



Validation of a commercial kit aimed to the detection of pathogenic anisakid nematodes in fish products

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ABSTRACT

Anisakids are parasitic nematodes responsible for a zoonosis that occurs following the ingestion of fish and fish products infected with larvae belonging to the genera *Anisakis* and *Pseudoterranova*. Rarely *Contracaecum* is found in association with gastric/intestinal illness, while *Hysterothylacium* is commonly considered not pathogenic. Although Real Time PCR assays have been recently used with the aim to detect and quantify these parasites in food products, methods applied did not undergo through extensive validation process, a feature highly desirable or mandatory in the case of testing laboratories accredited for the ISO EN 17025:2005.

Here, a comprehensive study has been performed to validate a commercial kit based on multiplex real time PCR for the qualitative detection of *Anisakis* and *Pseudoterranova*.

Inclusivity/exclusivity trials were carried out on DNA from species of the genera *Anisakis*, *Pseudoterranova*, *Contracaecum*, *Hysterothylacium* and *Ascaris*, on fish intentionally contaminated with *Anisakis* spp. and *Pseudoterranova* spp. and on marine organisms as fish, crustacean and squid to test the commercial kit on a large sample. The assay gave positive amplification for several *Anisakis* and *Pseudoterranova* species, while providing no signal for the members of the remaining genera. Each sample was correctly assigned either to *Anisakis* or *Pseudoterranova*, thus indicating that no cross-reaction occurred. The LOD was determined using two independent standard curves. Robustness was assayed by using two different thermocyclers in three distinct laboratories with different operators. The establishment of a validation dossier will permit the use of the commercial kit for the detection of *Anisakis* and *Pseudoterranova* DNA in fish and fish products intended for human consumption by public or private laboratories, following the requirements regarding the quality assurance processes described in the ISO EN 17025:2005.

1. Introduction

Anisakid nematodes comprise a parasitic group widely distributed at geographical level, with a complex life cycle depending on aquatic ecosystem and various intermediate, paratenic and definitive hosts (Anderson, 1992; Køie, 2001). Adult females parasites release eggs excreted in the aquatic environment through the faeces of definitive hosts, as fishes, piscivorous birds and marine mammals. Larvae hatched from the eggs are ingested by intermediate crustacean hosts. When infected crustaceans are ingested by suitable paratenic hosts, such as fish or squid, L3s penetrate the intestine and encapsulate in tissues, particularly viscera. Following ingestion of infected paratenic hosts by a

suitable host, L3s develop to fourth-stage larvae and subsequently to adult males and females.

The occurrence of larval nematodes in fish or fish-products intended for human consumption causes economic and medical problems. Alongside with the loss of marketability of fish, larval nematodes belonging to Anisakidae family may cause a fish-borne zoonosis known as Anisakidosis, while nematodes of Raphidascarididae family as *Hysterothylacium* spp. are considered not harmful to humans or of negligible concern (Klimpel and Palm, 2011). Humans may in fact become accidental hosts acquiring the infection by consuming raw or lightly cooked fish and cephalopods contaminated with third-stage larvae. The genera of major zoonotic interest in the family Anisakidae

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are *Anisakis* and *Pseudoterranova*, and in particular the *Anisakis simplex* and *Pseudoterranova decipiens* complexes of species. Larvae of *Contracaecum* have been rarely associated with the disease in humans (Hochberg and Hamer, 2010; Shamsi and Butcher, 2011).

Experts from the European Food Safety Authority released a scientific opinion on control of zoosanitary parasite of fishery products, indicating protection and prevention as priorities and recommending a continuous research in parasites of public health importance in fishery products (EFSA, 2010; Picó-Durán et al., 2016). In fact, anisakids are considered a predominant biological hazard associated to seafood, due to the high prevalence recorded in the main commercially relevant species worldwide, including cod, hake, sea bass, frog fish, ling, blue whiting, saithe, redfish, forkbeard, and greater forkbeard (Abollo et al., 2001; Costa et al., 2016; Gómez-Mateos et al., 2016).

Several methods for the detection of anisakid nematodes in fish and fish products have been so far used. Most of them are based on visual inspections of the coelomic cavity and fillets, such as candling and UV visualization, but chloro-peptic digestion is also used. The Baermann technique, based on active migration of larvae from the host under the positive hydrotropism, proved also to be effective (Cavallero et al., 2015).

The use of molecular approaches, besides to the potential to be more sensitive, has the advantage of discriminating between species and genera, according to the specificity of the probes selected. This is particularly relevant in the evaluation of risk related to anisakidosis, given that pathogenic and not pathogenic anisakid species may be present in fishes and fish products, and for revealing alimentary frauds as these nematodes may be used as valuable biological tags for stock discrimination (Mattiucci et al., 2014b).

Screening and survey methods to recover anisakids presence based on Real Time PCR assays targeting nuclear and mitochondrial DNA have been recently used with the aim to detect and quantify parasites in food products (Fang et al., 2011; Herrero et al., 2011; Lopez and Pardo, 2010; Mossali et al., 2010). However, such methods did not undergo through extensive validation processes, a feature highly desirable or mandatory in the case of testing laboratories accredited for the ISO EN 17025:2005.

Here we describe a comprehensive study, carried out to validate a commercial kit ("PATHfinder *Anisakis/Pseudoterranova* DNA detection assay"). The kit is based on multiplex real time PCR for the qualitative detection of *Anisakis* and *Pseudoterranova*, using a TaqMan Probe assay, targeted on the internal transcribed spacers of the nuclear ribosomal DNA.

2. Material and methods

2.1. Study design

The investigated molecular method entails of a process aimed to verify kit functionality, specificity for the parasites of interest, sensitivity, accuracy and robustness of the procedure (i.e. the ability to correctly identify a true positive and a true-negative sample). The validation of the entire kit was conducted according to protocol, experimental designs and calculation methods described for qualitative validations. The terminology herein used follows the "Principles and methods of validation of diagnostic assays for infectious diseases" as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2016).

The first step of the validation pathway has examined the fitness of the amplification kit for the purpose of correctly and specifically detecting and identifying nucleic acids from *Anisakis* and *Pseudoterranova*. The following parameters were considered: analytical specificity in term of inclusivity and exclusivity, analytical sensitivity (limit of detection - LOD), by establishment of a standard curve using quantified reference material available, followed by serial dilutions and subsequent interpolation.

The second step was focused on the evaluation of accuracy and robustness.

The validation steps to verify the specificity of probes differentially designed on a nuclear multicopy region (ITS) for *Anisakis* spp. and *Pseudoterranova* spp. have been performed using reference material (already characterized DNA) and testing: i) parasitic species of interest, ii) a mixture of parasitic DNA together with fish tissue, iii) fish tissue without parasitic DNA.

2.2. Inclusivity/exclusivity

With the aim to test the analytical specificity of the assay, several parasitic nematodes species belonging to the superfamily Ascaridoidea were screened considering the evolutionary relationships of species of interest and their probable genetic similarity.

Parasitic material used was previously characterized at species level using specific molecular diagnostic keys for species belonging to *Anisakis*, *Ascaris*, *Contracaecum*, *Hysterothylacium*, and *Pseudoterranova* genera. Methods were based on PCR-RFLP of nuclear ribosomal ITS (Abollo et al., 2003; Cavallero et al., 2011; D'Amelio et al., 2000), sequencing of mitochondrial *cox2* (Mattiucci et al., 2014a; Valentini et al., 2006) and High Resolution Melting profiles analyses (Cavallero et al., 2014).

As previously mentioned, the following experiments were performed on: i) parasitic species of interest, ii) contaminated fish fillets with purified DNA of parasitic origin, iii) marine organisms tissues (fishes, crustaceans and squids).

In the first experiment performed using genomic DNA, six samples of the following species were tested in duplicate, also including internal positive and negative controls provided with the kit: *A. pegreffii*, *Anisakis simplex* sensu stricto, *H. aduncum*, *P. decipiens* sensu stricto, *Contracaecum* spp. and *Ascaris* sp. An additional experiment included other phylogenetically related species belonging only to *Anisakis* and *Pseudoterranova* genera, such as: *A. pegreffii*, *A. simplex* sensu stricto, hybrid form of *A. simplex/A. pegreffii*, *A. typica*, *A. physeteris*, *H. aduncum*, *P. decipiens* sensu stricto, *P. krabbei*, *P. azarasi*, *P. cattani*, *C. rudolphii* A and *C. rudolphii* B.

The second experiment was performed on fish fillets contaminated with *Anisakis* spp. and *Pseudoterranova* spp. DNA, in triplicate, together with twelve positive and ten negative internal controls.

The last experiment was carried out on a large number of fish, crustacean and squid species (see supplementary Table S1), using 2.5 ng of isolated genomic DNA.

2.3. Development of the calibration function

To quantify samples using standard curves, in-house reference genetic material isolated from anisakids was selected and the genetic copy number per nanograms of material was calculated, taking into account the *Anisakis simplex* sensu lato genome length of about 126,869,778 bp. Information on *Anisakis* genome are available at the link http://parasite.wormbase.org/Anisakis_simplex_prjeb496

Standard curves were developed following the seven dilution points indicated in Table 1.

Table 1
Dilution points used to develop standard curves for *Anisakis* spp. and *Pseudoterranova* spp.

Dilution point	ng	Copy
1	100	730,200
2	20	146,040
3	4	29,208
4	0,8	5841
5	0,16	1168
6	0,032	233
7	0,0064	46

Calibration function was calculated by linear regression analysis of threshold cycles (Ct) measured for each amplification vs. the log₁₀ copy number for each standard dilution. The measured quantity for each dilution level was determined in retrospect by using the formula $\log_x 1/4 [Ct - b] / a$, where a is the slope, b is the y-intercept, and x is the quantity in term of molecular weight. For replicates of standard curves developed for *Anisakis* and *Pseudoterranova*, data are reported along with values of slope, R^2 and efficiency of amplification. Standard curves developed for the two parasites of interest were tested on ten samples run in triplicate (five each parasitic genus) together with positive internal control of the kit. Moreover, volume of sample indicated in the kit quick guide for Real-Time reaction is 5 μ l, a value considered in the conversion to genomic copies.

2.4. Limit of detection

The limit of detection (LOD), a measure of analytical sensitivity, is the smallest number of genomic copy per assay that gives a PCR positive result in at least 90% of cases. For the LOD determination, five separated series for *Anisakis* and five for *Pseudoterranova* containing 292, 58, 11.68 and 2.37 genomic copies were analyzed.

2.5. Accuracy and robustness

Considering that the proposed kit is a qualitative assay, accuracy is here intended as the ability to identify correctly already characterized reference material. Robustness was determined by performing the experiments in three different laboratories using two different Real-Time thermocyclers (StepOne Plus - Applied Biosystem and BIOER 9600 series - BIOER, Hangzhou, China).

3. Results

3.1. Analytical and diagnostic specificity

3.1.1. Analytical specificity on isolated genomic DNA

By the use of previously mentioned isolated genomic DNA, positive amplification was obtained for the following samples in the first experiment (Fig. 1): *Pseudoterranova decipiens* sensu stricto, *Anisakis pegreffii*, *Anisakis simplex* sensu stricto and positive internal control while samples of *Ascaris*, *Contracaecum*, *Hysterothylacium* and internal negative control gave no amplification. In the second experiment, positive amplification was obtained for *Anisakis* species (*A. pegreffii*, *A. simplex* sensu stricto, hybrid *A. simplex/A. pegreffii*, *A. typica*, *A. physeteris*) using the FAM fluorochrome, for *Pseudoterranova* species (*P. decipiens* sensu stricto, *P. krabbei*, *P. azarasi*, *P. cattani*) using the HEX fluorochrome and positive internal control while samples of *C. rudolphii* A and B, *H. aduncum* and internal negative control gave no amplification. In all experiments, *Anisakis* spp. showed Ct ranging from 15 to 28, while *Pseudoterranova* showed Ct from 21 to 34.

3.1.2. Diagnostic specificity on contaminated fish fillets with parasitic genetic material

Results obtained on contaminated fish fillets confirmed results obtained using only parasitic DNA: the kit was able to amplify contaminated material and positive controls, while no amplification was obtained on negative controls. *Anisakis* spp. showed Ct values ranging from 24 to 31, while *Pseudoterranova* showed Ct values from 25 to 29. Results obtained on fish tissues without parasitic DNA gave no amplification for the samples under investigation.

3.1.3. Diagnostic specificity tests in marine organism tissues

Tests of amplification conducted on 38 fish species, 5 crustacean species and 8 squid species gave negative results after amplification with both *Anisakis* and *Pseudoterranova* probes, with the exception of herrings (*Clupea harengus*) and mackerels (*Scomber scombrus*) which

gave positive signals with the *Anisakis* probe at 26,47 Ct and 32,65 Ct, respectively.

3.2. Quantification by calibration function

The development of standard curves intended to quantify parasitic material of *Anisakis* spp. and *Pseudoterranova* spp. allowed to obtain DNA quantification by interpolation.

Calibration functions were calculated by linear regression of the threshold cycles (Ct) versus the logarithm of the anisakids concentrations per reaction. Values resulted to be $y = -3.5$ and a R^2 of 0,978 corresponding to a validated reaction efficiency of 93% for *Anisakis* spp. and to be $y = -4.14$ and a R^2 of 0.989 corresponding to a validated reaction efficiency of 78% for *Pseudoterranova* spp.

3.3. Limit of detection

The *Anisakis* LOD is 0,00032 ng/ μ l corresponding to 11,85 genomic copies for the reaction volume of 5 μ l; LOD for *Pseudoterranova* is 0,0016 ng/ μ l corresponding to 58,4 genomic copies for the reaction volume of 5 μ l.

3.4. Accuracy and robustness

The kit “PATHfinder *Anisakis/Pseudoterranova* DNA detection assay” showed an accuracy of 100% due to the ability to identify correctly already characterized reference material. Robustness was determined by performing the experiments in three different laboratories and results obtained were concordant.

4. Discussion

The occurrence of anisakid larvae in fishes and fish products has determined an increasing concern in the public opinion for the perception of food quality, and in food safety authorities and operators, since the presumptive presence of infective third-stage larvae in a wide variety of commonly consumed fish is considered a natural condition and sensitive and specific diagnostic methods are needed. In the Mediterranean area, human anisakidosis is probably associated to the consumption of traditional preparations such as marinated anchovies. However, the recent spread of other exotic dishes, such as sashimi or ceviche, may contribute to an increase of human cases. Beside to prevention measures, such as mandatory deep-freezing before serving or public advertisements, the development of high-resolution detection methods in fish and fish products represents an additional objective to reach a high standard of consumers' safety awareness. The availability of commercial kits for the detection of anisakid larvae in fishes and fish products is therefore of high interest in this context, as it provides to public and private laboratories with a ready-to-use tool, easy to apply and reliable.

On the other hand, one of the main goal of public and private laboratories is to provide evidence of high reliability of procedures, reagents, equipment, personnel qualification and data quality assurance.

Commercial kits which underwent validation by the manufacturers are of great utility for laboratories, as this step is part of the overall validation dossier of the operative procedure applied.

As the commercial kit represents a qualitative assay, the validation parameters used were analytical specificity, analytical sensitivity (limit of detection), diagnostic sensitivity, accuracy and robustness.

Specificity tests have demonstrated that the kit is able to distinguish the two target genera, *Anisakis* and *Pseudoterranova*, and to discriminate those two genera with respect to species belonging to other related genera, whose pathogenic impact on humans is still under investigation, such as *Contracaecum* (family Anisakidae) and *Hysterothylacium* (family Raphidascarididae). Although the specific probes for *Anisakis* and *Pseudoterranova* have proved not to cross-react each other and with

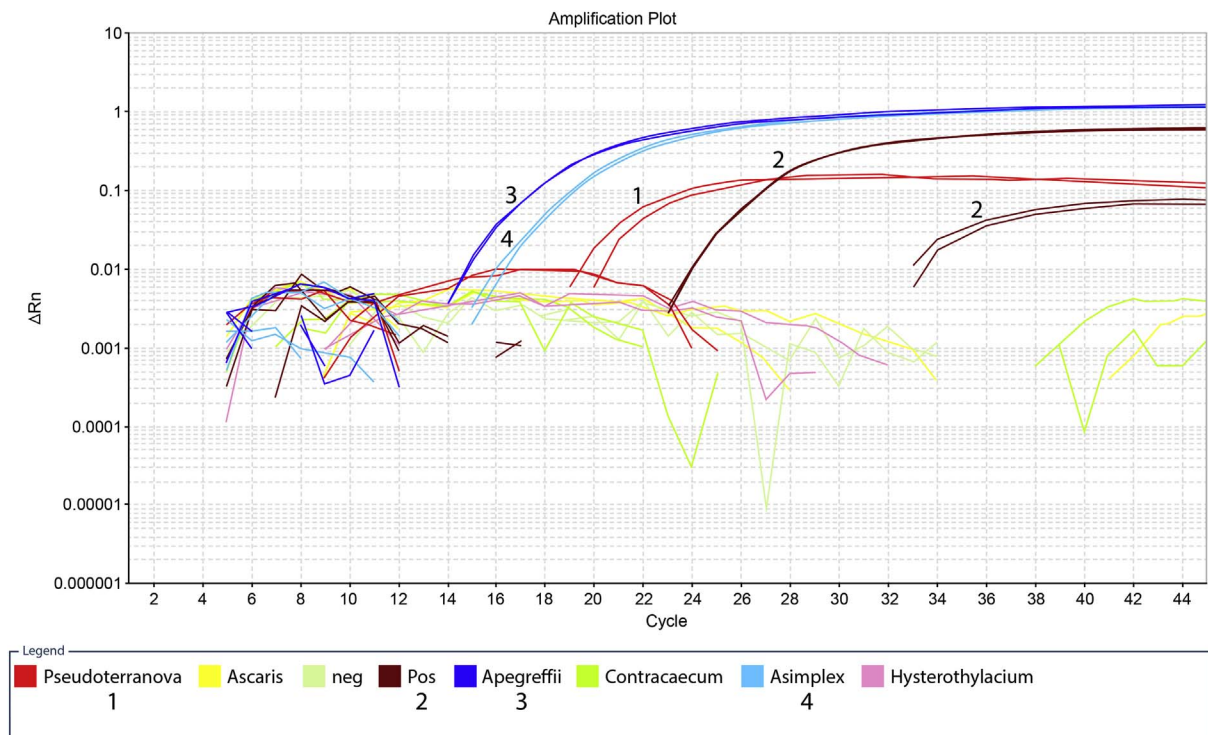


Fig. 1. Amplification plot of the parasitic DNA tested in the first experiment to verify specificity of the probes (DNA from the following parasitic nematode species: 1. *Pseudoterranova*; 2. positive internal control containing DNA from both *Anisakis* (left) and *Pseudoterranova* (right); 3. *Anisakis pegreffii*; 4. *Anisakis simplex* sensu stricto).

the other nematodes tested, the two reactions have shown to have different ability to recognize the targeted DNA region. This reflects also on the higher value of LOD detected for *Pseudoterranova*.

The selected probes resulted not to recognize DNA from different fish species tested, thus indicating a reliable direct application on fish and fish products matrices. The positive amplifications recorded using DNA from herrings and mackerels was likely due to the presence of *Anisakis* spp. DNA in the fish tissues, undetected after visual inspection.

The test evidenced a low number of genomic copies needed for the detection of the occurrence of anisakid DNA. The LOD of *Pseudoterranova* was around five-fold higher than that of *Anisakis*, probably as the result of a lower complementarity of the designed probes or due to different nucleotide composition of probes. In both cases, it appears that few cells may be sufficient to elicit the amplification process. This is of relevant importance, considering the allergenic potential of anisakid nematodes. In fact, anisakidosis may show different pathologic pictures, one due to the entire alive larva (gastric or intestinal anisakidosis) and another involving mainly an IgE mediated reactions presumptively exerted by antigenic residues.

Robustness was tested by applying the procedures in three different laboratories, using different thermocyclers and operators. This is of particular importance, as it provides evidence of the wide applicability of the kit, especially with regard to the fluorescent dyes used and the different filters available, based on overlapping emission spectra.

In conclusion, the increasing need to develop and assess diagnostic approaches for fish-borne parasites as anisakids should deal with the suitability for large-scale application in food market as well as with quality standards according to established international regulations elaborated to prevent these health problems.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jifoodmicro.2017.06.011>.

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