Optimization and validation of a loop-mediated isothermal amplification (LAMP) system for the detection of *Anisakis* spp. DNA in processed fish products

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INTRODUCTION

Anisakis is a marine nematode of health interests because of their high zoonotical potential, being responsible of a human disease called Anisakiasis. Anisakiasis is a zoonotic disease caused by the ingestion of raw or undercooked fish infested with *Anisakis* larvae. Furthermore, several authors have reported this parasite to be a relevant inducer of acute or chronic allergic diseases. *Anisakis* nematodes have a worldwide distribution and their life-cycle involves invertebrates, fishes, cephalopods and marine mammals so, these parasites can be found in the muscles and viscera of numerous fish and cephalopod species . Parasites could be considered as reliable markers for the traceability of fish products. The EC Regulation n. 178/2002 defines the term traceability and regulates the instructions for the labelling of fish products commercialised in the European Community in order to describe the geographical origin of the species. Despite these restrictions, consumers may determine fraudulent suppliers who compromise the veracity of the products. Therefore, the Anisakidae larvae could be another tool for the origin identification of fish products. The European Authority for the Food Safety (EFSA 2010) confirmed that all the wild seawater fish must be considered at risk of containing viable parasites of human health concern and no sea fishing grounds can be considered free of *A. simplex complex* larvae. EFSA also recommends further studies and methods to improve the surveillance and diagnostic awareness of allergic reactions to parasites in fishery products.

In this case, molecular biology methods are valuable tools in the identification of Anisakidae nematodes. Recently, loop-mediated isothermal amplification (LAMP) is considered a highly sensitive and rapid method for DNA amplification at constant temperature. LAMP method not requiring special reagents and sophisticated temperature control devices so the detection of LAMP products is also suitable on site conditions. The food sector operators must have systems and procedures that allow the competent authorities to access information on the product in order to guarantee its traceability and hygiene. In this work, a rapid LAMP commercial system for *Anisakis* spp. DNA detection was optimized and validated in order to obtain a simple, fast and cheap tool, which can identify possible risks to consumer health due to the presence of these organisms in transformed fish products and to safeguard the traceability of the fish products.

RESULTS

No positive and negative deviations were found, resulting in a total agreement between the reference method and the LAMP method proposed in this work, with relative accuracy, relative specificity and relative sensitivity equal to 100%. The LAMP method proposed in this work was able to amplify Anisakis spp. DNA from artificially infested fish samples, giving a sensitivity of 100% for each sample type analyzed (Fig. 2), whereas no amplification products were detected in uninfected samples, giving a very high specificity rate. The assay detected Anisakis spp. DNA to a dilution of 10^{-4} (1:10.000), giving an amplification for all the replicates with fluorescence intensity necessary for detection.

DISCUSSION & CONCLUSIONS

The LAMP system proposed in this work has proved to be very accurate and sensible, as confirmed by the validation procedure. The method optimized with the set of primers employed was able to amplify Anisakis spp. DNA in 35 min at 65°C from a considerable initial weight of the samples, giving a satisfactory specificity in comparison with the reference real-time PCR method. The LAMP assay optimized was able to amplify Anisakis spp. DNA from different sample type, suggesting that the type of fish product processing does not affect the quality of the assay by matrix interferences. Moreover, the LAMP system we describe here, detected very low concentration of Anisakis spp. DNA. This is about 102 times more sensitive than the reference method. The high sensitivity of a method is certainly an advantage, but needs special care to avoid false positive, but the utilisation of ready-to-use reagents allowed us to minimize any operator error and the occurrence of possible false positives. In conclusion, the great rapidity, sensitivity and ease of use suggest that LAMP assay can be a valid alternative for routine examination in the field, where sophisticated and expensive equipment are not sustainable

MATERIAL & METHODS

All the fish samples used for the method optimization came from a large-scale distribution in order to reduce any bias from local food specialties and extend the range of validation. Homogenized farmed trout (*Oncorhynchus mykiss*), farmed sea bream (*Sparus aurata*), farmed salmon (*Salmo salar*), anchovy (*Engraulis encreasicolus*) paste, anchovy in oil and salted sardines (*Sardina pilchardus*) samples were chosen as naturally negative (non-contaminated by *Anisakis*) and positive samples for the optimization and validation of the method. The Anisakidae larvae used for the artificial infestation of the samples were collected from *Lepidopus caudatus*, *Clupea harengus* and *Merluccius merluccius* samples after visual inspection and modified chloro-peptic digestion method. The larvae were cut into small pieces and then were carefully mixed with the fish samples. Genomic DNA was extracted from positive and negative fish samples, contaminated or not with *Anisakis* spp., respectively. DNA extraction was performed using extraction buffer contained in Anisakis Screen Glow kit. A portion of 250±50 mg of sample was directly placing into tubes containing 4 ml of the ready for use extraction buffer and then incubating for 60-90 minutes at room temperature.

To design the primer set targeting *Anisakis* spp. gene, the genomic sequences of internal transcribed spacer 2 gene from various species were collected from GenBank. Each primer set was designed from the consensus sequence using Primer Express v3.0.1 software. A set of six primers, two outer (F3 and B3), two inner (FIP and BIP) and two loop (LF and LB), which recognize eight distinct regions of the target gene was designed. The analytical and diagnostic assays to recognize *Anisakis* spp. DNA have been performed using Anisakis Screen Glow commercial kit (Enbiotech Group S.r.l., Palermo, Italy) with ICGENE mini portable instrument (Fig. 1). Anisakis Screen Glow commercial kit includes ready-to-use reagents to achieve a rapid LAMP amplification of DNA template. The amplification protocol to obtain the specific amplification of the target Anisakis spp. was carried out in a mixture of a final volume of 55 µl, including 22 µl of LAMP mix, 30 µl of mineral oil and 3 µl of the extracted DNA samples. The amplification was performed at 65°C for 35 minutes. The method was validated taking into account the relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE) parameters described on the ISO 16140:2016 for qualitative methods (ISO 16140-2:2016). The Real-Time PCR method proposed by Cavallero et al. (2017) was chosen as reference method for AC, SP and SE assessment. Based on the evolutionary relationships and their feasible genetic similarity, parasitic material belonging to the Anisakidae family were screened by the method proposed in order to have further evidence on the diagnostic specificity of the LAMP assay. The limit of detection (LOD) was established as the lowest concentration of DNA of Anisakis species which provides a fluorescent signal significantly different from the negative control. The determination of the LOD of the LAMP method was assessed by diluting the DNA extracted from artificially infested samples with nuclease free water. All measurements were performed in ten replicates from each sample type independently.

Figure 1

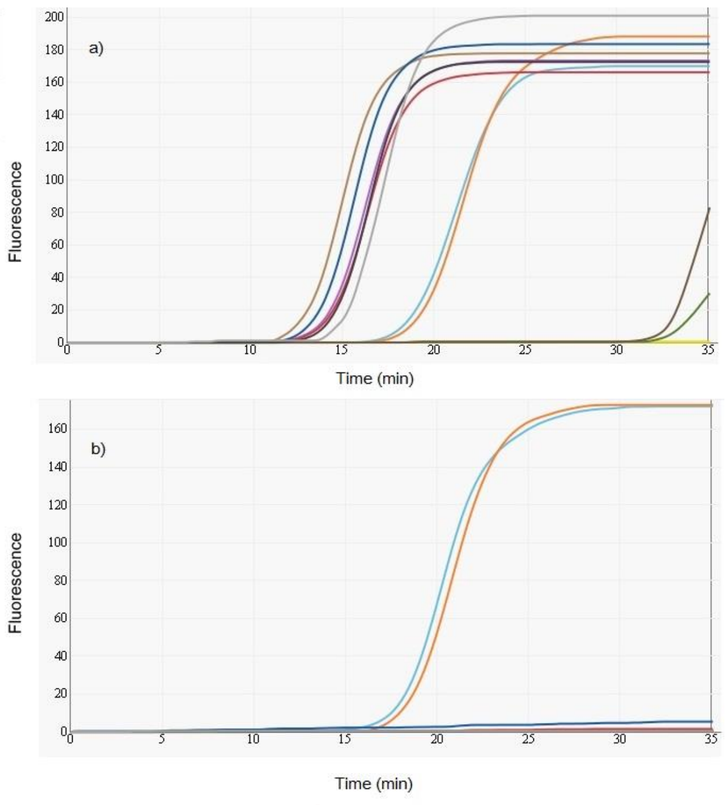

Figure 2

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