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Detection of anisakids in fish and seafood products by real-time PCR

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ABSTRACT

Anisakids are a group of widely distributed nematodes which have acquired high social relevance due to their involvement in foodborne infections caused by consumption of raw or undercooked seafood. A TaqMan®-LNA probe real-time assay targeting the *cytochrome oxidase* subunit I (COI) was developed allowing the simultaneous detection of the most important anisakids species present in fish and seafood products.

The determination of the detection limit in terms of ppm was 1 ppm

For the validation of method developed, twenty fish and cephalopod samples were experimentally contaminated with anisakid. It was checked that in cases in any anisakids species was present, it was detected because the Ct was always less than 35 and did not produce any case false negatives. The main novelty of this work lies in the fact that it can be applied to all kinds of processed products, including those undergoing intensive processes of transformation, as for instance canned foods. The proposed methodology is rapid, robust, highly sensitive and readily adaptable in routine molecular diagnostic laboratories, and can be employed as molecular screening method in order to assess the food security.

1. Introduction

Anisakids are a group of nematodes widely distributed at geographical level, parasitizing numerous hosts (fishes, cephalopods, mollusks, crustaceans, birds, reptiles, and mammals) (Anderson, 1992). This group includes many common parasites of marine mammals and marine fish-eating birds. *Anisakis, Pseudoterranova*, *Contracaecum* and *Hysterothylacium* are the most common genera that infect fish. Anisakids larvae have been found in numerous fish species destined to human consumption. According to the literature prevalence levels as well as parasitism degrees are very variable and they are dependent onsome aspects such as the fish species tested (Holst, Nielsen, & et al, 1993; Stromnes & Andersen, 1998), the geographical area (Arthur & Albert, 1993; Boily & Marcogliese, 1995; Moser & Hsieh, 1992), time of the year (Sewell & Lester, 1995), and the specimen individual characteristics (Smith & Wootten, 1978). They have acquired a high social relevance due to their involvement in foodborne infections mainly caused by the consumption of raw or undercooked seafood. Humans can only be considered accidental hosts of the larvae in stage 3 (L3) by eating raw or undercooked contaminated seafood (as for instance smoked, marinated, semi-preserved, salted or dried fish) (Ruitenberg, van Knapen, & et al, 1979). The disease caused by these parasites is well known (Chen, Yu, & et al, 2008; Dick, Dixon, & et al, 1991; Smith & Wootten, 1978). Symptomatic intestinal anisakiasis is often originated by the penetration of larvae into the gastric mucosa wall (Sakanary & JH, 1989; Ishikura, Kikuchi, & et al, 1993). Moreover, it can cause allergic reactions in sensitized patients as a result of its parasitism in the gastrointestinal tract, even if fish has undergone transformation process such as canning (Audicana et al, 1997, 2002).

The anisakidosis has become a public health problem due to two reasons: the increase in prevalence of parasitism in fish (Darwin & Fried, 2007), which can affect between 40% and 80% of the catch, depending on the fish species and origin (Cabezas, García, & et al, 2007; Yubero, Auroux, & et al, 2004); and the increase of new gastronomic trends based on consumption of raw or undercooked fish as, for instance, sushi (Pereira, 1992; Puente, Anadón, & et al, 2008).

Consideringthis situation, different Administrations have established regulations to prevent these health problems (EEC/140, 1993; Powers, Todd, & et al, 1997; EC/854, 2004; EC/853, 2004; EC 2074/2005).

The authorities of different countries perform visual inspections and controls of fish in order to detect any visible parasites and check the fulfillment of these regulations. At present, there are a lot of methods used for the detection of anisakid larvae in fish: non-destructive analyses, as visual control and transillumination with UV (Dixon, 2006, p. 7), white light (Levsen, Lunestad, & et al, 2005); or destructive methods (Jackson, Bier, & et al, 1981). These methods have drawbacks: the first ones are with low effectiveness and the second ones are more laborious and expensive and all of them have the disadvantage that cannot be applied for the analysis of

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processed products (such as surimi and precooked, canned or salted products) because the detection is based on whole parasites. In order to solve this situation, the immunological methods were tested. However, the long testing times, laborious procedures, cross-reaction with antigens from other nematodes (Akao, Ohyama, & et al, 1990; Iglesias, Leiro, & et al, 1996) and the protein degradation as consequence of the thermal treatment applied to processed products prevent the application of these techniques.

In this context, the molecular techniques offer an alternative to the previous methods. Different genetic techniques for the identification of Anisakis and other parasites have been developed in last years PCR–RFLP (Abe, Tominaga, & et al, 2006; Espiñeira, Herrero, & et al, 2010; Kijewska, Rokicki, & et al, 2002; Szostakowska, Myjak, & et al, 2002), multiplex or specific PCR assay (Chen et al., 2008; Santos, Sasal, & et al, 2006; Umeharaa, Kawakamia, & et al, 2007) SSCP analysis (Zhang, Hu, & et al, 2007; Zhu et al., 2007) and ALF (Espiñeira et al., 2010).

With the exception of methodologies alternatives developed by Espiñeira et al the great majority of genetic methods developed were proposed for the identification of some species which belong to genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*, but all of them have great drawbaks. The majority of them include a low number of species, generally belonging to anisakis genera (Farjallah, Slimane, & et al, 2008; Marques, Cabral, & et al, 2006; Umeharaa et al., 2007, 2008; Zhu et al., 2007; Zhu et al., 2007;). Other works include a higher number of species, but can not be applied to all kinds of processed products, including those undergoing intensive processes of transformation, as for instance canned foods (Chen et al., 2008; Kijewska et al., 2002). This is due to the large size of the PCR products amplified. The thermal treatment produces DNA fragmentation, and this fact prevents DNA amplification. In this sense, Quinteiro, Sotelo, & et al, 1998 determined that the maximum size of the DNA fragment amplified in canned products is around 170 bp (Quinteiro et al., 1998). Other authors achieved amplification of fragments with higher sizes (Santaclara, Espineira, & et al, 2006; Terol, Mascarell, & et al, 2002).

A novel genetic technique for species identification is the application of specific DNA probes with the method of RT-PCR. This methodology has become an important technique in many fields of food industry due to its reliability, sensitivity, specificity and rapidity. Owing to these reasons, in the present study a methodological alternative based in RT-PCR method has beendeveloped to make the detection of anisakid larvae in any fish or seafood products possible. The RT- PCR systems are easily disposal in control laboratories. Therefore, this molecular system will be suitable to ensure the absence of the studied parasites in fish, cephalopods and seafood, contributing to improve the safety of fish products and consequently consumers' health.

2. Materials and methods

2.1. Samples materials and DNA extraction

L3 stage larval worms of *Anisakis* spp., *Pseudoterranova* spp., *Hysterothylacium* spp., *and Contracaecum* spp. were isolated from the viscera of fish destined to human consumption (Table 1). These samples were obtained from different fish markets and shops in Spain. The larvae were isolated and washed in physiological saline serum (pH 7.3) *(Braun)* and classified on the basis of morphological characteristics (Berland, 1961, 1989; Fagerholm, 1982; Koie, 1993; Olson, Lewis, & et al, 1983; Smith, 1983) using a Leica MZ16 FA stereomicroscope. Anisakid larvae and fish samples were preserved in 70° ethanol and stored at −80 °C until processing for DNA extraction.

Genomic DNA of parasites was isolated and purified from individual larvae according to a standard CTAB phenol–chloroform protocol of Rogers and Bendich with slight modifications (Rogers & Bendich, 1985).

The DNA was extracted from 300 mg of fish muscle tissues and mixtures fish parasite in order to detect the presence of anisakid larvae in the analyzed samples. The DNA obtained was diluted in 100 μL of 1X TE buffer (Tris–EDTA, *Sigma*).

The quality and quantity were determined by measuring the absorbance at 260 nm and the 260/280 nm and 234/260 ratios using a *NanoDrop™ ND-1000* spectrophotometer *(Thermo Scientific)* (Winfrey, Rott, & et al, 1997). DNA extractions were appropriately labeled and stored at −80 °C for subsequent tasks.

2.2. PCR amplification and DNA sequencing

A fragment of 699 bp that includes a partial region of *cytocrome oxidase* subunit I (COI) was amplified by PCR using the primers *COIFALT/COIR-ALT* (Mikkelsen, Bieler, & et al, 2006).

In all cases the PCR reactions were carried out in a total volume of 50 μL with the following composition: 100–300 ng of DNA template were added to a PCR mix consisting of 0.8 mM dNTP mix *(Bioline)*, 5 μL 10X buffer, 2 mM $MgCl_2$, 0.75 units of *BioTaq™* DNA polymerase *(Bioline)*,

0.8 μL of a 10 μM solution of each primer *(Sigma Genosys)* and molecular biology grade water *(Eppendorf)* up to adjust to the final volume.

Polymerase chain reactions were carried out in a *MyCycler™* thermocycler *(BIO-RAD)*. Conditions of cycling were as follows: a preheating step at 95 °C for 3 min, 35 cycles of amplification (95 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min) and a final extension step of 72 °C for 3 min.

Polymerase chain reaction amplicons were visualized on agarose gels *(Sigma)* at 2% in TBE buffer with 5 μg/mL of ethidium bromide *(Sigma)* under UV light, using a *Molecular Imager Gel Doc XR System* transiluminator and the software Quantity One® *v 4.5.2 (Bio-Rad)*. Next, double-stranded DNA were purified using the *Nucleospin© 96 Extract II (Macherey–Nagel)* according to the manufacturer's instructions. The concentration and purity were measured by means of the *Nano-Drop™ ND-1000* spectrophotometer *(Thermo Scientific)* as described for DNA extraction. Subsequently, sequencing reactions of both DNA strands were carried out with the primers previously described in a final volume of 10 μL with *BigDye Terminator cycle sequencing ready reaction v1.1 (Applied Biosystems)* and sequenced on an ABI Prism 3130 *(Applied Biosystems)*. Next, these sequences were analyzed with *Sequencing Analysis Software* v5.3.1. *(Applied Biosystems)*and aligned with *Clustal W* (Thompson, Gibson, & et al, 1997) available in the program BioEdit 7.0 (Hall, 1999). The nucleotide sequences obtained were submitted to the *GeneBank* database of the *National Centre for Biotechnology Information* (NCBI).

2.3. Design of a specific RT-PCR method to detect anisakids

2.3.1. Design of PCR primers

From the resulting alignment specific primers/probe set for the four genera included in this study were designed by using *Primer Express software (Applied Biosystems)*. ANISAKIS COI F (5′ GGK CYA TTA AYT YTA TRA CWA CTA C 3′), ANISAKIS COI R (5′ AAA GAW GTA TTM ARR TTA CGR TCV G 3′) and a LNA™ TaqMan™ probe labelled with ANISAKIS COI PROBE (5′ (FAM TCT ATT TCT TTG GAR CAY A 3′ TAM), the LNA nucleotides are underlined.

The tool Basic Local Alignment Search Tool (BLAST) available at NCBI, especially the Megablast algorithm was used to verify the maximum specificity of the primers/probe set with anisakid species

Table 1
Anisakids and hosts used in the present work. The parasites used as references in the present work belonged to the following host species: *Merluccius merluccius*, *Scomber scombrus*, *Gadus morhua*, *Solea solea*, *Lophius piscatorius*, *Brama brama*, *Salmo salar*, *Dicentrarchus labrax*, *Sparus aurata*, *Anguilla anguilla*, *Micromessistius poutassou*, *Thunnus obesus*, *T. alalunga* and *Katsuwonus pelamis*.

	Organism	Family	Species	Samples
Parasites		Anisakidae	*Anisakis simplex*	30
			A. pegreffii	15
			A. typica	18
			Pseudoterranova decipiens	27
			P. krabbei	12
	Nematodes		*P.cattani*	8
			Hysterothylacium aduncum	16
			Contracaecum osculatum	14
			C.rudolphii	9
		Dracunculidae	*Anguillicola crassus*	5
		Capillariidae	*Capillaria* spp.	2
		Gymnorhynchidae	*Gymnorhynchus gigas*	6
	Cestods	Bothriocephalidae	*Bothriocephalus* spp.	3
	Acanthocephalans	Neoechinorhynchidae	*Neoechinorhynchus* spp.	2
	Digeneans	Diplostomidae	*Diplostomum* spp.	5
	Myxozoans	Myxobolidae.	*Myxobolus cerebralis*	6
		Spraguidae	*Spraguea lophii*	8
	Microsporidans	Tetramicriidae	*Tetramicra brevifilum*	5
		Ceratomyxidae	*Ceratomyxa shasta*	2
	Myxosporeans	Myxidiidae	*Myxidium* spp.	2
	Coccids	Sarcocystidae	*Hemogregarina* spp	3
		Scincidae	*Eimeria* spp.	2
	Kinetoplastids	Trypanosomatidae	*Trypanosoma* spp	5
	Trematode	Dactylogyridae	*Dactylogyrus ramulosus*	3
Hosts		Scombridae	*Thunnus alalunga*	5
			Euthynnus lineatus	5
			Sarda orientalis	5
			Rastrelliger kanagurta	5
			Scomber colias	5
			Auxis thazard	5
		Engraulidae	*Engraulis encrasicolus*	5
		Pleuronectidae	*Limanda aspera*	5
	Fishes		*Microstomus pacificus*	5
		Bramidae	*Brama australis*	5
		Salmonidae	*Salmo salar*	5
			Oncorhynchus mykiss	5
		Moridae	*Saliota asutralis*	5
		Carangidae	*Decapterus macarellus*	5
		Gadidae	*Gadus morhua*	5
		Merluccidae	*Merluccius merluccius*	5
	Cephalopods	Loliginidae	*Loligo vulgaris*	5
		Octopodidae	*Octopus vulgaris*	5
		Ommastrephidae	*Todarodes sagittatus*	5
		Sepiidae	*Sepia officinalis*	5

belonging to the four genera included in this work and the minimum nucleotide similarity with other fish parasites and fish hosts.

2.4. PCR optimization

The PCR reactions were carried out in a total volume of 25 μL containing 50 ng of DNA template, 12.5 μL of *SsoFast™ Probes Supermix (BIO- RAD)*, the amount of primers and probe that were optimized and molecular biology grade water *(Eppendorf)* up to adjust to the final volume.

Optimal amount of primers and probe were evaluated by preparing dilution series. A common range of working stock concentrations of 50, 100 and 300 nM of each primer and 50, 200 and 400 nM of the LNA™ TaqMan™ probe was used to determine the optimal concentrations.

The reactions were performed in *iQ 96-well PCR plates (BIO-RAD)* covered with *iCycler iQ™ Optical Tape (BIO- RAD)* and reactions were run in triplicate on *B*io-Rad *iCycler iQ™* Real Time PCR instrument. The annealing temperature of real-time PCR assay is one of the most critical parameters for reaction specificity. In order to find the optimal annealing temperature of reaction, a range of temperatures was tested with the following thermal cycling protocol: 95 °C for 3 min followed by 40 cycles of 95 °C for 30 s and 50 °C-62 °C for 30 s.

2.5. Analytical specificity and detection limit (LDO)

Anisakid specific primers/probe set was tested on DNA extracts from fish parasites not belonging to the family Anisakidae and different species of marine fish and cephalopods suitable for human consumption, which act as hosts of the third larval stage of the four genera included in this work (Table 1).

The determination of the LDO of the methodology developed was established from DNA dilutions and mixtures of tissues of specimens belonging to the genera *Anisakis, Pseudoterranova*, *Contracaecum* and *Hysterothylacium* and fish and cephalopods species. All measurements were performed in triplicate from three processed samples independently. The limit of detection (LOD) was established as the lowest concentration of DNA of anisakids species which yields a fluorescent signal significantly different from the negative control.

Table 2
Kinds of transformation process applied to samples experimentally contaminated with anisakids for the validation of the methodology.

Samples	Parasite	Host	Transformation process	Ct
1	*A. simplex*	*Thunnus albacares*		29
2	*A. typica*	*Thunnus obesus*		27
3	*P. decipiens*	*Katsuwonus pelamis*		25
4	*H. aduncum*	*Scomber scombrus*	Canned	22
5	*C. osculatum*	*Loligo vulgaris*		30
6	*C. rudolphii*	*Dosidicus gigas*		31
7	*A. simplex and P. decipiens*	*Octopus vulgaris*		20
8	*A. pegreffi*	*Salmo salar*	Smoked	28
9	*P. catani*	*Gadus morhua*		22
10	*A. simplex*	*Merluccius merluccius*		17
11	*P. decipiens*	*Thunnus albacares*	Frozen	18
12	*H. aduncum and A. typica*	*Loligo vulgaris*		18
13	*C. osculatum*	*Solea solea*		22
14	*A. simplex and C. osculatum*	*Uroteuthis japonica*	Fried	23
15	*H. aduncum*	*Thunnus albacares*	Cooked	21
16	*A. pegreffi*	*M. merluccius*		22
17	*A. simplex and P. decipiens*	*G. morhua*	Salted	19
18	*H. aduncum*	*Clupea harengus*		21
19	*C. rudolphii and P. cattani*	*G. morhua*	Dried	16
20	*A. simplex and A. typica*	*Brama brama*		17

In the first case the range of extracted DNA from anisakid varied between 50 ng/μL and 1 pg/μL. The dilutions were prepared by adding DNA from different fish species. In the second case the mixtures parasite-fish were prepared using percentages from 100% to 0.016% of anisakid tissue. The DNA extraction was performed from these mixtures of tissue to evaluate the minimum ratio anisakid/fish tissue that can be detected with the developed method.

2.6. Methodological validation

Twenty fish and cephalopod samples were experimentally contaminated with anisakid. For this task eviscerated fish and cephalopods and anisakid were cut into small pieces and then were carefully mixed. These samples were later subjected to different transformation processes. Canned, smoked, frozen, fried, cooked, salted and dried products were elaborated in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology). The most extreme treatments applied were sterilization in a horizontal retort steel-air, at 115 °C for 50 min with 1.2 bar of overpressure (cans of 125 mL), and the smoking process, which combined two effects: on the one hand salting and drying steps and on the other the effect of temperature. The temperature corresponding to smoke treatment of the fillets was raised to 121 °C, while inside the product was 60 °C. The cooking time depended on the thickness of the samples.

The amount of parasite tissue used to contaminate the samples employed in the methodological validation corresponded to the minimum amount detected during development of the methodology.The processing to which each sample has been subjected is detailed in Table 2.

3. Results

From sequences obtained (accession numbers HQ268717-HQ268729) one internal region for designing the primer/probe set ANISAKID COI was selected. This set generated PCR products of 176 bp allowing the detection of anisakid species.

BLAST analysis showed that the species specific primers and probe gave a 100% match to species targeted by the assay.

The conditions that allow obtaining best results were established by means of primers and probe matrix. The concentrations of 100 nM for both primers, 400 nM for the probe yielded the best results in terms of specificity and sensitivity.

3.1. Specificity and sensitivity of PCR

Assay specificity of developed RT-PCR was confirmed when amplification in different species of fishes, cephalopods and parasites not belonging to the family Anisakidae was not detected Fig. 1. No cross-reactivity was detected with any of the tested samples. In this way the optimal annealing temperature of 52 °C was established, in order to assure the highest sensitivity and specificity of the developed methodology.

Serial dilutions of genomic DNA extracted from anisakids were tested to assess the sensitivity of the RT-PCR assay. The total DNA quantities used for the PCR reactions were obtained by mixing fish DNA with decreasing amounts of DNA from anisakids. The

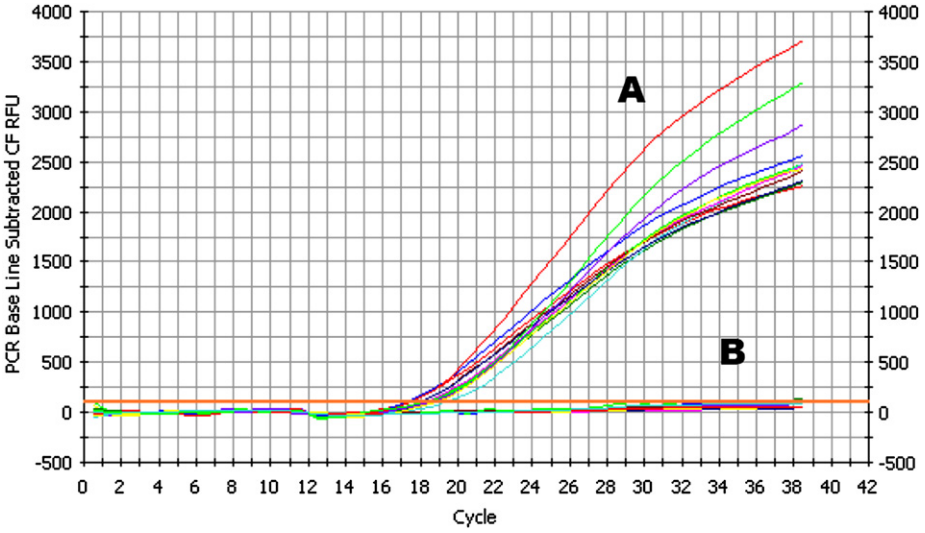

Fig. 1. Specificity of the RT-PCR assay. A: amplification pattern shown by anisakids species, with C_t values about 18 ± 2. B: amplification pattern shown by others parasites and host fish and cephalopods with C_t values > 35.

detection limit for species belonging to the four genera included in this work, employing dilutions of genomic DNA was 2 pg.

Also, DNA extractions were performed from mixtures of fish and anisakids to estimate the optimal amount of tissue necessary to obtain a high sensitivity. Specifically DNA was extracted from 300 mg of mixtures of fish and parasite in different proportions. The diagnostic method herein designed was applied to these mixtures, allowing the establishment of the minimum amount of anisakid tissue which can be detected in seafood products in the conditions previously described. The detection limit is lower than 0.016% of anisakid tissue using 300 mg of tissue for DNA extraction. It has not been possible lower measures due to the restrictions in analytical weight. The determination of the detection limit in terms of ppm was 1 ppm.

In all samples that contained anisakids species the average Ct values obtained were ranged from 16 to 35. On the other hand, in the cross-reactivity analysis no false positive results were observed, under the stringent assay conditions used, as documented by Ct values >35. Significant differences were not found in the Ct values for each of anisakids species analyzed.

The efficiency of the developed method was calculated basingon the slope of the standard curve obtained using DNA serial dilutions with template of known concentration (tenfold dilutions from 10 ng to 10 pg) as templates for RT-PCR (Pfaffl, 2004, pp. 87–120).

The amplification plot of the experiment using primer/probe set ANISAKIS COI generated a slope of −3.35 or 99% efficiency, with a correlation coefficient of 0.999. The amplification efficiency (E) was calculated using the equation $E = (10^{(-1/slope)}\text{-}1) \times 100$. These values of Ct and efficiency demonstrated the utility of the RT-PCR system to identify anisakids.

3.2. Methodological validation

The aim of the methodological validation was to check whether the manufacturing process which the food had undergone had no influence on the detection of anisakids species. The elaborated products in the pilot plant of CECOPESCA were analyzed by the proposed methodology. In the samples elaborated in controlled conditions and experimentally contaminated with anisakids, the added parasites were detected independently of the kind of processed product considered.

However, the high temperature and pressure to which the autoclaved products, such as canned fish, are subjected produces DNA fragmentation and will make the increment of the Ct value in these products. Despite this, it was checked that in cases in which any anisakids species was present, it was detected because the Ct was always less than 35 and did not produce in any case false negatives. Therefore, the RT-PCR system might be applied to fresh, frozen, precooked and canned fish.

4. Discussion

Mitochondrial gene has been targeted in multiple PCR systems because it is present in multiple copies and is highly conserved allowing to design specific primers-probe sets and, in a parallel way, to increase the sensitivity of the PCR. For these reasons, COI sequences of anisakids species were utilized to design a specific RT-PCR method for the detection of these parasites in fish and seafood products.

The introduction of RT-PCR assays to the microbiological control has significantly improved the diagnostic of bacteria (Donofrio, Bestervelt, & et al, 2010; Kobayashi, Oethinger, & et al, 2010; Lee & Levin, 2010), viruses (Godoy, Kibenge, & et al, 2010; Panzarin, Patarnello, & et al, 2010; Yilmaz, Bostan, & et al, 2010), and parasites (Collins, Kerr, & et al, 2010; Grabner & El-Matbouli, 2009; True, Purcell, & et al, 2009). This technique is employed as molecular screening method in order to assess food security. This is due to the high level of sensitivity of RT-PCR, its rapidity and readily adaptable in routine molecular diagnostic laboratories.

In the great majority of previous studies the specificity of primers used were not tested with other related parasites or host fish (Abe et al., 2006; Kijewska et al., 2002; Szostakowska et al., 2002), among them worth mentioning, the work of Santos et al. (2006) which can lead to false positive assignment for unspecific or unexpected bands (Santos et al., 2006).On the contrary, in both our previous (Espiñeira et al., 2010) and current studies in which the specificity is checked with related parasites and numerous host fish and cephalopods belonging to different families and orders.

In most works the sensibility of the methods were not tested either, in some cases dilutions of DNA for each species are used for this task (Chen et al., 2008). The results obtained in the evaluation of sensitivity of methodology developed show that this technique is highly efficient as detection method, the sensitivity of RT-PCR of the methodology developed allows detection of the target in 2 pg.

The sensibility of this method is higher than the one obtained in our previously work, in which it was necessary 25 and 2.5 pg for ETB stained-AGGE and ALF methodologies for positive results respectively. In RT-PCR methodology, the highhest sensitivity is corresponded with earlier Crossing threshold (Ct) values. The Ct is the point at which fluorescence is first detected as statistically significant above the baseline or background. It is inversely correlated to the logarithm of the initial copy number. The threshold should be set above the amplification baseline and within the exponential increase phase. The higher the initial amount of sample DNA, the sooner the accumulated product is detected in fluorescence plot, and the lower the Ct value (Sigma–Aldrich). It is necessary to find the lowest Ct value and the highest final fluorescence value by means of appropriate concentrations of primers and probe.

The aim of methodological validation was to evaluate the correct operation of the system herein proposed. In this validation it was proved that the DNA fragmentation in fresh or frozen fish is not significant. However, in the case of fish that have undergone different treatments, the thermal and pressure processing generates DNA fragmentation. This was the case of cans or smoked products, where fragments of little sizes were formed (Espiñeira, Vieites, & et al, 2009; Quinteiro et al., 1998). We can conclude that the type of processing does not affect the correct operation of the methods, and these ones can be applied for systematic analysis.

The work by Espiñeira et al allows the simultaneous detection and identification of the most important anisakids species present in fish products. However,since all the parasites studied originate the same disease and allergic reactions the species identification is less relevant. Because of this, it is necessary the use of more efficiency, accuracy, simplicity, cost- and time- effectiveness methods. In this sense RT-PCR techniques are the most adequate for screening (presence/absence). If positive results are obtained, and it is necessary to identify the present species the methodology developed by Espiñeira et al., 2010 can be applied (Espiñeira et al., 2010).

Altogether, this work describes the development of an RT-PCR method for the detection of anisakids species in fish and seafood products (fresh, frozen, precooked, canned, or any other foodstuff, including those that have undergonean intensive thermal treatment). RT-PCR has becomean important technique in many fields of food industry. Although this technique is more costly than traditional PCR, this is offset by savings in subsequent costs and time (the entire procedure can be completed within 4 h), as post PCR processing steps are no longer required for detection of the PCR product.

This methodology is a powerful tool forthe food quality and security control for prevention of gastrointestinal anisakiasis and anisakis allergy.

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