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EVALUATION OF A CONVENTIONAL PCR TECHNIQUE FOR THE MOLECULAR DETECTION OF ANISAKID PARASITES IN THREE DRAINING LIQUIDS OF CANNED MACKEREL

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Abstract
Anisakids, which cause severe food allergies in children and the elderly, cannot be detected in hydrobiological commodities using sensory studies. Since parasites in canned fish are difficult to spot visually, this technique is not recommended. There needs to be an evaluation of molecular approaches to detecting Anisakid bugs in canned fish. Anisakid parasites were cultured and then fed to fish that had been experimentally infected. Total genomic DNA was extracted using the modified CTAB method from the control fluid that had been drained from the preserve following a thorough mixing of the contents. Two-fold amplification PCR was utilised to locate a 629bp region of the cytochrome c oxidase subunit II (COXII) gene. In total, 48 inoculation (with parasites) preserves, 48 control (uninoculated) preserves, and 48 preserves with additional parasites were examined. This demonstrated that the test's diagnostic sensitivity was 100% for preserves prepared using oil and commercial tomato sauce as the governing liquid, and 93.33%; for those prepared using water canning. Analysis and diagnosis were at the heart of the procedure.
Keywords: Anisakids, canned fish, PCR, CTAB method, COXII gene

1. Introduction

Nematodes from the family Anisakidae are ascaroids that parasitize fish and marine mammals, but when consumed raw by people, they can become zoonotic. Around 24 genera make up the Anisakidae family, the most well-known of which are Anisakis, Phocascaris, Contracaecum, and Phocanamea—especially when you consider that the Anisakis genus is the largest and is in charge of parasitosis and food allergies in fish (Bilska et al 2015). Since there is currently only one test for "Detection of Foreign Matter" (Mohd Khairi, et al 2018) for canned fish, which is based on physical sensory tests that are ineffective, it is necessary to evaluate new techniques for the molecular detection of Anisakids in canned fish in Iraq (which turn out to be complex samples because they have been exposed to high temperatures and the DNA in these conditions is degraded) (Yemmen, et al 2020). It is crucial that these techniques address the issue of taking multiple samples from the same container, which is why it is suggested to use various canned fish preparations as a study reference, such as grated fish in various sauces, dressings, and governing liquids, in order to identify the presence of the parasite in these fluids. Even after heat treatment, proteases with the potential to cause allergies have been found in canned fish, despite the fact that the Anisakis parasite is dead and no longer contagious. This suggests that canned fish may not be safe for humans. consumers who had previously developed parasite sensitivities. The chance to show the presence of these parasites, subjected to conditions degradation due to the sterilisation process, from the drained government liquids: water, oil, and commercial tomato sauce arises because there are few research studies where Anisakid detection techniques are evaluated from canned fish government liquids, and because this is an easily obtained sample (Debenedetti Lopez, 2019).

Since it won't be necessary to take multiple samples from a single container, as is suggested in other PCR methodologies from tissue samples, the detection of anisakids in government liquids

by means of a PCR technique (Herrero, et al 2011) will have the advantage of being easily accessible. Otherwise, you run the risk of analysing an uncontaminated portion and receiving false negative results. The homogenization of the entire container sample of the public liquid would be the solution to this. The information produced is anticipated to act as a foundation for the verification of detection strategies and batch quality assurance.

Anisakid nematodes were found in a batch of canned whole mackerel in tomato sauce that had a health certificate from the People's Republic of China's (AQSIQ) Health Authority on November 17, 2017, prompting SANIPES to issue the AQSIQ an international health alert (SANIPES, 2020). Based on studies showing that some Anisakids proteins maintain certain allergenic properties even after heat treatment due to the presence of heat-resistant antigens, the health alert was issued because canned mackerel with Anisakids could put those who consume these products at risk (Moneo et al., 2005). It must be remembered that in order for the host's body to synthesise IgE and allergic symptoms to be triggered in a subsequent human-human interaction, there must be an initial infestation in the stomach or intestine of a man with a live larva (L3) of the Anisakid. Because the protease enzymes and protease inhibitors the larva secretes when invading the host act as allergens, the parasite is considered to be a parasitic. According to Ador et al. (2021), the traditional polymerase chain reaction (PCR) technique is a quick and accurate way to find and identify fish parasites. Three species—*A. simplex*, *A. pegreffi*, and *Hystherothylacium aduncum*—were found in a total of seven species of fish in the Cantabrian Sea according to a genetic analysis study of the Anisakis genus. All of these studies were conducted on fresh and frozen fish. Studies (Kijewska, et al 2001) of restriction fragment length polymorphisms, or "PCR-RFLP," through the Internal region were conducted for the purpose of detecting Anisakids in a variety of hydrobiological products. Transcripted spacer 1 (ITS-1) proved that even in the most severe case of DNA degradation, such as heat treatment used on canned goods, correct DNA amplification is possible.

A highly sensitive and quickly developed real-time PCR assay targeting Subunit I of Cytochrome Oxidase (COI) was developed to enable the simultaneous detection of the most significant Anisakid species in fish and hydrobiological products like canned foods able to adapt in typical molecular diagnostic labs. Herrero et al (2011) study for the detection of Anisakids in fresh cod livers and canned cod livers using PCR allowed the identification of a high presence of *A. simplex* and *P. decipiens* in fresh livers and a lower presence of these parasites in canned. According to the works of Herrero et al. (2011), it is one of the most commonly used in the detection of Anisakids in hydrobiological products. This technique uses Cetyl Trimethyl Ammonium Bromide (CTAB), a substance that is suitable to process tissues with a high concentration of polysaccharides and polyphenols, to extract DNA from plants and foods derived from plants. The recommendations for the extraction and purification of DNA (Beck 2002) were used in the current study to extract DNA from positive controls and samples. These recommendations were modified to be used in the preserves' draining liquid, which is covered in more detail in the materials and methods and results sections.

An essential part of the electron transport chain in the process of aerobic respiration is the cytochrome oxidase subunit II (COX II). Although this sequence contains a high percentage of A+T bases, which is also true of other nematode mitochondrial sequences, it has been demonstrated that the amino acid sequence of cytochrome oxidase II (COX II) is conserved. The genetic makeup of Anisakid populations has been investigated using this sequence (Ramilo, et al., 2023). In light of this, this work was created to assess the sensitivity and specificity of a traditional PCR molecular detection method for Anisakid parasites in 3 different governing fluids (water, oil, and tomato sauce) in artificially contaminated canned mackerel.

2. METHODOLOGY

2.1. Study design and sample size

The sample size was one of the first steps in this experimental prospective investigation, which involved manipulating the variables (inoculation of parasites) under controlled circumstances. The sample size was calculated using the WinEpi 2.0 Programme (Working in Epidemiology) through the estimation of proportions, which gave us a total of 16 samples (canned mackerel) for an unknown population of canned fish with a confidence level of 90%, a margin of error of 10%, and an expected ratio of 94%. A plan for the preparation of inoculated and non-inoculated preserves was created based on the sample size and the research objectives (Table 1).

Table 1: Plan for the preparation of inoculated and non-inoculated preserves aligned with the objectives of the investigation and the sample size.

determine sensitivity	Presentation Canned Mackerel in Oil	Presentation Canned Mackerel in Water	Presentation Canned Mackerel in Tomato Sauce	sample size
diagnostic	Inoculated with Anisakids	Inoculated with Anisakids	Inoculated with Anisakids	16 preserves x presentation positive samples
Determine analytical specificity	Inoculated with other parasites and portions of other fish	Inoculated with other parasites and fish	Inoculated with other parasites and fish	16 preserves x presentation negativity samples
Determine diagnostic specificity	without inoculating	without inoculating	without inoculating	16 canned x presentation negative samples
TOTAL				144 preserves in total

Based on this plan and in order to improve the understanding of the objectives, steps and activities of this investigation, a research design consisting of three Phases was proposed for alignment with the specific objectives that are proposed in the investigation.

2.2. Phase A: Extraction, amplification and visualization of positive controls

The steps of DNA extraction, amplification, and visualisation were worked on in this phase with the aim of assessing the validity of the detection method under DNA degradation conditions using samples that will serve as positive controls (whole Anisakis). The work was completed in accordance with the steps of the following scheme, and you can read a detailed description of each step in the lines below.

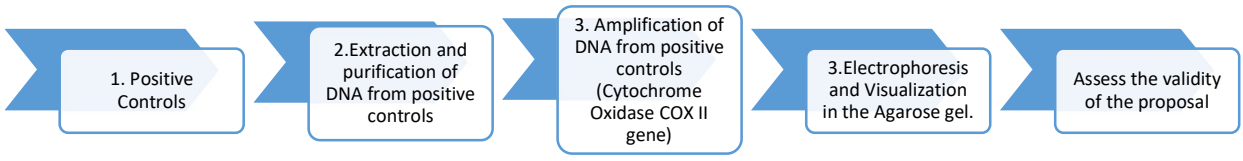

Figure 1: Work scheme for Phase A of the work

2.2.1. Positive controls: Anisakids from the Parasitology Laboratory Repository, which correspond to a parallel work of sequencing and phylogeny of Anisakid parasites in the Sea in charge of another parallel investigation, were used as positive controls for the PCR. These parasites had previously been identified by taxonomic keys (Bray, et al., 2008) and through the sequencing of the cytochrome oxidase II (COX II) gene. In our study, visualising an amplicon of the predicted size (629 bp) allowed us to determine the parasite's identity. Positive controls are used to evaluate the reliability of the detection technique through visualisation on the

agarose gel.

2.2.2. Extraction and purification of DNA from positive controls

In the current study, DNA was extracted from positive controls and samples in accordance with guidelines provided by Ramilo, et al., (2023). The parasites were washed with distilled water for roughly two hours to remove excess alcohol before moving forward with the DNA extraction from the positive controls (which were preserved in 70% alcohol). A precision balance (Mettler Toledo-Spain) was used to weigh 300 mg of anisakid individuals, and the CTAB-Cetyl Trimethyl Ammonium Bromide protocol (Clarke, 2009) was slightly modified to include vigorous homogenization of the parasites. For about ten minutes, using a mortar. Then, 1 mL of 2X CTAB was added, heated to 60°C, mixed by inversion, and incubated for 10 minutes at room temperature and then for 10 minutes at 0°C with the crushed parasites in an Eppendorf tube. Proteinase K (Promega, USA) (10 mg/µL) was then added to 10 µL of the mixture and mixed by inversion before being incubated at 65°C for 25 minutes and then at 0°C for 5 minutes. Both the phenol and chloroform-based cell component separation step and the DNA precipitation step were left unaltered. Finally, 50 µL of 10Mm Tris1Mm EDTA buffer (1X TE buffer) was used to rehydrate the DNA. Thermo Scientific's Nano Drop TM ND-10 Spectrophotometer was used to assess the quantity and quality of the extracted DNA, after which dilutions with nuclease-free water were made until the concentration reached 150 ng/L. Until they were used as positive controls, these DNAs were kept at -80 °C.

2.2.3. Amplification of the DNA of the positive controls (Cytochrome Oxidase COX II gene)

The cytochrome c oxidase subunit II (COX II) gene was amplified by PCR based on the recommendations of (Bello et al 2021), using the primers A211 (5′-TTTAGATATATATTYRTATGTGTTCT-3′) and A210 (5′-CACTCCTTATACAATCATAA-3′) described by Steven et al (2000), which generate a 629 bp amplicon. The reaction was carried out in a final volume of 30µL, 150ng of DNA, containing 0.4 uM of each primer, Promega GoTaq™ G2 Green Master Mix (which contains 3mM $MgCl_2$), completing the final solution with ultrapure water. The amplification times were: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 47 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. In all PCRs, nuclease-free ultrapure water was used as a negative control (Promega-USA).

2.2.4. Electrophoresis and visualization on the Agarose gel

The PCR products were separated by agarose gel electrophoresis with 1% TAE, at 90 V for 1 h, stained with 5 mg/mL of ethidium bromide (Sigma-USA), visualized under ultraviolet light in a transilluminator. The 1kb DNA Ladder (Promega-Brazil) was used as a molecular weight marker.

2.3. Phase B.-Extraction, amplification and visualization of Anisakid DNA in canned fish

In this phase, the steps are detailed to be able to extract, purify and amplify the DNA of Anisakids that have been artificially inoculated in the preparation of canned fish and therefore have been subjected to the most extreme conditions of DNA degradation. The variants of protocols suggested in other investigations are detailed, which served as the basis for the development of the present investigation. The work was carried out following the steps of the following scheme:

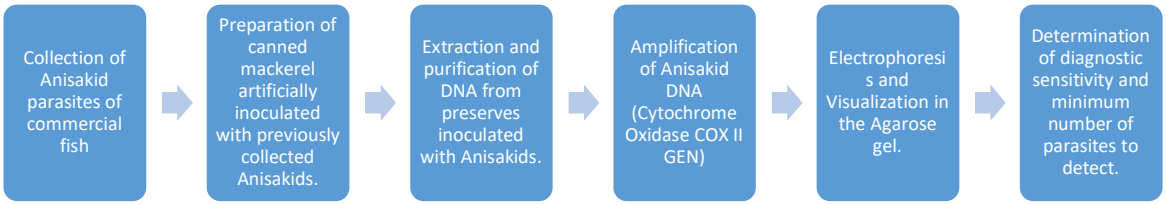

Figure 2: Scheme for Phase B of the work

2.3.1. Collection of Anisakid parasites of commercial fish

The Anisakids in stage (L3) were collected by necropsy of the stomach and viscera of fish for direct human consumption marketed in the fishing terminal of Umm Qasr, Basra, Iraq (Table 2). Anisakid larvae (L3) (approximately 240 larvae) were isolated and identified by taxonomic evaluation, using a stereoscope, and then preserved in 70% alcohol for use.

Table 2. Number of Anisakids collected from commercial fish

S.No	Scientific name of the Fish	Family	Number of Anisakids collected
1	*Scomber scombrus*	Scombridae	16
2	*Scomber japonicus*	Scombridae	42
3	*Rastrelliger kanagurta*	Scombridae	160
4	*Mugil cephalus*	Mugilidae	15
5	*Hemilutianus macrophthalmus*	Serranidae	9

Before proceeding with the artificial inoculation of the parasites and the preparation of the preserves, the parasites were washed with distilled water for approximately two hours to remove excess alcohol.

Figure 3. Anisakids collected from commercial fish preserved in 70% alcohol.

2.3.2. Preparation of canned mackerel artificially inoculated with previously collected Anisakids and Mackerel fillets being processed for the production of preserves

The preserves were prepared with "mackerel" (*Scomber japonicus peruanus*), purchased in local markets. After the washing, cutting and gutting processes, the fish were analyzed using the transillumination method to ensure that the fillets were free of parasites and, under strict biosafety parameters, they were inoculated with previously collected and identified Anisakid specimens. In glass containers with hermetic screw caps, 50 g of fish (83.39% of the total weight of the product), 10 mL of public liquid (12.6% of the total weight of the product) were placed, the liquids being water, oil and sauce. commercial tomato and 5 Anisakid individuals (approximately 30 mg), as shown in Table 3.

Table 3: Design of Elaboration of Inoculated and Non-Inoculated Preserves

Design of Elaboration of Inoculated and Non-Inoculated Preserves	Canned Mackerel in Oil inoculated with Anisakids	Water Mackerel Preserves inoculated with Anisakids	Canned Mackerel in Tomato Sauce inoculated with Anisakids
Mackerel	50g	50 g	50g
public liquid	10 mL Commercial oil	10 mL Water	10 mL Commercial Tomato Sauce
Anisakids	5 individuals (30 mg)	5 individuals (30 mg)	5 individuals (30 mg)

The flow of the elaboration of the preserves included the stages of evisceration and cutting of the fish, inoculation of parasites (as an experimental model), packaging, precooking, cooling, sterilization of the preserves and finally the storage of the preserves.
Pre-cooking was carried out by submitting the containers to a water bath for 10 minutes. Each container was labeled (type of product, date, weight, inoculation or control, public liquid) and sterilized in an autoclave (121 °C, 15 atmospheres, for 55 minutes) (Jildeh, et al, 2021).

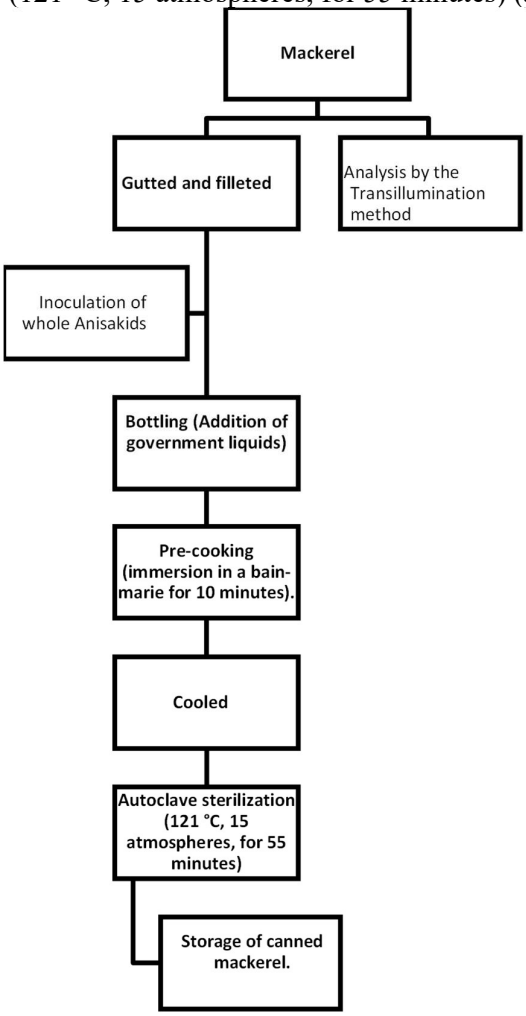

Figure 4. Processing flow of canned mackerel

A total of 48 preserves inoculated with Anisákidos larvae were elaborated, in detail 16 mackerel preserves with Anisákidos larvae in water, 16 mackerel preserve with Anisákidos larvae in oil and 16 mackerel preserves with Anisákidos larvae in sauce were elaborated. commercial tomato in order to obtain a minimum expected sensitivity of 95%.

2.3.3. DNA extraction using the CTAB protocol applied to canned fish inoculated with Anisakids

For all the DNA extraction and purification steps of the samples and controls, the traditional CTAB methodology recommended by Ramilo et al. (2023), with slight modifications, which are detailed in each point. For the extraction and purification of DNA from the samples, it was necessary to previously sterilize mortars, pestles, microcentrifuge tubes, and micropipette tips. In the present work we prepare in advance the solutions to be used for DNA extraction. The DNA of the canned samples previously described was extracted from the drained liquid, product of the homogenization of the canned food in its entirety (water, oil and tomato sauce). There was a modification of the traditional CTAB Protocol, recommended by Ramilo et al.

(2023), where instead of spraying the sample with liquid nitrogen, the entire contents of the unit (fish preserves) were vigorously homogenized by using a mortar for approximately 20 minutes (due to the fact that the parasite is heterogeneously distributed in the canned). Since the objective of our work was to extract the DNA from the homogenate of canned mackerel and to ensure its recovery by the proposed method, the tests detailed below were carried out.

2.3.3.1. Variant of the extraction method: Experimentation in the draining liquid

To extract the draining liquid, the mortars containing the homogenized preserve mixture were inverted at an angle of 45° for approximately 10 minutes. At this stage, a small experiment was carried out in which this draining liquid was subjected to centrifugation. of 10,000 rpm for 10 minutes in order to verify if it was the supernatant or the precipitate that contained the best quality and quantity of DNA (once the extraction process was finished), once the experimentation process was finished, the phase that contained better concentration and quality of DNA, this could be evaluated in the NanoDrop™ Spectrophotometer ND-10 equipment (Thermo Scientific, Germany). Unlike the protocol (Ramilo et al., 2023) where 100 mg of tissue samples were used, 300 µL of centrifuged draining liquids were used (See results for more details on this step) in an Eppendorf tube and 1 mL was added. of 2X CTAB, prewarmed to 60 °C, was mixed by inversion and incubated for 10 minutes at room temperature, followed by 10 minutes at 0 °C. Next, 10 µL of proteinase K (Promega, USA) (10 mg/µL) was added, mixed by inversion and incubated at 65°C for 25 minutes and then incubated for 5 min at 0°C and consisted of adding 600 µL of phenol:chloroform:isoamyl (25:24:1) and shake by inversion. Then it was centrifuged at 10,000 rpm at 8 ºC for 12 min. After carefully recover 200 µL of the supernatant and place it in a new 1.5 mL microcentrifuge tube. 50 µl of 10 M ammonium acetate was added and mixed by inversion several times. Then 500 µL of cold (-20 ºC) isopropanol was added and mixed by inversion several times. The mixture was kept at -20ºC for 2 h, to favor DNA precipitation, and then centrifuged at 10,500 rpm at 8 ºC, for 5 min. Finally, it was rehydrated in 50 µL of TE 1X buffer, the quantity and quality of the extracted DNA was evaluated in a Nano Drop ™ Spectrophotometer ND-10 (Thermo Scientific) equipment and stored at -80 °C until further use.

2.3.4. Variation in DNA Amplification

During the DNA amplification stage by means of the Polymerase Chain Reaction (PCR), the first DNA amplification tests of the Anisakids were carried out based on the recommendations of (Bello et al 2021) with some variants in terms of the number of amplification stages (which are detailed in the results of this investigation). The cytochrome c oxidase subunit II (COX II) gene was amplified by PCR, using the A211 and A210 primers, described by Steven et al (2000), which generate a 629 bp amplicon.

Table 4. Primers A 211 and A 210 Steven et al (2000)

COX II	F5′-TTTAGATATATATTYRTATGTGTTCT-3′R	R5′-CACTCCTTATACAATCATAA-3′R

The PCR reactions were carried out in a thermocycler (Thermo Scientific, USA), containing in a final volume of 30µL, 150ng of DNA, containing 0.4 uM of each primer, Promega GoTaq™ G2 Green Master Mix (which contains 3mM $MgCl_2$), completing the final solution with ultrapure water. DNA from Anisakid specimens from the parasitology laboratory collection was used as a positive control, and nuclease-free water was used as a negative control. The amplification times were: 95 °C for 5 min; 35 cycles of 94°C for 30 s, 47°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. As a variant to the recommendations of Gea (2015), after the first PCR amplification, 3 µL of the final product were used as a template for a second reaction (double amplification). The same primers were used as in the

first PCR: A211 and A210, described above. The amplification times were the same: 95 °C for 5 min; 35 cycles of 94°C for 30 s, 47°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were visualized by agarose gel electrophoresis according to the specifications of section 2.2.4.

2.3.5. Determination of the performance criteria of the test used: Sensitivity: The diagnostic sensitivity of the test was determined by positive detection of the 48 preserves inoculated with parasites in the three government liquids (oil, tomato sauce, and water) and by the diagnostic sensitivity calculation formula recommended by (Stralen et al. , 2009).

diagnostic sensitivity= $\frac{True\ Positives}{True\ Positives + False\ Negatives}$ X 100

In the present work, the analytical sensitivity (detection limit) was determined through the method of serial dilutions of Anisákidos DNA that were subjected to the same transformation conditions of canned fish.

In addition, different fish-parasite proportions were determined by preparing preserves of (50 g) inoculated with different numbers of parasites (1-5) individuals, approximately 6 mg each, and it was distributed according to the plan for determining different fish-parasite proportions. parasite. (Table 5).

Table 5: Plan for the determination of different proportions of fish-parasite.

Sample 1	sample 2	Sample 3	Sample 4	Sample 5
1 whole parasite	2 whole parasites	3 whole parasites	4 whole parasites	5 whole parasites
50g mackerel	50g mackerel	50g mackerel	50g mackerel	50g mackerel

2.4. Phase C. Extraction, amplification and visualization of DNA in canned fish inoculated with other parasites

Analyses were performed on samples that were contaminated with parasites other than Anisakids in order to establish that there is no cross reaction (analytical specificity). Additionally, canned mackerel without immunisation was prepared in order to assess the alleged diagnostic specificity. In this phase, the procedures are described in detail in order to establish the analytical and diagnostic specificity. These procedures include collecting additional parasites and fish for later inoculation in the making of preserves, as well as extracting, amplifying, and visualising the DNA of the samples. The following scheme was followed in order to complete the work.

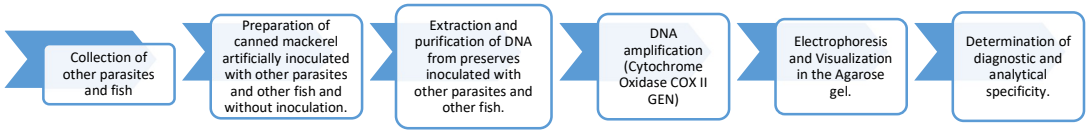

Figure 5. Scheme for Phase C of the work

2.4.1. Collection of other parasites: The parasites were collected by necropsy of the stomach and viscera of fish for direct human consumption marketed in the fishing terminal of Umm Qasr, Basra, Iraq (Table 5). Parasites were isolated and identified by evaluation of taxonomic characteristics using a stereoscope, and then preserved in 70% alcohol for use.

Table 5. Parasites and fish used to determine the analytical specificity of the assay.

Scientific name	Family	Organismos
Diphyllobothrium latum	Diphyllobothriidae	cestodes
Diphyllobothrium lanceolatum	Diphyllobothriidae	cestodes
Echinorhynchus trichiuri	Sphyriocephalidae	cestodes
Nybelinia coryphaenae	tentaculariidae	cestodes
Nybelinia surmenicola	tentaculariidae	cestodes
Rhadinorhynchus mariserpentis	Rhadinorhynchidae	Acanthocephalos

Scomber scombrus	Scombridae	Fish
Scomber japonicus	Scombridae	Fish
Mugil cephalus	Mugilidae	Fish
Rastrelliger kanagurta	Scombridae	Fish

In addition to this, before inoculating the preserves, 30 mg of tissues from other fish (other than mackerel) were collected in order to inoculate them and evaluate if there was a cross-reaction with these fish.

2.4.2. Preparation of canned mackerel artificially inoculated with other parasites and other fish and without inoculation: In glass jars with hermetic screw caps, 50 g of fish (83.39% of the total weight of the product), 10 mL of public liquid (16.6% of the total weight of the product) were placed, the liquid being water and approximately 30 mg of other parasites and tissues of other fish, as shown in Table 6. The flow of the preparation of the preserves was exactly the same as the flow of preparation of the canned mackerel inoculated with Anisakids, which were detailed lines above.

Table 6. Design for the preparation of preserves inoculated with other parasites and fish and non-inoculated preserves

Design of Elaboration of Preserves Inoculated with other parasites and not Inoculated	Canned Mackerel in Water inoculated with other parasites and fish	Uninoculated canned mackerel
Mackerel	50g	50g
Public liquid	10 mL Water	10 mL Water
Other parasites and fish	(30 mg)	0 individuals

2.4.3. Extraction, amplification and electrophoresis: DNA extraction was performed according to section 2.3.3.1. The DNA amplification step by means of the Polymerase Chain Reaction (PCR) was carried out according to section 2.3.4. The electrophoresis and visualization in the agarose gene were carried out according to section 2.2.4.

2.4.4. Determination of the performance criteria of the test used: Specificity: To determine that there is no cross reaction (analytical specificity), samples contaminated with parasites other than Anisakids were analyzed (Table 3). To determine the parameters of diagnostic sensitivity, analytical and diagnostic specificity, the data obtained were analyzed by means of descriptive statistics using the Excel program and by means of the following work formula (Stralen et al., 2009).

$$\text{Diagnostic specificity} = \frac{True\ blanks}{True\ blanks + False\ Positives} \text{ X } 100$$

3. RESULTS

3.1. DNA Extraction Results of Positive Controls

The DNA extractions from the positive controls by homogenizing the crude tissue of the preserved parasites yielded DNA concentration values greater than 800 ng.µL-1 and an 260/280 factor greater than 1.8, after which serial dilutions were made with free water. of nucleases until reaching a concentration of 150 ng.µL-1, which allows obtaining clear amplifications of the expected size in the development stage of the agarose gel. These positive controls were used to verify the visualization of the amplicons in the agarose gel in comparison with the blank controls (Mix of PCR + Water in replacement of the DNA) and the conserved samples that were analyzed.

Table 7. Concentration and purity of DNA extracted from Anisakids used as positive controls

S.NO	MUESTRA	Concentration (ng.µL-1)	Factor 260/280
1	Raw anisakids at 70% Alcohol used as positive controls	904.3	1.85
2		894.5	1.87
3		857.1	1.95
4		804.5	1.86
5		959.1	1.8

3.2. Results of the DNA Extraction of preserves inoculated with Anisakids

3.2.1. Results of DNA extraction tested in the supernatant and precipitate of the sample: An experiment was carried out in which this drained liquid was subjected to a 10,000 rpm centrifugation for 10 minutes in order to verify if it was in the supernatant or the precipitate. the one that contained the best amount of DNA, once the experimentation process was finished it was determined that the supernatant of the sample (drained liquid) was the one that contained the best concentration and quality of DNA.

Table 8: Concentration and purity of DNA extracted from the supernatant liquid and precipitate of canned fish inoculated with Anisakids.

		Supernatant		Precipitate	
S.NO	DNA concentration Sample	(ng.µL-1)	Factor 260/280	(ng.µL-1)	Factor 260/280
1	Drained liquid from canned mackerel inoculated with Anisakids	208.6	1.55	15.8	1.5
2		162.7	1.94	5.88	1.42
3		364.5	1.47	29.9	1.32
4		267.2	1.85	18.2	1.52
5		305.6	1.9	8.6	1.43

The DNA extractions carried out from 300 µL of the supernatant of the drained liquid product of the vigorous homogenization of the preserves inoculated with parasites, yielded much lower values of DNA concentration than that of the positive controls and the values of the 260/280 factor they were also minor.

3.2.2. Variant Amplification Results: For the amplification process, repetitions were experimented with only one amplification process in which, during the visualization process in the agarose gel, the bands could not be observed. Based on these results, it was decided to carry out a second amplification using 3 uL of the product of the first PCR, using the same primers as in the first PCR: A211 and A210, described above. The amplification times were the same: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 47 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 10 min. With this second amplification, it was possible to observe bands that evidenced the presence of Anisakids in these samples. Together with the DNA samples from the canned fish, blank samples were amplified, containing a final volume of 30µL, 1µL of each primer, 15µL of Promega GoTaq™ G2 Green Master Mix, 7.5 µL of ultrapure water and 3 µL of ultrapure water. instead of template DNA.

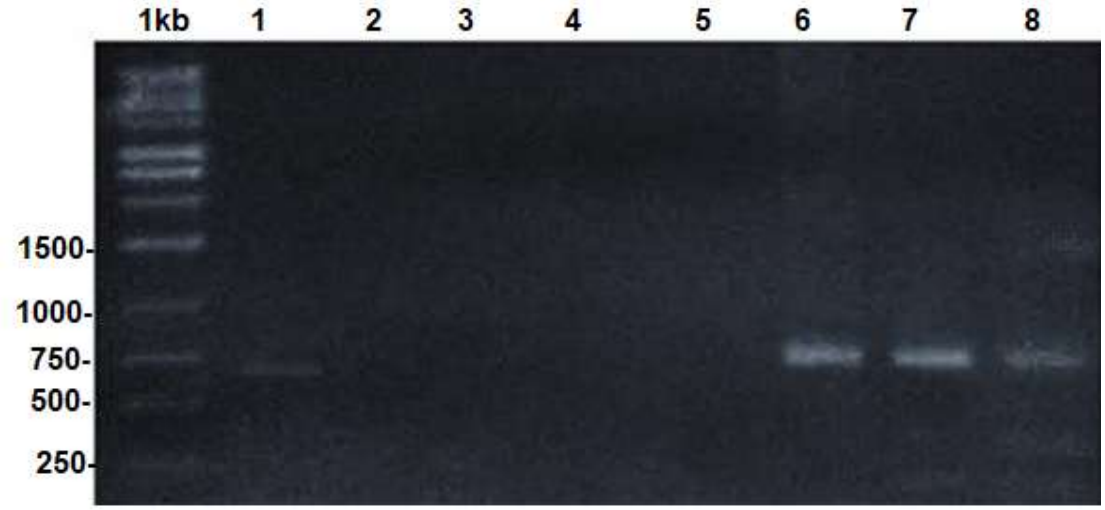

Figure 6. Detection of Anisakids in preserves inoculated from different government liquids differences between the first and second amplification. 1% agarose, stained with ethidium bromide. 1kb: Molecular weight marker; (1) Positive control; (2) Blank, (3) First amplification of Canned fish in Oil; (4) First Amplification of Canned Fish in Water (5) First amplification of Canned Fish in Tomato Sauce. (6) Second amplification of Canned fish in Oil; (7) Second amplification of Preserved fish in water; (8) Second amplification of Canned fish in tomato sauce.

Table 9. Table of data for the calculation of diagnostic sensitivity and diagnostic specificity in the detection of Anisakids in Canned Mackerel in Oil Inoculated and Not Inoculated with Anisakids.

Confidence level %: 95%		true diagnosis	
Canned Mackerel in Oil		inoculated	not inoculated
Proof	Positives	16	0
evaluated	negatives	0	16

- 16/ (16+0) x 100= 100% diagnostic sensitivity of the evaluated test of canned foods inoculated with Anisakids.
- 16/(16+0) x 100= 100% diagnostic specificity of the evaluated test of preserves in oil not inoculated with any parasite.

Data from Table 9 were used to determine the diagnostic sensitivity and specificity for finding anisakids in canned mackerel in tomato sauce that had been inoculated with the parasite or not.

3.2.3. Results of the determination of performance criteria of the present study

A 100% diagnostic sensitivity was found for the preserves in oil and tomato sauce, as can be seen in (Table 9) and (Table 10), and in their respective mathematical calculations. The diagnostic sensitivity of the test was determined by the positive detection of the 48 preserves inoculated with parasites in the three official liquids (oil, tomato sauce, and water). Based on the non-detection of DNA amplificates in negative controls (canned fish that had not been vaccinated with Anisakid parasites), the diagnostic specificity of the protocol was 100%.

Table 10. Table of data for the calculation of diagnostic sensitivity and specificity in the detection of Anisakids in Canned Mackerel in Tomato Sauce Inoculated and Not Inoculated with Anisakids.

Confidence level %: 95%		true diagnosis	
Canned mackerel in tomato sauce		inoculated	not inoculated
Proof	Positives	16	0
evaluated	negatives	0	16

- 16/ (16+0) x 100= 100% diagnostic sensitivity of the evaluated test of preserves in Tomato Sauce inoculated with Anisakids.
- 16/ (16+0) x 100= 100% diagnostic specificity of the evaluated test of preserves in Tomato Sauce not inoculated with any parasite.
- Only in one case of canning in water was a false negative result obtained, giving a final sensitivity of 93.33 with this public fluid.

Table 11. Data table for the analytical specificity in the detection of Anisakids in Canned Mackerel in Water Inoculated and Not Inoculated with Anisakids.

Confidence level %: 95%		true diagnosis	
Canned Mackerel in Water		inoculated	not inoculated
Proof	Positives	14	0
evaluated	negatives	1	16

14/(15+1) x 100= 93.33% of diagnostic sensitivity of the evaluated test of preserves in water inoculated with Anisakids.
16/ (16+0) x 100= 100% diagnostic specificity of the evaluated test of preserves in water not inoculated with any parasite.
Analytical sensitivity was determined by means of a sample of 5 Anisakid parasites that were subjected to 121°C for 55 minutes. It was used to carry out the DNA dilutions, for this the sample had an initial DNA concentration of 346.5 ng.µL-1 and a factor of absorbance of 1.47 and serial dilutions at 182.25 ng.ul-1, 91.125 ng.ul-1, 45.5 ng.ul-1 and 23.2 ng.ul-1 were carried out observed that first three values found positive and last two are found to be negative, from which it was determined that the technique evaluated in this investigation can detect up to 91.125 ng.µL-1, which is equivalent to 1.25 individuals of Anisakids.
In the present work, different fish-parasite ratios were determined by making preserves of (50 g) inoculated with different numbers of parasites (1-5) individuals, approximately 6 mg each. This test was able to detect up to 1 parasite (6 mg) per 50,000 mg of fish. The analytical specificity of the protocol proposed in this study was 100% for the three public fluids proposed, based on the non-detection of DNA amplifications from different species of fish and parasites that did not belong to the Anisakidae family (Figure 7). In (Table 3) the parasites and fish that were used for the specificity tests of the test are specified.

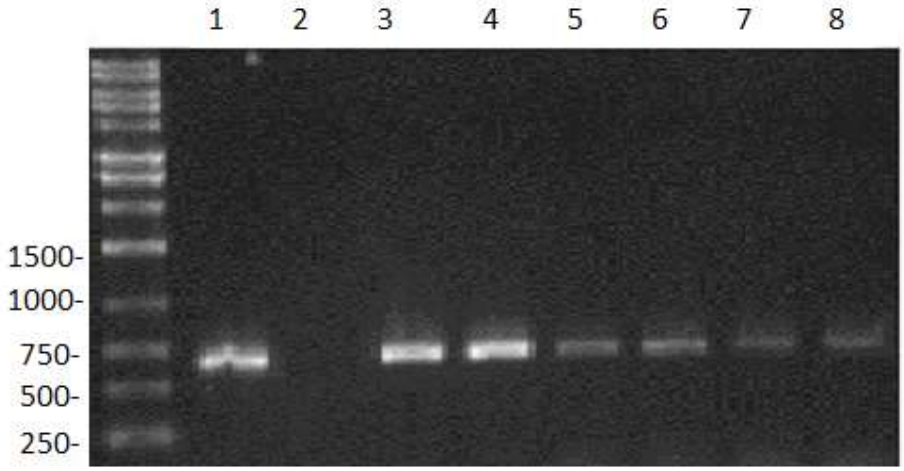

Figure 7. Determination of diagnostic sensitivity in preserves inoculated with Anisakids. 1% agarose, stained with ethidium bromide. 1kb: molecular weight marker; (1) Positive control; (2) Blank (3) Canned fish in oil inoculated with Anisákidos (I) (4) Canned fish in oil inoculated with Anisákidos (II); (5) Preserves fish in water inoculated with Anisakids (I); (6) Preserves fish in water inoculated with Anisakids (II); (7) Preserved fish in tomato sauce inoculated with Anisákidos (I) (8) Preserved fish in tomato sauce inoculated with Anisákidos (II).

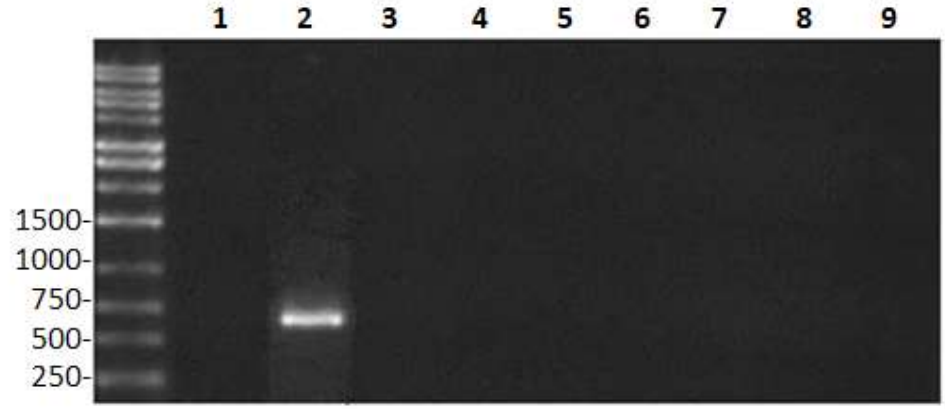

Figure 8. Determination of analytical specificity in preserves inoculated with other parasites distinct from the Anisakids. 1% agarose, stained with ethidium bromide. 1kb: Molecular weight marker;(1) Blank;(2) Positive control, (3) *Diphyllobothrium latum*; (4) *Diphyllobothrium lanceolatum*; (5) *Echinorhynchus trichiuri* ;(6) *Rhadinorhynchus mariserpentis*; (7) *Scomber japonicus*; (8) *Rastrelliger kanagurta*;(9) *Mugil cephalus.*

4. DISCUSSION

Studies (Kijewska et al 2001; Espineira et al 2010) on the molecular detection of Anisakids in various hydrobiological products have shown that it is possible to find parasites in samples that are undergoing extremely rapid degradation, like canned fish. According to a study by According to Espieira et al. (2010), even after canned goods were heated (at 115 °C for 50 minutes), proper DNA amplification could still be assessed. The objective of the current study is to show that anisakids can be found in canned fish samples using more traditional methods, such as the CTAB extraction method and DNA amplification using a thermocycler. The success of this goal will depend on the findings. results from the current study allowed for the detection of amplicons of 629 bp from the mitochondrial region of COXII subunit II. Instead of a mixture of tissues as in the works previously mentioned, a variation of the other studies was to detect the presence of Anisakids from the drained liquid product of vigorous

homogenization of the entire preserve. The issue of taking multiple samples from the same container is resolved by this variant, enabling multiple samples to be processed simultaneously (pools).

The use of a double amplification was another variation on the conventional techniques. Given this, it's important to note that the extraction process takes around 6 hours, followed by two PCR amplification phases that last 2.5 hours each (for a total of 5 hours), and then a process that takes 2 hours to run and visualise the sample on agarose gel. On the other hand, since handling these substances exposes people to UV rays and ethidium bromide, it is up to the researchers to decide whether to use more advanced techniques like real-time PCR or more conventional ones.

Mossali et al. (2010) used a maximum of 8 samples per product in their study of the detection and quantification of Anisakids in baby food and canned fish, while Lopez and Pardo (2010) used 4 canned fish samples and 1 baby food sample for the real-time PCR detection of Anisakis simplex. In the current study, we used a sample size of 16 canned fish per presentation, which was determined by estimating the proportions for an unidentified population of canned fish and arriving at an expected proportion of 94% with a 95% confidence level. 240 Anisakid parasites had to be collected for our study, which required a margin of error of 10%, making a total of 16 samples (canned mackerel in various public liquids). This was one of the limitations of previous research on the inoculation of parasites.

In numerous studies (Mishra, et al 2019; Aboul-Maaty et al 2019), the CTAB protocol is employed to isolate the genomic DNA of anisakid parasites from fish tissues and processed foods. The innovative aspect of this work was the use of the CTAB protocol from the drained liquid following the thorough homogenization of the entire preserve. The high lipid content of mackerel is responsible for the oily characteristics of the supernatant of the liquid produced by vigorous homogenization in the three governing liquids examined in this study. The work by Yunjing et al. (2018), which compared five DNA isolation methods with 25 commercial vegetable oils made from soybean, peanut, corn, sunflower, rapeseed, and mixed oils in order to establish an effective method of DNA isolation from vegetable oils, is evidence that DNA extraction methods in oils are more successful with the CTAB extraction method. Only the DNA samples isolated using the cetyl trimethyl ammonium bromide (CTAB) method were able to amplify the tRNA-Leu gene, according to Nanodrop 2000 spectrophotometry, which assessed the quality of the isolated DNA. Additionally, the Ct values of the endogenous reference genes specific to each species were higher than 36.

5. Conclusions and Recommendation

5.1 Conclusions

In the present study, analytical sensitivity was determined through DNA dilutions, at an initial DNA concentration of 346.5 ng/L and an absorbance factor of 1.47, from which it was calculated that the analytical sensitivity was 4.8 mg of parasite in 300 mg of fish tissue using the Espineira et al. (2010) and Herrero et al. (2011) methods. However, they did not specify the methodology to determine analytical sensitivity. The proportion method (fish/parasite) also allowed for the detection of up to a full 6 mg of parasite per 50,000 mg of fish. Since the test's sensitivity and specificity indices did not significantly differ from those of the RT-PCR method reported by Herrero et al. (2011) and Espieira et al. (2010), who reached 99% efficiency in their studies despite using different genes and PCR models, it was determined that the government liquids do not serve as limitations in the extraction and amplification of DNA. Since the primary goal of the current study was the detection of Anisakids DNA in them, no tests were conducted to determine the expression of heat-resistant proteins of Anisakids following a sterilisation process (autoclaving) (121°C x 60 minutes). Due to the extreme conditions of polymerase chain reaction degradation, we are unable to conclusively state that canned mackerel contaminated with parasites can be toxic to individuals who have already

developed a sensitivity to it. The evaluated molecular technique detected the presence of artificially inoculated Anisakid parasites in canned mackerel, through 3 different government liquids (water, oil and tomato sauce). The molecular technique evaluated was able to amplify fragments of 629 bp, through a double amplification PCR protocol, even after processes such as commercial sterilization. The diagnostic sensitivity of the evaluated technique was 100% for preserves whose governing liquid was oil and commercial tomato sauce and 93.33% in the case of preserves in water. The analytical sensitivity of the evaluated technique was 91.125 ng.µL-1 of parasite, which was determined by serial dilutions. likewise, using the proportions method (fish/parasite), it was possible to detect up to a whole parasite of 6 mg per 50,000 mg of fish. The evaluated technique had 100% analytical and diagnostic specificity However, because of the parasite inoculation methodology and autoclaving conditions, we are able to compare our findings with studies relating to the detection of heat-resistant proteins of Anisakids (Carballeda et al., 2014), which after autoclaving processes However, Ani S4, which is also resistant to high temperatures and pepsin screening tests, is the other allergen that more than 27% of patients with allergic symptoms have identified.

Due to its accessibility and lack of the need to collect multiple samples from a single container as in other PCR methodologies for tissue samples, the technique proposed in this study is expected to be used in Iraq for quality control in the industry. This eliminates the risk of collecting non-contaminated tissue and prevents false negative results. This problem was resolved by homogenising all samples from the container.

5.2 Recommendations

- Examine whether the production of canned fish has an impact on the molecular detection of parasite DNA and proteins. Although it cannot be demonstrated that allergenic proteins are discovered when the DNA is degraded to its maximum, this study examined the molecular detection of anisakid DNA.
- It was challenging to collect enough parasites to validate the reviewed technique because 240 Anisakid parasites were used.
- Because fish sold in markets is already postmortem, the ideal research of the customary detection method in preserves is constrained. Before processing, the parasite should be "alive" in the muscle, secreting its juices and toxins. Due to this restriction, all of the anisakid larvae used in this study were dead.

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