* larvae in mackerel fish sold in Astana markets, focusing on specimens imported from Norway and Iceland. The high prevalence of *A. simplex* (s.s.) larvae in the examined mackerel indicates the significance of this fish as a potential source of human anisakiasis.

Materials and Methods. Morphological analyses were deemed insufficient for accurate identification of Anisakis larval species, highlighting the necessity for more advanced molecular tools. Through partial sequencing of the 5.8S gene, the isolated Anisakis larvae from Norwegian Sea mackerel and Atlantic mackerel were classified as *A. simplex* (s.s.), demonstrating the efficacy of molecular methods in distinguishing closely related species.

Results. A high prevalence of *A. simplex* (s.s.) larvae was observed in 47 out of 50 examined mackerel, indicating the significance of this fish as a potential source of human anisakiasis.

Conclusion. These findings significantly contribute to understanding *Anisakis* larvae in fish within Kazakhstan and are pertinent to public health. The study underscores the importance of molecular-genomic analysis in this field and emphasizes the potential risks associated with inadequate expertise. Importantly, the study calls for further research on the genetic variability and infection rates of these parasites to monitor the status of *A. simplex* in Kazakhstan.

**Keywords:** Anisakis larvae; fish market; mackerel; human anisakiasis; molecular method.

### Introduction

In Kazakhstan, where the annual fish consumption per capita is approximately 3.4 kg, the import of 43.000 tons of frozen fish in 2023-primarily salmon, mackerel, and herring from Norway, Iceland, and Russia-highlights the importance of understanding the risks associated with consuming raw fish. Anisakiasis, a parasitic infection caused by Anisakidae nematodes, can be contracted by humans through the consumption of raw or undercooked fish [1].

The *Anisakida*e family comprises 12 genera, seven of which are parasitic, including *Anisakis spp., Contracaecum spp., Phocascaris spp., Pseudoterranova spp., Pulchrascaris spp., Terranova spp.,* and *Sulcascaris spp.* [2, 3]. These parasites are distributed globally, predominantly in the Atlantic and

Pacific oceans and coastal waters, with cold and temperate climates, closely following the distribution of their marine hosts [4, 5].

The life cycle of *Anisakidae* nematodes involves four larval stages (L1–L4), with marine mammals such as whales, dolphins, and seals serving as the definitive hosts. Female nematodes can release up to 1.5 million eggs into the intestines of their hosts. In the L1 stage, eggs are expelled into the environment through the host's excrement. In the sea, they develop into the L2 stage and are ingested by small crustaceans and copepods. Inside these intermediate hosts, the larvae molt into the L3 stage. Copepods are consumed by marine fish, which function as paratenic hosts. The larvae embed themselves in the internal tissues of fish, particularly the liver and peritoneum, causing inflammation. When a marine mammal eats an infected fish, its life cycle is completed, and the larvae mature into adult L4 forms [2, 6].

Humans can become infected by consuming raw fish containing L3 larvae, leading to anisakiasis. Symptoms can range from gastric to intestinal, and allergic reactions are possible because of allergenic proteins produced by the nematodes [2, 5]. Mild infections can be managed with symptomatic treatment, whereas severe infections might require surgical intervention to remove the larvae. Primary treatments for anisakiasis include endoscopic and surgical removal, with some success reported for albendazole treatment [5, 7-10].

The aim of this work is to raise awareness about the risks associated with raw or undercooked fish consumption, especially in regions with significant import and consumption of frozen fish. The focus is on understanding and highlighting the parasitic infection caused by *Anisakidae* nematodes and identifying the species of the anisakid parasite. Through this work, our goal is to promote safer consumption practices and minimize the incidence of anisakiasis.

**Materials and Methods**

*Fish sample collection and processing*

To perform the research, 50 samples of imported mackerel from Norway and Iceland, destined for the fish markets of Astana, were selected. Parasitological examinations were conducted at the Laboratory of Parasitology, Faculty of Veterinary Medicine, S.Seifullin Kazakh Agrotechnical Research University. Molecular and genetic research was performed at the Laboratory of Biodiversity and Genetic Resources within the National Center for Biotechnology. Quantitative assessment of larval occurrence in fish was conducted by calculating the following metrics: prevalence = number of infected fish/numbers of examined fish, mean intensity (MI) = total number of isolated larvae per fish species/number of infected fishes of that species, and mean abundance = total number of isolated larvae per fish species/number of examined fish of that species [11]. All collected larvae were processed for microscopic examination to differentiate between type I (anisakid larvae) and type II larvae.

*Morphological examination of the isolated larvae using a light microscope*

The larvae isolated from each fish species were washed in saline solution for 30 min, incubated in lactic acid for 15 min, and examined using ordinary light microscopy (Fig. 1).

*DNA extraction*

For molecular analysis, five samples of contaminated mackerel imported from Norway and Iceland were selected. A small piece of each specimen was cut and homogenized, and the homogenate was subjected to the standard phenol–chloroform method [12], supplemented with proteinase K, to extract genomic DNA (gDNA). DNA was precipitated with ethanol, purified, dissolved in dd $H_2O$, and stored at −70 °C for subsequent analysis.

*Polymerase chain reaction analysis*

Polymerase chain reaction (PCR) was performed using the 5.8S primer pair (NC13 Forward: ATCGATGAAGAACGCAGC and NC2 Reverse: TTAGTTTCTTTTCCTCCGCT) to amplify worm gDNA [13]. PCR was conducted in a 25 µL reaction mixture containing 10× Taq buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase, 200 µM dNTPs (Thermo Scientific, Carlsbad, California, USA), 10 pmol of each primer, and 20 ng of extracted gDNA as the template. DNA segments were amplified by thermal cycling for 30 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s). The resulting amplification products were separated by electrophoresis on a 1.5% agarose gel prepared with 1× TAE buffer and stained with 8 ng/µL ethidium bromide.

*Sequencing*

The PCR-amplified target gene fragment was purified using a QIAquick PCR Purification Kit (QIAGEN, Germany, Cat. No. 28106) following the manufacturer's instructions. Sequencing was conducted using a Seq Studio Genetic Analyzer (Thermo Fisher Scientific Applied Biosystems) according to the manufacturer's instructions. The resulting nucleotide sequences were visually inspected using BioEdit software version 7.0. These sequences were then compared with other sequences in the NCBI GenBank database using BLAST. The nucleotide sequences of the studied species were deposited in the NCBI GenBank database.

*Phylogenetic analysis*

The obtained sequences were manually edited, and sequence similarity searches were performed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov) to compare them with GenBank reference sequences. Nucleotide sequences were aligned using the MUSCLE multiple sequence alignment program for the partial cox1 gene. A maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model with MEGA v11 software [14], with *Pseudoterranova decipiens* (AB201790) serving as the outgroup.

For detailed analyses, a pairwise distance method was used with the maximum composite likelihood model [15]. This analysis involved six nucleotide sequences with codon positions including first, second, third, and noncoding regions. For each sequence pair, all ambiguous positions were removed using the pairwise deletion option. The final dataset included 458 positions.

**Results**

This study analyzed 50 mackerel samples and presented findings related to anisakid larvae. Table 1 presents the prevalence, MI, and mean abundance of the larvae.

Table 1- Prevalence (P), mean intensity (MI), and mean abundance (MA) of isolated anisakid larvae in the examined fish

| Fish host | No. examined | No. infected | Larvae found | P, % | MI | MA |
|---|---|---|---|---|---|---|
| Norway mackerel (*Scomber scombrus*) | 45 | 42 | 899 | 93.33 | 21.40 | 19.97 |
| Iceland mackerel (*Scomber scombrus*) | 5 | 5 | 70 | 100 | 14 | 14 |

The overall prevalence of anisakid larvae was >93.33% in Norwegian mackerel and 100% in Icelandic mackerel. The MI of Norwegian mackerel was 21.40, whereas that of Icelandic mackerel was 14. The MA of Norwegian and Icelandic mackerel was 19.97 and 14, respectively.

Most anisakid larvae were encapsulated in fish muscle and visceral organs, including the peritoneum (Fig. 1A and B). Using an ordinary light microscope, all isolated larvae displayed features characteristic of Anisakis larval type 1, particularly the long ventriculus and mucron at the posterior end (Fig. 1).

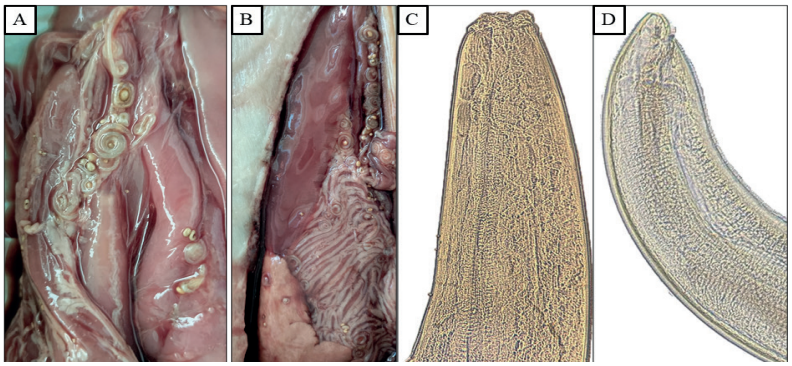


Figure 1 - Anisakis helminths observed inside mackerel. Morphology of the isolated larvae

A and B: Observed *A. simplex* helminths in the visceral organs; C: anterior end of *A. simplex*; and D: posterior end of *A. simplex*

Because the larvae within six to seven pools of each fish species exhibited nearly identical sequences of the 5.8S gene, we used three gene sequences from a single larval pool of each fish host for this analysis. Phylogenetic analysis (Fig. 2) revealed that all studied larvae belonged to anisakid larvae. Larvae isolated from Norwegian Sea mackerel (PQ047832, PQ047833, and PQ047834) and Atlantic Ocean mackerel imported from Iceland (PQ047828, PQ047829, and PQ047830) clustered closely together and were classified as A. simplex (s.s.) because they grouped near other A. simplex (s.s.) and A. pegreffii larvae (e.g., Accession numbers: AM706346.1, LC536534.2, HF680316.1, and AJ937671.1). In contrast, A. berlandi was found in separate clades [16]. Pseudoterranova decipiens (AB201790) was used as the outgroup for the rooted tree.

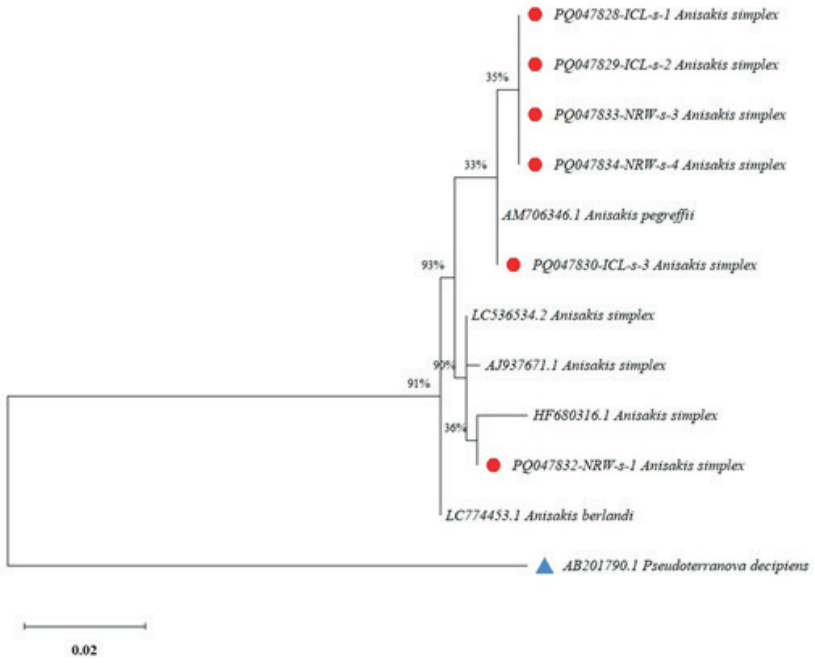


Figure 2 - Phylogenetic relatedness of the study isolates (red dot beside) to other isolates belonging to the anisakid family (divergence = 0.02)

To obtain more detailed data on nucleotide sequences, Table 2 presents the number of base substitutions per site between sequences. The data show the close relationships between the studied isolates.

Table 2 - Estimation of evolutionary divergence between sequences

| | PQ047828 | PQ047829 | PQ047830 | PQ047832 | PQ047833 | PQ047834 |
|---|---|---|---|---|---|---|
| PQ047828-ICL-s-1 *A. simplex* | | | | | | |
| PQ047829-ICL-s-2 *A. simplex* | 0.0000 | | | | | |
| PQ047830-ICL-s-3 *A. simplex* | 0.0000 | 0.0000 | | | | |
| PQ047832-NRW-s-1 *A. simplex* | 0.0000 | 0.0000 | 0.0000 | | | |
| PQ047833-NRW-s-3 *A. simplex* | 0.0000 | 0.0000 | 0.0000 | 0.0000 | | |
| PQ047834-NRW-s-4 *A. simplex* | 0.0000 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | |

**Discussion and Conclusion**

The aim of this study was to determine the occurrence of *Anisakis* larvae in mackerel fish widely sold in Astana markets. *A. simplex* (s.s.) larvae were found to be highly prevalent in the examined mackerel imported from Norway and Iceland. Morphological analyses alone may not be suitable for identifying Anisakis larval species [17, 18]. Although *A. simplex* (s.s.) is a well-known fish parasite associated with human disease, no prior studies have investigated the prevalence of this nematode in fish sold in Kazakhstan. Our study revealed a prevalence of *A. simplex* (s.s.) in marketed mackerel of 94%, with an MI of 20.61 larvae per infected fish (Table 1), underscoring the importance of this fish as a potential source of human anisakiasis. The high MI of *A. simplex* (s.s.) in the examined mackerel indicates significant risk of infection in humans.

Fish of the genus *Scomber* (mackerel) are widely distributed and exhibit considerable diversity. However, Atlantic and Norwegian mackerel (*Scomber scombrus*) are the most consumed *Scomber* species in the Astana market. This study provides the first insights into the occurrence of A. simplex in fish imported to and sold in Kazakhstan.

Our research demonstrated that morphological analysis can provide preliminary information about larval species, whereas advanced molecular tools offer more definitive species identification. Through partial sequencing of the 5.8S gene, we classified the isolated *Anisakis* larvae. We found that larvae from Norwegian Sea and Atlantic Ocean mackerel grouped with *A. simplex* (s.s.) and were distinct from other *Anisakis* species. This underscores the effectiveness of molecular methods in distinguishing closely related species and enhances our understanding of larval species identification.

It is evident that molecular methods such as sequencing are most effective for defining larval species. These findings make a significant contribution to our knowledge of *Anisakis* larvae in fish in Kazakhstan and have important public health implications.

Although *Anisakis* has been known since the 19th century, research on anisakiasis has notably increased recently [19]. Publications from 1970 to 1990 were limited, and research during this period was relatively stagnant. The literature output began to rise steadily between 1990 and 2000, reflecting growing interest in A. simplex. Despite this, molecular and genomic analysis of this subject remains underexplored.

Cipriani et al. [20] provided valuable insights into the distribution of Anisakis parasites in the Atlantic Ocean and Norwegian Sea, sources of mackerel imported to Kazakhstan. This report highlights the potential risks associated with a lack of awareness in this field, emphasizing the challenges posed by insufficient expertise.

Anisakiasis is a disease closely associated with local dietary habits [21]. Most reported cases (>90%) occur in Japan, with additional cases occurring primarily in European countries where raw fish dishes are common [22, 23]. There are significant gaps in research on imported fish in Kazakhstan.

Future research should focus on estimating the genetic variability and infection rates of these parasites as indicators for monitoring the status of *A. simplex* in Kazakhstan.

In conclusion, our study sheds light on the prevalence of *A. simplex* (s.s.) larvae in mackerel fish sold in Astana markets, highlighting the potential risk of human anisakiasis associated with the consumption of infected fish. Our study demonstrates the importance of using advanced molecular tools to definitively identify species in *Anisakis* larvae. Furthermore, the findings underscore the need for increased awareness and expertise in this field, particularly regarding imported fish in Kazakhstan. Future research efforts should prioritize estimating the genetic variability and infection rates of these parasites to effectively monitor the status of *A. simplex* in Kazakhstan.

**Authors' Contributions**

Conceptualization, VK.; methodology VK. and RU.; validation, RU., AS., AN. and AB.; formal analysis, VK., RU. and NM.; investigation, VK.; resources, VK.; data curation, AB. and AS.; writing–original draft preparation, RU., NM., VK.; writing–review and editing, VK and RU.; visualization, AB., TT. and AN.; project administration, VK.; funding acquisition, VK. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest**

Authors have no conflict of interest to declare.

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