CPQ Microbiology (2018) 1:4
Research Article

CIENT PERIODIQUE

Evaluation of a New Loop-Mediated Isothermal Amplification (LAMP) Assay for the Detection of *Anisakis* spp.

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Received: 08 August 2018

Published: 20 August 2018

Keywords: *LAMP; Anisakis; Fish Products; Diagnostic Tool; Baby Food; Minimum Concentration Detected*

Abstract

Objective of the present study was to test the performances of a real-time LAMP-based field-friendly tool system for the detection of *Anisakis* spp., with particular focus on fish products. The specificity of the method was evaluated on *Anisakis* spp. larvae from internal collection. 100% of the *Anisakis* spp. strains tested were recognized, while, correctly, no amplification occurred for non-pathogenic *Hysterothylacium* spp. The sensitivity was evaluated in three independent trails conducted on intentionally infested at several intensities salmon fish fillets homogenate, on seabream commercial baby food and on domestic seabream baby food.

Results obtained showed a detected minimum intensity of 20larvae/kg in the first trial (infected salmon fillets homogenate), while in intentionally infected commercial and homemade seabream baby foods this minimum intensity was 4larvae/kg, in agreement with the limit suggested by the Codex Alimentarius for instruments intended for the identification of the presence of larvae in fishery products (5larvae/kg). The system did not give the same performances in the equivalent matrixes after thermal treatment inactivation. This LAMP method can be considered a very useful tool for the application to fish raw matrixes as it is a cost-effective and easy-functioning method, while in the detection of inactivated larvae for the prevention of possible allergic reactions, other studies should be performed.

Introduction

Parasitic nematodes of the genus *Anisakis* are all over the world considered as one of the biological hazard of major concern in seafood products [1]. Anisakidae larvae, at their third larval stage (L3), may be responsible for the zoonotic human infection known as anisakidosis, with Anisakis and *Pseudoterranova* as the two genera associated with this disease. In particular, among the nine species of the genus *Anisakis, A. simplex* complex and *A. pegreffii* were identified as responsible of several human infections, with *A. pegreffii* recognized as the most widespread in Italian waters [2]. Human ingests the parasite through the consumption of infected raw or lightly cooked seafood products. The subsequent migration of larvae through the gastrointestinal walls can determine the formation of an eosinophilic granuloma in the mucosa with severe non-specific symptoms [3]; for this reason, the disease is often misdiagnosed.

Moreover, *A. simplex* is the only parasite associate with fish consumption that could cause clinical allergic responses [1]. The main allergic response reported is the gastro-allergic anisakiasis (GAA), an IgE-mediated generalized reaction in which allergic manifestations (urticaria, angioedema-anaphylaxis, eosinophilic gastroenteritis, rheumatological and dermatological symptoms) are generally associated to the acute gastric symptomatology. The symptom generally arises after the consumption of raw or undercooked fish products that contain live larvae; in some cases, the gastric symptoms are mild or completely absent. In addition, in some cases, dead or inactivated larvae were previously described as cause of sensitization and immunoglobulin E (IgE)-dependent hypersensitivity in humans [4]. Furthermore, other immunoglobulin isotypes (IgG4) or non-immunological event, were also reported by Daschner *et al.* (2012) [5]. As showed by Caballero and Moneo (2004) [6], the cause of reactions and symptoms after the ingestion of well-cooked or canned fish was likely due to the presence of heat-resistant and/or pepsin-resistant allergens derived from *A. simplex*. Episodes of allergy and GAA were reported in several countries including Spain, Croatia, Italy and Morocco [4,7-11]. Moreover, gastro-allergic reactions due to the presence of Anisakis pegreffii have been reported in recent years also in Italy [12].

Currently, two methods for the evaluation of the presence of larvae are used: the non-destructive one based on visual inspection of fish fillets, and the destructive one based on artificial chloric-peptic digestion of fish muscle.

In recent years, many different screening molecular-based methods including SYBR green qPCR and multiplex PCR have been developed. Thanks to the potential high sensitivity and the ability to discriminate pathogenic from non-pathogenic nematodes larvae, different systems have been developed and evaluated for the detection of anisakid nematodes in fish and fish products [13-18].

Loop-mediated isothermal amplification (LAMP) is a relatively new real time DNA amplification technique, first described by Notomi *et al.* [19]. The main advantage of LAMP consists in the absence of complex thermal cyclers and its results could be obtained within few hours [20,21]. Thus, in recent years, the LAMP technique has been deeply studied and used as an easy, rapid and economic field-friendly tool for the identification of several potential pathogenic bacteria like *Salmonella* spp. and *Listeria monocytogenes* [22-26]. Anyway, only one preliminary study, focused on the suitability of LAMP technology when applied for the detection of *Anisakis simplex* [27].

The aim of the present study was to evaluate the performances of a LAMP-based method for the detection of *Anisakis simplex* in fish based matrix with particular attention to baby food, where heat-inactivated larvae could be present and responsible for allergic reactions in infants.

Materials and Methods

Evaluation of the Specificity of the Method

Eight *Anisakis spp.,* in particular three *Anisakis simplex sensu strictu,* four *Anisakis pegreffii,* one *A. ziphidarum* and one *Hysterothylacium* spp. were used to evaluate the specificity of method. These specimens belonged to the internal laboratory collection and were previously isolated from fish and identified by PCR technique. Parasites were kept at -80 °C until use. The producer declared that the instrument was able to detect the following species: *Anisakis simplex, Anisakis pegreffii, Anisakis physeteris, Anisakis typica and Anisakis ziphidarum.*

Protocol of Lamp Diagnostic Test for *Anisakis* spp.

For the tests, the system "ICGENE mini" (Enbiotech, Palermo, Italy) was used. The system is based on a LAMP technology, and is composed by a fluorescence amplifier, able to detect the fluorescence produced by the samples (it allows the simultaneously analyses of 12 samples), and a device that worked with Radio Frequency Identification technology (RFID), that guides all the activities step by step and is able to interpret in real-time the results obtained. For this research, specific kits named "ICGENE *Anisakis*" were tested. The operative protocol of the instrument, provided by the producer, is described in the sections below.

DNA Extraction

An aliquot of about 1 mg of each parasite was collected and suspended in extraction buffer and immediately homogenized by vortex to allow the extraction of nucleic acid by chemical lysis. The homogenate was then incubated at room temperature for at least 10 minutes and newly

Amplification (LAMP) Phase

Three µl of homogenate were inserted into primer mix tubes with 22µl of LAMP mix (Enbiotech, Palermo, Italy) containing a lyophilized primer and a master mix of reagents useful to carry out the test (enzyme, Magnesium chloride, nucleotides and reaction buffers) and 30µl of mineral oil. Then, tubes were vortexed with the aim to obtain a homogenous solution, and inserted into the amplifier for 60 minutes at 65°C; the positivity was assessed graphically by the development of sigmoid curve by the instrument. Positive and negative DNA control, included in the kit were also used.

Experimental Infestations

Harvest of Anisakis spp. Larvae

Larvae used for the intentionally infection were isolated from commonly consumed fish purchased in retail stores in Milan (Italy); in particular, *Anisakis* was obtained from mackerels *(Scomber scombrus),* caught in Atlantic Ocean (FAO zone 27). To isolate the larvae, body cavity and belly flaps were examined by visual inspection and the larvae isolated were observed by light microscopy in order to identify the genus [28] (6320D spectrophotometer, Jenway, Staffordshire, UK) and the preserved in saline solution until use as reported below.

Evaluation of the Sensitivity of the Method on Fish Matrix

For the evaluation of the sensitivity of the LAMP method, farmed salmons were chosen at this stage to avoid the presence of the parasite in fish muscle. Anyway, salmon samples were checked before use for absence of larvae. Salmon fish muscle (Sa) was intentionally infected with 8 known larvae concentration: 3000larvae/kg (Sa3000), 2000larvae/kg (Sa2000), 200larvae/kg (Sa200), 100larvae/kg (Sa100), 50larvae/kg (Sa50), 20larvae/kg (Sa20), 10larvae/kg (Sa10) and 4larvae/kg (Sa4); then infected fish muscle was homogenised. Afterwards, to assess the sensitivity of the technique, LAMP analyses were carried out in triplicate on each matrix. Control samples (not infected homogenised fish muscle) were also tested.

Evaluation of the Sensitivity of the Method on Seabream Commercial Baby Food

At this stage, to evaluate the sensitivity of the LAMP method, a commercial seabream baby food (Cs), was used. Each of baby food samples was intentionally infected with the larvae isolated from other fishes, as reported in section 2.5.1; five known intensity of larvae infection were tested: 40larvae/kg (Cs40), 20larvae/kg (Cs20), 10larvae/kg (Cs10), 4larvae/kg (Cs4) and 1larvae/kg (Cs1). Afterwards, to assess the sensitivity of the technique, LAMP analyses were carried out on each matrix at different infection intensities in triplicate. A control sample (not infected baby food) was also tested in triplicate.

Evaluation of the Sensitivity Of the Method on Homemade Baby Food

At this stage, farmed sea breams (farmed in Greece) were purchased. Fish muscle was homogenised and homemade seabream baby food (Hs) was produced following a common recipe [200g of fish muscle, 250 g of vegetables (potatoes and carrots in equal concentration), 400mL of water, 2g of oil, 30g of rice flour]. Fish muscle was then intentionally infected with larvae at 6 known concentration: 200larvae/kg, 100larvae/kg, 50larvae/kg, 20larvae/kg, 5larvae/kg and 1larva/kg, obtaining a final infection intensity in baby food equal to 40larvae/kg (Hs40), 20larvae/kg (Hs20), 10larvae/kg (Hs10), 4larvae/kg (Hs4), 1larva/kg (Hs1) and 0.2larvae/kg (Hs0.2), respectively. All the baby foods infected at each intensity were then dispensed in 80 g glass boxes and afterwards, LAMP analyses were carried out on each matrixes in triplicate. Control samples (not infected homogenised fish muscle) were also tested in triplicate.

Evaluation of the Sensitivity of the Method on Homemade and Commercial Sterilized Baby Food Fish Based Matrix

At this stage, to evaluate the sensitivity of the LAMP method, sea bream based baby foods were chosen. Each of these baby foods was intentionally infected with the larvae isolated from other fishes, as reported in section 2.3.1; five known intensity of infection were considered: 40larvae/kg (Css40), 20larvae/kg (Css20), 10larvae/kg (Css10), 4larvae/kg (Css4) and 1 larva/kg (Css1). Afterwards, baby food was sterilized at 105 °C for 75 minutes. Then LAMP analyses were carried out on each matrix at different infection concentration in triplicate. Control samples (not infected homogenised fish muscle) sterilized were also tested in triplicate. Homemade baby food was produced as described in section 2.3.4. Fish muscle was then intentionally infected with larvae at 6 known intensity: 200larvae/kg, 100larvae/kg, 50larvae/kg, 20larvae/kg, 5larvae/kg and 1larva/kg, final infested intensity in baby food equal to 40larvae/kg (Hss40), 20larvae/kg (Hss20), 10 larvae/kg (Hss10), 4larvae/kg (Hss4), 1larva/kg (Hss1) and 0.2larva/kg (HssA0.2), respectively. Baby foods infected were sterilized at 105°C for 75 minutes. Afterwards, LAMP analyses were carried out on each matrix in triplicate. Sterilized control samples (not infested homogenised fish muscle) were also tested in triplicate.

Results

Evaluation of the Specificity of the Method

By using the LAMP system, positive amplification was obtained for all Anisakis species tested *(A. simplex sensu strictu, A. pegreffi, A. ziphidarum and A. simplex/A. ziphidarum). Hysterothylacium* spp., correctly, was not identified by the LAMP system. Times of amplification ranged from 16 to 25 minutes.

Evaluation of the Sensitivity of the Method on Fish Matrix

Results obtained in infected fish fillets are reported in Table 1. The kit was able to amplify the parasites when present from 2000 to 20larvae/kg (3/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 300larvae/kg, probably due to the great amount of DNA present in the sample, and for samples below 20larvae/kg (10 and 4 respectively). Amplification times varied from 16 to 35 minutes.

Table 1: *Sensitivity of LAMP technique in intentionally infested raw salmon fish muscle (Sa) at different intensities.*

	Sa3000	Sa2000	Sa200	Sa100	Sa50	Sa20	Sa10	Sa4
Trial 1	-	+	+	-	+	+	-	-
Trial 2	-	+	+	+	-	+	-	-
Trial 3	-	+	+	+	-	+	-	-

SaA=3000larvae/kg;
SaB=2000larvae/kg;
SaC=200larvae/kg;
SaD=100larvae/kg;
SaE=50larvae/kg;
SaF=20larvae/kg;
SaG=10larvae/kg;
SaH=4larvae/kg

Evaluation of the Sensitivity of the LAMP Method on Homemade Baby Food

Results obtained in infested homemade fish based baby food are reported in Table 2. The kit was able to amplify the parasites when present from 40 (1/3 positive samples) to 4 larvae/kg (1/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 1 larva/kg of baby food. Amplification was not successful for the baby food infested with intensities equal to 10 larvae/kg, probably due to the variability during homogenization of the larvae. Amplification times varied from 20 to 35 minutes.

Table 2: *Sensitivity of LAMP technique in intentionally infested homemade seabream baby food (Hs) at different intensities.*

	Hs40	Hs20	Hs10	Hs4	Hs1	Hs0.2
Trial 1	+	+	-	-	-	-
Trial 2	-	+	-	+	-	-
Trial 3	-	+	-	-	-	-

HsA= 200larvae/kg fish= 40larvae /kg baby food;
HsB= 100larvae/kg fish = 20larvae /kg baby food;
HsC= 50larvae/kg fish = 10larvae /kg baby food;
HsD= 20larvae/kg fish = 4larvae /kg baby food;
HsE= 5larvae/kg fish = 1larvae /kg baby food;
HsF= 1larva/kg fish = 0.2larvae /kg baby food.

Evaluation of the Sensitivity of the Lamp Method on Commercial Baby Food

Results obtained in infested commercial fish based baby food are reported in Table 3. The kit was able to amplify the parasites when present from 40 (1/3 positive samples) to 4larvae/kg (1/3 positive samples), while no amplification was reported for samples characterized by infestation intensities of 0.2larvae/kg baby food. Amplification times varied from 20 to 35 minutes.

Table 3: *Amplification by LAMP technique in intentionally infested commercial seabream baby food (Cs) at different intensities.*

	Cs40	Cs20	Cs10	Cs4	Cs1	Cs0.2
Trial 1	+	+	+	+	+	-
Trial 2	+	+	+	-	-	-
Trial 3	-	-	+	-	-	-

CsA= 40larvae /kg baby food;
CsB= 20larvae /kg baby food;
CsC= 10larvae /kg baby food;
CsD= 4larvae /kg baby food;
CsE= 1larvae /kg baby food;
CsF= 0.2larvae /kg baby food.

Evaluation of the Sensitivity of the Method on Sterilized Homemade Baby Food and Sterilized Commercial Baby Food

The kit was never able to amplify the parasites in homemade or commercial baby food at each of the six intensities considered when sterilization happened after infestation at fixed intensities (data not shown).

Discussion

About 20,000 human anisakiasis cases were diagnosed worldwide before 2010; over 90% of notifications came from Japan, where around 2000 cases were described per year [1]. Also in Italy, 144 number of cases were stated in the period 2009-2013 [29]: in all the cases, the patients reported the consumption of raw or marinated fish. The incidence of this zoonosis is considerably increased in the last years due to the growth of the international market of fish and fish products and the development of new diagnostic tests that have proven anisakiasis as a worldwide zoonosis. Human anisakiasis is commonly frequent especially in those countries where culinary habits provide the consumption of large amounts of raw or undercooked fish (sushi, sashimi, ceviche or carpaccio). In the Mediterranean countries, human anisakiasis were related also with the consumption of traditional preparations (marinated fish, especially anchovies).

Food business operators and competent authority are particularly sensitive to "Anisakiasis" thematic, and, as the presumptive presence of larvae in many commonly consumed fishes is considered a natural condition, they feel the need of specific, fast and easy to use diagnostic systems, able to identify the presence of larvae of *Anisakis* in fish matrixes and fish-based products.

In the present study, a LAMP based system was evaluated with the aim to provide useful information in terms of specificity and sensitivity in different fish based matrixes; this real time, rapid and cost-effective field-friendly tool, may be convenient for public and private laboratories as results on the presence of larvae in a product may be available in a short time (less than 1 hour).

Specificity tests demonstrated that, the system was able to detect correctly all *Anisakis* species without amplification of non-pathogenic *Hysterothylacium* spp. In terms of sensitivity, the system showed good performances: in the first experiment where larvae were added to homogenate salmon fish fillets, the minimum intensity detected by the system was 20larvae/kg. Other study evaluated the performances of real time PCR assay with limit of detection of 1larva in 25g of fish tissue, corresponding to 40larvae in 1kg of fish (Lopez and Pardo 2010). The minimum intensity determined in the first experiment, was slightly higher if compared to the limits suggested by Codex Alimentarius (5larvae/kg fish) and to the suggested British commercial limit (3larvae in 3,2kg of fish).

When applied to homemade baby foods, the minimum infestation detected was 4larvae/kg of whole baby food (20larvae/kg in the fish muscle as it represents 20% of a baby food), in agreement with the limits reported above. Results obtained from commercial baby food were generally in agreement with those reported above with a minimum intensity detected established at 4larvae/kg of product, although in one of the three replications, the test was able to amplify the concentration equal to 1larva/kg of baby food. Positively, the system tested, did not show any interference or inhibition due to the presence in the food matrix of fat, minerals, polysaccharides, enzymes, glycogen, effects reported by previous studies on PCR assays [30].

Furthermore, the attention of the medical community has been moved because of the possible implications caused by *Anisakis* allergy in hypersensitive individuals. Kasuya *et al.* [31] in 1990, reported the first case of urticaria not related to a direct allergy to fish but due to sensitization to *Anisakis simplex*. *A. simplex* is, by now, the only species described to be able to cause allergic responses after consumption of fish products (EFSA 2010) [1].

As already stated by Ivanovic *et al.* (2017), food allergies are increasing faster if compared to the other allergic syndromes worldwide and due to the seafood industry globalization, the risk of acquiring anisakiasis in developed countries is miscalculated [32]. In addition, as reported by Moneo *et al.* (2017), several patients with no clear symptoms are underdiagnosed in endemic areas; this underestimation should be further investigated by the research community [33].

To match this goal, a constant and continue deepening especially in the development and the evaluation of tools useful for the research of these parasites in fish products, is strategical for the knowledge of the prevalence and intensity of alive but also dead parasites in fish products.

The second part of the tests was performed on the same concentrations of infested larvae in domestic and commercial baby foods submitted to a sterilization process. In this case, the thermal treatment that was mimicked by our sterilization process (105≈C for 75 minutes), determined the DNA degradation that impeded the amplification, and dead larvae were not recognized by the system. This fact was already reported for other systems for samples submitted to other thermal treatments [34].

Conclusions

The performances in terms of specificity and intensity of infection detected by the LAMP based system evaluated, indicated its appropriateness in the use in infected raw fish products with the aim to be a quality control point of care system advantageous for food operators and competent authority with the aim to avoid the infection with parasites. The detection of *Anisakis* genetic material in thermally treated products can be still considered a challenge for the performance of PCR-based methods, due to the modifications of DNA molecules, and the food industries should consider the control of the raw matter used for the production as a key phase in their control programs.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Funding

The present study did not receive any funding.

Conflict of Interest

Author Erica Tirloni declares that she has no conflict of interest. Author Simone Stella declares that he has no conflict of interest. Author Chiara Drago declares that she has no conflict of interest. Author Giuseppe Stampone declares that he has no conflict of interest. Author Mauro Vasconi declares that he has no conflict of interest. Chiara Coppola declares that she has no conflict of interest. Author Monica Caffara declares that she has no conflict of interest. Author Andrea Gustinelli declares that he has no conflict of interest. Author Cristian Bernardi declares that he has no conflict of interest.

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