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Quantitative SYBR Green qPCR technique for the detection of the nematode parasite *Anisakis* in commercial fish-derived food

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A B S T R A C T

The extensive presence of anisakids in fish for human consumption has become a problem of food safety and quality. The aim of this study was to develop and assess the performance of a quantitative SYBR Green qPCR assay for the detection and quantification of *Anisakis* DNA in fish by-products. L3 nematode larvae of *A. simplex* (*s.l.*) (n = 510), *A. physeteris* (n = 3), *Hysterothylacium* sp. (n = 10) and *Pseudoterranova* sp. (n = 1), isolated from blue whiting, horse mackerel and monkfish, were used for the optimization of the molecular assay. In addition, molecularly typed larvae of *A. simplex* (*s.s.*) (n = 10) and *A. pegreffii* (n = 5) of the complex *A. simplex* (*s.l.*) were used for the specificity assay. Primers targeting the mitochondrial cytochrome *c* oxidase subunit II gene (COII) were selected. Analytical sensitivity and reproducibility were evaluated in a food matrix consisting of commercial fish-derived food spiked with larvae of *A. simplex* (*s.l.*). The assay proved to be specific for the three analyzed *Anisakis* species. A high reproducibility and sensitivity was detected, with a 95% limit of detection (LOD) of 0.30 ng ($_{95\%}$CI 0.15–1.50) of *A. simplex* (*s.l.*) DNA per gram of food matrix and an operative LOD of 1.50 ng after a PROBIT analysis. The assay was applied to study the presence of *Anisakis* in four types of processed commercial food, namely crab sticks, "gulas", croquettes and burgers. Overall, 180 food samples from 15 commercial brands were studied, detecting *Anisakis* DNA in over half of them. The analyzed surimi-based products, "gulas" and crab sticks, showed the highest *Anisakis* burden (5.86 ± 0.69 and 4.68 ± 0.73 ng of *Anisakis* DNA per gram of food, respectively). Our results indicate that the optimized SYBR Green qPCR technique is an accurate and sensitive method that may improve detection of *Anisakis* in fresh and processed products.

1. Introduction

International trade in food products has grown rapidly as a result of expanding urbanization, an increase in large-scale food production and changes in consumer tastes and preferences. In addition, greater knowledge of foodborne pathogens has heightened the concern of consumers and authorities about the safety and quality of food available in the marketplace, and has presented new challenges in terms of safety control measures (Ruzante et al., 2010).

The presence of anisakids in fish for human consumption has become a public health problem due to their role in gastrointestinal infections and allergic reactions (EFSA, 2010). These nematodes display a complex life cycle in the marine ecosystem, their hosts including a broad variety of commonly consumed fish, which transport the third-stage larvae (L3) responsible for human anisakiosis after accidental ingestion (Nieuwenhuizen and Lopata, 2014). Species of *Anisakis,* widespread among many fish species and geographical locations (Farjallah et al., 2008), are described as the main etiological agents, particularly two molecularly characterized sibling species of the complex *A. simplex* (*sensu lato*): *A. simplex* (*sensu stricto*) and *A. pegreffii*. Species of *Pseudoterranova* have also been involved in clinical cases, mainly in North and South America (Hochberg and Hamer, 2010; Torres et al., 2007), while species of *Contracaecum* have been rarely associated with the human disease (Shamsi and Butcher, 2011). Although *Hysterothylacium* species, very frequent in edible fish, have been described as the causative agent in one non-invasive clinical case (Yagi et al., 1996), it is currently accepted as non-pathogenic for humans (Angelucci et al., 2011).

Due to the difficulties in the prevention of *Anisakis* contamination throughout the food supply chain, as no maritime area can be presumed

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free of *Anisakis*, the presence of larvae in fishery products is considered a natural condition, with a "presumption of infection", according to the European Food Safety Authority (EFSA, 2010). There is therefore an important need to develop simple and effective methods to ensure the quality and safety of fishery products. Food safety authorities currently perform different analytical methods for anisakid detection in fish: non-destructive techniques such as candling and the use of ultraviolet light, or destructive methods such as artificial digestion. These methods are based on visual detection and identification of whole or large fragments of larvae in fish (Fraulo et al., 2014; Levsen et al., 2005; Yang et al., 2013) and are therefore not suitable for determining the presence of the parasite in processed fish-based food products, due to the mechanical and temperature conditions that the production of this type of food usually entails.

Molecular methods, mainly based on real time PCR methodology (qPCR), are being increasingly applied for pathogen detection and quantification in fresh and processed food due to their high sensitivity, specificity and speed, being efficient even with damaged material. Bacteria such as *Salmonella* spp. (Malorny et al., 2004) and *Listeria monocytogenes* (O'Grady et al., 2008), and parasites like the protozoa *Toxoplasma gondii* (Gomez-Samblas et al., 2015), *Cryptosporidium parvum* and *Giardia duodenalis* (Kumar et al., 2016) have been detected in different food and water samples by qPCR using specific labeled probes. More recently, fluorescent dyes such as SYBR Green qPCR have been used for food pathogen analysis as an economical alternative to the use of specific probes. SYBR Green has proved to be a sensitive and specific method for the identification of different microorganisms, including Shiga toxin-producing *Escherichia coli* in meat (Brusa et al., 2015) and emetic *Bacillus cereus* in a variety of food, such as hamburger, cow milk or vegetable salad (Ueda et al., 2013).

PCR-based assays have been widely used for the taxonomic identification of larvae at the genus and species level (Chen et al., 2008; Zhu et al., 1998), but qPCR has been applied only recently for anisakid detection in fish and fish-derived products (Herrero et al., 2011; Lopez and Pardo, 2010; Mossali et al., 2010). In the present study, a SYBR Green qPCR-based assay was developed for the detection and quantification of *Anisakis* DNA and evaluated in a food matrix for direct use in fish by-products. The qPCR technique was applied in different types of fish-derived food samples acquired in retail stores to detect and quantify the *Anisakis* DNA burden.

2. Materials and methods

2.1. Nematode larval material

L3 of anisakid species, *A. simplex* (*s.l.*) (n = 510), *A. physeteris* (n = 3) and *Pseudoterranova* sp. (n = 1) and L3 of the raphidascaridid *Hysterothylacium* sp. (n = 10) were used for the optimization of the molecular assay. Larvae were isolated from commonly consumed fish purchased in retail stores in Barcelona. *Anisakis* and *Hysterothylacium* larvae were obtained from blue whiting (*Micromesistius poutassou*) and horse mackerel (*Trachurus trachurus*) specimens, and *Pseudoterranova* from a monkfish (*Lophius piscatorius*). To isolate the larvae, the body cavity and belly flaps of fish were examined by visual inspection, and internal organs were observed under a stereomicroscope. Larvae were identified morphologically (Koie, 1993; Petter and Maillard, 1988) and stored in physiological solution until DNA extraction. For specific identification, 15 *A. simplex* (*s.l.*) larvae were typed by PCR-RFLP of the ITS region (D'Amelio et al., 2000) as *A. simplex* (*s.s.*) (n = 10) and *A. pegreffii* (n = 5).

2.2. Food matrix

For analytical sensitivity and repeatability studies, samples of commercial fish-derived food spiked with larvae of *Anisakis* were prepared as follows. 40 *A. simplex* (*s.l.*) larvae were lysed in 400 μL of phosphate buffer solution (PBS, pH = 7.2) with 40 μL of proteinase K (Roche Diagnostics, Spain) at 55 °C overnight. From the lysed larvae, ten-fold serial dilutions were prepared using PBS, and each dilution was homogenized with a sample of 25 g of verified anisakid-free crab sticks and 100 mL of PBS. As a result, different concentrations ranging from 1.36×10^4 to 1.36×10^{-3} ng of *A. simplex* (*s.l.*) DNA per gram of processed food were obtained, calculating the mean DNA concentration of a single larva as 85 ng/μL after the analysis of 30 specimens. This procedure was performed 12 times, carrying out 2 DNA extractions in each case, which resulted in 24 DNA extractions from each *Anisakis* concentration per gram of processed food.

2.3. Food samples

Different commercial fish-derived food products were studied: 6 brands of crab sticks, 5 of "gulas", 3 of croquettes, and 1 of burgers; two commercial batches of each one were bought in different retail stores. According to the manufacturers' labels, the crab sticks and "gulas" were surimi-based, the burgers were salmon- and hake-derived, two brands of croquettes were described as hake products, and in one the fish species was undeclared. The purchases were immediately transferred to the laboratory and kept in the fridge or freezer as recommended by the manufacturer until analysis. All the analyzed commercial brands were assigned with letters from A to I; brands A, B, D, E and H were refrigerated products, while brands C, F, G and I were frozen foods. Twenty-five grams of the commercial fish-derived food were homogenized with 100 ml of PBS using a Stomacher-400 (Seward, UK) for 30 min. This sample preparation was carried out in triplicate. Afterwards, two DNA extractions were performed from each food homogenate, obtaining 6 DNA extractions from each batch of product, which resulted in 180 analyzed samples.

Verified larvae-free flesh of blue whiting and horse mackerel, as well as non-seafood products such as chicken and beef were used as extraction negative controls (ENCs) in the molecular assays. Flesh samples of 200 mg were processed for DNA extraction in triplicate in all cases.

2.4. DNA extraction

Genomic DNA was isolated and purified from anisakid larvae specimens, the food matrix, food samples and ENCs using the Wizard Genomic DNA Purification Kit (Promega, Spain), according to the manufacturer's instructions with some modifications. Before DNA extraction, a volume of 200 μL of a single larva in PBS or food homogenate was mixed with 600 μL of Nuclei Lysis Solution and 17.5 μL of 20 mg/mL Proteinase K (Roche Diagnostics, Spain) and incubated overnight at 55 °C. DNA was separated by precipitation and centrifugation, purified using ethanol at 70% (Sigma-Aldrich, Spain), and resuspended in the rehydration solution (10 mM Tris-HCl, 1 mM EDTA). The DNA concentration was measured by absorbance at 260 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, USA). DNA extracts were stored at − 20 °C until molecular analysis.

2.5. SYBR Green qPCR technique

A quantitative SYBR Green qPCR technique, targeting a small fragment of 96 pb of the mitochondrial cytochrome *c* oxidase subunit II gene (COII), was developed using primers QCYTCII forward (5′-AGTAA-GAAGATTGAATATCAGTTTGGTGA-3′) and QCYTCII reverse (5′-AAGTAA- ACTCAAAGAAGGCACCATC-3′) (Lopez and Pardo, 2010). The reaction mixture, a total of 10 μL, contained 1× SYBR Green Master Mix (Roche Diagnostics, Spain), 300 nM of each QCYTII primer and 2.5 μL of DNA as the template. All reaction mixtures were prepared in 384 optical well plates and amplifications were run in the LightCycler 480 Instrument II (Roche Diagnostics, Germany) with the following thermal conditions: 50 °C for 2 min, 95 °C for 10 min,

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