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PREVALENCE, INFECTION INTENSITY, AND ALLERGENIC RISK OF ANISAKIS SPP. IN READY-TO-EAT FISH PRODUCTS

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ABSTRACT

Anisakis spp. are foodborne parasites of major public health concern due to their dual role as infectious agents and potent allergens. This study investigated the prevalence, infection intensity, and allergenic potential of *Anisakidae* in ready-to-eat fish products available on the Astana market (Kazakhstan). A total of 27 commercial fish product brands representing various species and processing methods were examined using artificial digestion and molecular analysis. Third-stage larvae (L3) of *Anisakis* were detected in 10 products, including cod liver, pink salmon, tuna, mackerel, smoked herring, lightly salted herring, and smoked mackerel. Infection prevalence ranged from 7.5% to 100%, while mean infection intensity varied markedly, with the highest values observed in smoked and lightly salted herring products. PCR assays targeting the *18S rRNA* and *COX II* genes confirmed the presence of *Anisakidae* DNA in all parasitologically positive samples. Notably, several products without detectable larvae by artificial digestion tested positive by PCR, indicating the persistence of *Anisakis*-derived genetic material associated with thermostable allergenic proteins. These findings demonstrate that the absence of visible larvae does not necessarily indicate the absence of allergenic risk and highlight the importance of combining parasitological and molecular methods for comprehensive food safety assessment of ready-to-eat fish products.

Keywords: *Anisakis* spp.; foodborne parasites; fish products; allergenic proteins; PCR detection

INTRODUCTION

Anisakis spp. are unique among foodborne parasites in that they act not only as infectious agents but also as potent food allergens [1]. To date, 14 *Anisakis* allergens (Ani s 1-14) have been registered, several of which demonstrate marked thermostability [2, 3]. It is commonly found in commercially significant fish species, such as tuna, cod, anchovy, and herring, making it prevalent in seafood products. In particular, Ani s 1, 4, 5, 8 and 9 are recognized as heat-stable allergens, capable of retaining IgE-binding activity after cooking, freezing, or canning. Consequently, sensitized individuals may develop allergic reactions to cooked or canned fish, even in the absence of morphologically detectable third-stage larvae (L3) during routine parasitological inspection, which does not necessarily indicate the absence of *Anisakis*-derived allergenic material [4-7]. Most heat-stable allergens belong to the excretory/secretory protein fraction of *Anisakis*, and some show additional resistance to pepsin digestion, further increasing their clinical relevance. These characteristics explain why processed fish products may remain allergenic despite adequate thermal treatment [8, 9].

Consuming infected fish, whether raw or undercooked, can lead to gastrointestinal issues and allergic reactions. Studies have shown that some *A. simplex* allergens can provoke immune responses even after cooking and are resistant to heat and pepsin treatments [11-14]. Food allergies are becoming a major public health issue, particularly in developed countries, leading to the widespread use of allergen detection methods such as SDS-PAGE, immunoblotting, and ELISA. While PCR-based techniques are effective for species differentiation, their utility for allergen detection in processed foods remains under exploration [15]. Recent findings indicate that

ELISA and PCR offer comparable sensitivity and specificity in detecting allergens, with DNA-based methods providing advantages in specificity and stability against environmental influences [16-18].

Ready-to-eat/processed fish products contain “traces” of *Anisakis* and/or are in frozen or processed formats; emphasis is placed on the complexity of control in these products (including some canned products) and the need to combine control methods [19].

Effective detection of parasites in seafood is crucial for food safety and quality. Legislation requires freezing seafood for raw consumption to eliminate viable larvae; however, heat-stable antigens can still trigger allergic reactions in sensitive individuals [20]. Current inspection methods are inadequate for detecting parasites in modern seafood products, necessitating the development of more accurate testing frameworks to address evolving market demands and ensure food safety [21]. The aim of the study was to assess the level of contamination of finished fish products with *Anisakidae* larvae and their allergenic residues using PCR analysis.

MATERIALS AND METHODS

Sample preparation

Table 1 summarizes the commercial fish products analyzed in the present study. A total of 27 product brands (78 batches) representing various fish species and processed forms, including canned and tinned products (cod liver, tuna, mackerel, anchovies, sardines, pink salmon, herring, and Alaska pollock fillet [back cut]) and local smoked and salted products (lightly salted herring fillet pieces, smoked capelin, smoked herring, lightly salted herring, and smoked mackerel), were purchased

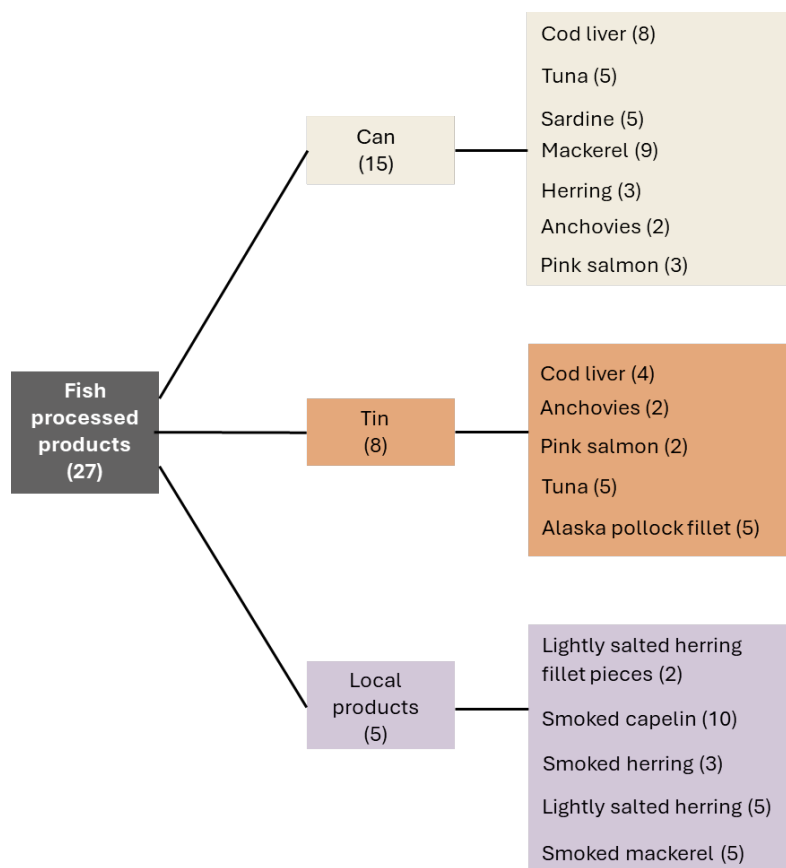


Figure 1. Commercial fish products analyzed in the present study

from local retail markets (Figure 1). The study of fish products included a parasitological examination of fish tissues and an artificial digestion procedure. This was followed by molecular analyses, including DNA isolation and PCR, to identify residues of food allergen proteins.

Parasitological examination

Parasitological examination revealed the occurrence of third-stage larvae of *Anisakis* in fish products. Third-stage larvae of *A. simplex* were removed from the abdominal cavity with tweezers. Nematodes were characterized according to taxonomic keys [22, 23]. Residual proteins from the host fish were removed by washing in 1% (w/v) NaCl. After this process, the larvae were stored at -80°C until use.

Artificial digestion

From each sample, 20g was used per product, depending on the product type. The following ingredient ratios were used to prepare the digestion liquid: 2 L of tap water and 10.8 mL of hydrochloric acid (HCl, 37%). 10 g of pepsin powder (Fisher Chemical, USA) was incorporated into the mixture. The prepared sample was placed in a 1 L glass beaker containing the digestion liquid. A sample-to-digestive fluid ratio of 1:10 was maintained during the AD process. The beaker was covered with aluminium foil to maintain a stable temperature of 40–42 °C and to minimize evaporation. The fluid temperature was monitored regularly with a thermometer, and the stirring was adjusted to produce a deep vortex without splashing. The sample was digested for 30 min. At the end of the procedure, the AD product was passed through a sieve, and the detected parasites were collected, counted and stored in saline solution at -20 °C for further examination.

DNA Extraction and Clean-up

Before DNA extraction from commercial fish products, excess salt and oil were removed by rinsing and blotting with filter paper, respectively. Defatted and unsalted tissues were stored at -20°C until use. For DNA isolation, six different protocols were performed. In all cases, except for the commercial kits with a specific lysis buffer, the larvae were previously digested with 300 µL of extraction buffer [1% (w/v) SDS, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and Tris-HCl at pH 8.0] supplemented with 30 µL of proteinase K and subsequently incubated at 56 °C overnight. After centrifugation for 5 min at 16,000 g, the supernatant was purified using the NaCl method [24] and the phenol-chloroform method [25]. The Monarch Genomic DNA Purification Kit was performed after substituting its lysis buffer with the digestion procedure.

PCR analysis

Primers used to detect *Anisakis* were designed based on the first internal transcribed spacer regions of rDNA and the mitochondrial gene, which allowed identification of the presence of the *Anisakis* complex (Table 1); neither the host organisms (fish) nor other nematodes that may contaminate food from sources other than fish (e.g., *Ascaris* spp.) were amplified using these primers. PCE mix contained: 10 mM Tris-HCl at pH 9.0, 50 mM KCl, 0.2 mM dNTPs, 2.5 mM MgCl₂, 1 µM primer, and 100 ng of template DNA. Amplification reactions were conducted in a Mastercycler Personal (Eppendorf). The PCR cycled as follows: 1 min at 50°C, then 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Electrophoretic separation of nucleic acids was performed in 1% agarose gel using 1× TBE buffer.

Statistical analysis

Table 1. Primers used to detect the presence of *Anisakis*

Target	Name	Sequence (5'-3')	Amplicon size	Reference
18S rRNA	EUKA F	GGTGT- CAGAAAAGTTAC- CACAGG	139	[26]
	EUKA R	AGTGGGTGAA- CAATCCACG		
COX II	CYTII-F	TTTCTAGTTATATG- GATTGATTCCATAA	260	[27]
	CYTII-R	GACCAGT- GACTTTCACAGT- CAAATTAC		

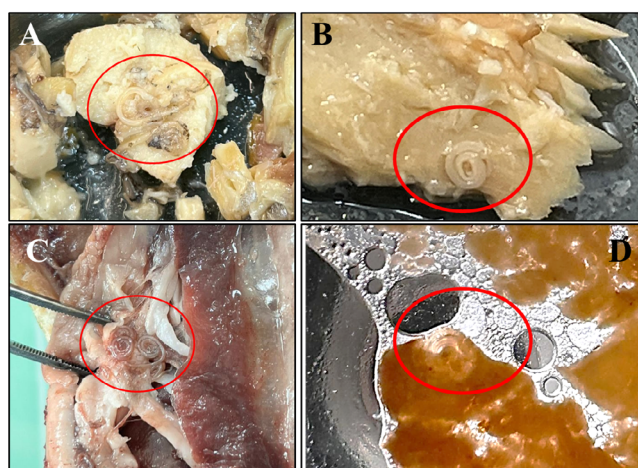


Figure 2. Parasitological examination of commercial fish products. (A-D) Macroscopic detection of *Anisakis* larvae in different fish products during routine parasitological inspection.

The observed prevalence, mean intensity and abundance of each species were calculated according to the standards of parasitological statistics [28, 29].

RESULTS

To evaluate the assay, we purchased 78 different commercial seafood products from local markets. These samples included industrially prepared salted and smoked fish, canned goods, and ready-to-eat products (Table 2). During parasitological examination of the analyzed fish products, *Anisakis* larvae were detected in several samples. Macroscopic inspection revealed the presence of tightly coiled nematode larvae embedded in fish tissues. The detected larvae were morphologically consistent with third-stage *Anisakis* larvae (L3). The appearance of *Anisakis* larvae in different fish products is shown in Figure 2.

Larvae are visible as tightly coiled, whitish nematodes embedded in fish tissues. Panels illustrate *Anisakis* larvae detected in processed fish products, including canned and raw fish tissues, demonstrating their persistence in commercially available products.

To confirm the presence of *Anisakis* parasites in the examined fish products, molecular analysis was performed using PCR amplification of the mitochondrial *COX II* and ribosomal

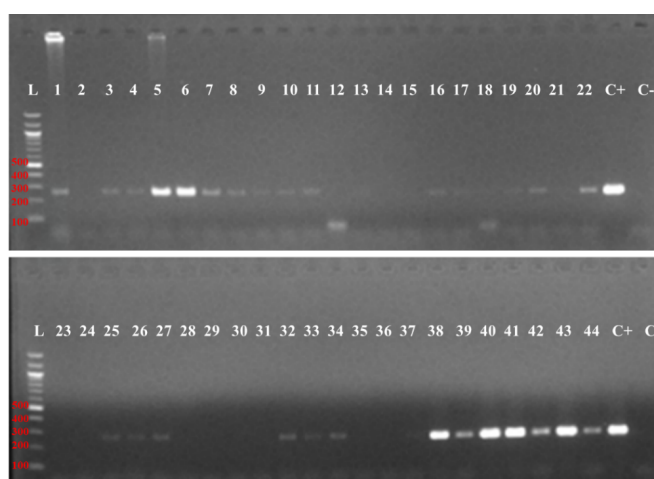


Figure 3. L – ladder 100-1000 bp; lines 1-44 PCR analysis of samples studied fish products using the *COX II* gene; C+ – positive control for *Anisakis simplex* DNA; C- – uncontaminated fish as negative control

18S rRNA gene (Table 2). DNA extracted from the analyzed samples was subjected to PCR, and the amplification results were evaluated by agarose gel electrophoresis. PCR results obtained for the studied fish products, together with positive and negative controls, are presented in Figure 3. DNA from uncontaminated fish served as a negative control.

PCR products obtained using the *COX II* gene were analyzed by agarose gel electrophoresis. Specific amplification bands 260 bp corresponding to the expected fragment size were observed in samples and in the positive control, while no amplification was detected in the negative control.

PCR amplification of the *18S rRNA* gene produced a specific band of the expected size (139 bp) in several fish product samples and in the positive control, while no amplification was observed in the negative control.

An overview of *Anisakidae* infection parameters in ready-to-eat fish products, including prevalence, infection intensity, and PCR-based confirmation of the presence of the thermostable protein, is presented in Table 3.

Table 3 highlights the findings on the prevalence of *Anisakidae* larvae in ready-to-eat fish products. A total of 27 product brands were examined. *Anisakidae* larvae were detected in 10 products, while the remaining products were negative by artificial digestion.

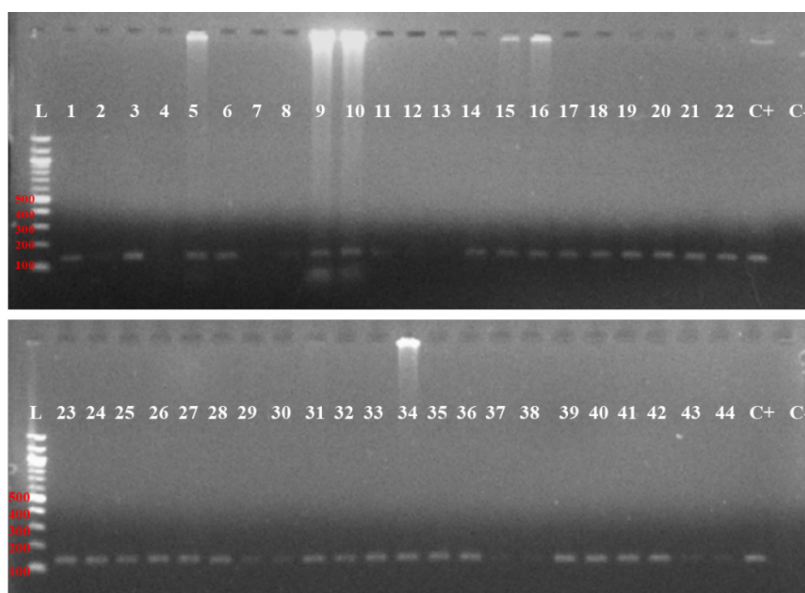


Figure 4. L – ladder 100-1000 bp; lines 1-44 PCR analysis of samples studied fish products using the *18S rRNA* gene; C+ – positive control for *Anisakis simplex* DNA; C- – uncontaminated fish as negative control

Positive samples were identified among cod liver, pink salmon, sardine, tuna, mackerel, smoked herring, lightly salted herring, and smoked mackerel. Prevalence varied widely between products, ranging from 7.5% in smoked herring to 100% in cod liver and pink salmon, although confidence intervals were generally broad due to limited sample sizes.

Mean infection intensity differed markedly across products. The lowest mean intensities were observed in cod liver and pink salmon ($2.0\text{--}2.5$ larvae per sample), whereas higher intensities were recorded in mackerel products ($2.5\text{--}4.3$ larvae per sample) and especially in smoked and lightly salted herring products, which showed the highest mean intensities (13.3 ± 2.9 and 9.3 ± 1.2 larvae per sample, respectively). Smoked mackerel also showed relatively elevated intensity (5.5 ± 0.6 larvae per sample). The intensity ranges indicated substantial aggregation of larvae within individual samples.

All detected larvae were identified as third-stage larvae (L3). Molecular analysis confirmed the presence of *Anisakidae* DNA in all parasitologically positive samples, with amplification of both *18S rRNA* and *COX II* targets, supporting the morphological identification.

From Table 3, we can see that in several samples, no third-stage larvae (L3) of *Anisakidae* were detected by artificial digestion; however, molecular analysis yielded positive results. PCR assays targeting the *18S rRNA* and *COX II* genes detected *Anisakidae*-specific signals in a number of products that were negative by morphological examination.

In several fish products, no third-stage larvae (L3) of *Anisakidae* were detected by artificial digestion; however, molecular analysis revealed positive PCR results. Specifically, tuna (Yellowfish) showed no detectable larvae, yet was positive for both *18S rRNA* and *COX II* targets. Similarly, cod liver products from the Pishchevik and Pelican brands, as well as mackerel (Pervomayskiy), were negative by parasitological examination but yielded positive PCR signals for both genes. In addition, PCR positivity in

the absence of observed larvae was recorded for sardine (Kubley), herring (Dobroflot), pink salmon (Russkiy Rybnyy Mir), and tuna (FishMaster), where amplification was detected for one or both molecular targets.

DISCUSSION

The present study confirms that ready-to-eat fish products may contain *Anisakidae*-derived allergenic material even in the absence of morphologically detectable larvae. Although artificial digestion did not detect third-stage larvae (L3) in several samples, PCR analysis targeting the *18S rRNA* and *COX II* genes demonstrated the presence of *Anisakidae*-specific genetic material. This discrepancy highlights the limitation of morphology-based inspection alone for assessing allergenic risk in processed fish products [26, 27].

The use of *18S rRNA* and mitochondrial *COX II* marker genes proved effective for detecting *Anisakidae* residues in such cases [30]. DNA-based methods are particularly advantageous in processed foods, where proteins may be denatured and larvae morphologically unrecognizable, yet DNA fragments remain detectable [31, 32].

The results obtained in this study clearly demonstrate this phenomenon. Several products, including tuna (Yellowfish), cod liver (Pishchevik and Pelican), mackerel (Pervomayskiy), sardine (Kubley), and herring (Dobroflot), were negative for L3 larvae but positive by PCR for one or both marker genes. These findings indicate that the absence of intact larvae does not equate to the absence of allergenic risk, particularly in ready-to-eat products that undergo non-lethal processing methods.

Importantly, products with detectable larvae often showed high infection intensities, especially smoked and lightly salted herring, further increasing the potential allergenic burden. However, the detection of *Anisakidae* DNA in larva-negative samples suggests that even low-intensity or degraded infections may be clinically relevant, as allergic reactions can be triggered by minute quantities of allergenic proteins.

Table 2. Ready-to-eat fish products found infected with *Anisakidae* larvae, infection intensity per sample and results of molecular analysis

№	Type of product	Brand	No. of samples	Prevalence (CI 95 %)	No. of larvae per sample (Presence of L3)	Range of intensity	Mean (SD) intensity	PCR analysis for the presence of the thermostable protein	
								18S rRNA	COX II
1	Cod liver	Khozyain Baltiki	2	-	-	-		-	-
2	Tuna	Pelican	2	-	-	-		-	-
3	Tuna	Sajo	2	-	-	-		-	-
4	Cod liver	Kapitan Vkusov	2	100 (15.81* – 100)	5	2-3	2.5 ± 0.7	+	+
5	Cod liver	Ultramarin	2	66.7 (9.4 – 99.1)	4	1-3	2.0 ± 1.4	+	+
6	Cod liver	Pishchevik	3	-	-	-		+	+
7	Cod liver	Pelican	3	-	-	-		+	+
8	Mackerel	Pervomayskiy	3	-	-	-		+	+
9	Anchovies	Roland	2	-	-	-		-	-
10	Sardine	Kubley	2	-	-	-		+	+
11	Pink salmon	Roskom	2	100 (15.81* – 100)	2	2	2.0 ± 0.0	+	+
12	Herring	Dobroflot	3		-	-		+	+
13	Pink salmon	Russkiy Rybnyy Mir	3	21.4 (4.6 – 50.8)	14	3-6	7.0 ± 0.0	+	+
14	Tuna	Yellowfish	2	-	-	-		+	+
15	Tuna	Lorado	2	-	-	-		-	-
16	Tuna	FishMaster	1	-	-	-		+	-
17	Tuna	Celeste	1	33.3 (0.8 – 90.6)	3	3	3.0 ± 0.0	+	+
18	Sardine	Russkiy Rybnyy Mir	3	-	-	-		+	+
19	Mackerel	Russkiy Rybnyy Mir	3	60.0 (14.6 – 94.7)	5	1-3	2.5 ± 0.7	+	+
20	Mackerel	FishMaster	3	23.1 (5.0 – 53.8)	13	4-8	4.3 ± 0.6	+	+
21	Anchovies	Azovchanka	2	-	-	-		+	-
22	Lightly salted herring fillet pieces	VICI	2	-	-	-		-	-

23	Alaska pollock fillet	Local product	5	-	-	-	-	-	-
24	Smoked capelin	Local product	10	-	-	-	-	-	-
25	Smoked herring	Local product	3	7.5 (1.6 – 20.4)	40	5-15	13.3 ± 2.9	+	+
26	Lightly salted herring	Local product	5	17.8 (6.0 – 36.9)	28	4-12	9.3 ± 1.2	+	+
27	Smoked mackerel	Local product	5	22.7 (7.8 – 45.4)	22	5-8	5.5 ± 0.6	+	+

*One-sided 97.5% confidence interval; No. – number; CI – confidence interval; SD – standard deviation

Overall, the combined parasitological and molecular approach applied in this study provides a more comprehensive assessment of *Anisakidae* contamination and allergenic potential in fish products. The findings emphasize that food safety evaluations should incorporate molecular screening alongside traditional inspection methods, particularly for processed and ready-to-eat seafood intended for direct consumption.

AUTHOR CONTRIBUTIONS

RU and VK: conceptualisation, study design, data validation, and writing—original draft preparation. KJ, RU, AI, AA: collection of biological material. RU, KJ, LL and MK: data curation and laboratory experiments. RU and KJ: data analysis. VK: funding acquisition. RU, AB, and VK: revising and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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ДАЙЫН ТҮТЫНУҒА АРНАЛҒАН БАЛЫҚ ӨНІМДЕРІНДЕГІ ANISAKIS SPP. ТАРАЛУЫ, ИНВАЗИЯ ҚАРҚЫНДЫЛЫҒЫ ЖӘНЕ АЛЛЕРГИЯЛЫҚ ҚАУІП

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АННОТАЦИЯ

Anisakis spp. – тағам арқылы жұғатын паразиттер болып табылады және олардың инфекциялық агент әрі күшті аллергия ретіндегі қосарлы рөлі қоғамдық денсаулық сақтау үшін елеулі қауіп тудырады. Бұл зерттеуде Қазақстанның Астана қаласы нарығында сатылатын дайын тұтынуға арналған балық өнімдеріндегі *Anisakidae* тұқымдасы өкілдерінің таралуы, инвазия қарқындылығы және аллергиялық әлеуеті зерттелді. Өртүрлі балық түрлері мен өңдеу әдістерін қамтитын 27 коммерциялық балық өнімі бренді жасанды қорыту әдісі және молекулалық-генетикалық талдау арқылы зерттелді. *Anisakis* паразитінің үшінші даму сатысындағы (L3) дернәсілдері 10 өнімнен анықталды, оның ішінде треска бауыры, қызғылт албырт, тунец, скумбрия, ысталған майшабақ, аздап тұздалған майшабақ және ысталған скумбрия. Инвазияның таралу деңгейі 7,5%-дан 100%-ға дейін өзгерді, ал орташа инвазия қарқындылығы айтарлықтай айырмашылық көрсетті және ең жоғары көрсеткіштер ысталған және әлсіз тұздалған майшабақ өнімдерінде тіркелді. *18S* рРНҚ және *COX II* маркерлік гендерін нысанаға алған ПТР талдауы паразитологиялық тұрғыдан оң нәтиже көрсеткен барлық үлгілерде *Anisakidae* ДНҚ-сының бар екенін растады. Айта кетерлігі, жасанды қорыту әдісімен дернәсілдер анықталмаған кейбір өнімдер ПТР арқылы оң нәтиже көрсетті, бұл термостабильді аллергиялық қауыптармен байланысты *Anisakis*-ке тән генетикалық материалдың сақталуын көрсетеді. Алынған деректер көзге көрінетін дернәсілдердің болмауы аллергиялық қауіптің жоқтығын білдірмейтінін дәлелдейді және дайын тұтынуға арналған балық өнімдерінің қауіпсіздігін кешенді бағалау үшін паразитологиялық және молекулалық әдістерді біріктіре қолданудың маңыздылығын көрсетеді.

Түйін сөздер: *Anisakis* spp.; тағам арқылы жұғатын паразиттер; балық өнімдері; аллергиялық қауыптар; ПТР анықтау

РАСПРОСТРАНЁННОСТЬ, ИНТЕНСИВНОСТЬ ИНВАЗИИ И АЛЛЕРГИЧЕСКИЙ РИСК ANISAKIS SPP. В ГОТОВЫХ К УПОТРЕБЛЕНИЮ РЫБНЫХ ПРОДУКТАХ

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АННОТАЦИЯ

Anisakis spp. являются пищевыми паразитами, представляющими серьёзную угрозу общественному здравоохранению вследствие их двойной роли – как инфекционных агентов и как мощных аллергенов. В настоящем исследовании изучены распространённость, интенсивность инвазии и аллергенный потенциал представителей семейства *Anisakidae* в готовых к употреблению рыбных продуктах, реализуемых на рынке г. Астаны (Казахстан). Было исследовано 27 коммерческих брендов рыбной продукции различных видов и способов переработки с использованием метода искусственного переваривания и молекулярно-генетического анализа. Личинки третьей стадии (L3) *Anisakis* были выявлены в 10 продуктах, включая печень трески, горбушу, тунец, скумбрию, копчёную сельдь, слабосоленную сельдь и копчёную скумбрию. Распространённость инвазии варьировала от 7,5% до 100%, тогда как средняя интенсивность инвазии существенно различалась, достигая наибольших значений в продуктах из копчёной и слабосоленной сельди. ПЦР-анализ с использованием маркерных генов *18S* рРНҚ и *COX II* подтвердил наличие ДНК *Anisakidae* во всех паразитологически положительных образцах. Примечательно, что в ряде продуктов, в которых личинки не были обнаружены методом искусственного переваривания, ПЦР дала положительный результат, что указывает на сохранение генетического материала *Anisakis*, ассоциированного с термостабильными аллергенными белками. Полученные результаты свидетельствуют о том, что отсутствие визуально обнаруживаемых личинок не обязательно означает отсутствие аллергического риска, и подчёркивают необходимость сочетания паразитологических и молекулярных методов для комплексной оценки безопасности готовых к употреблению рыбных продуктов.

Ключевые слова: *Anisakis* spp.; пищевые паразиты; рыбная продукция; аллергенные белки; ПЦР-диагностика