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CASE REPORT

MOLECULAR IDENTIFICATION OF *Pseudoterranova azarasi* LARVAE IN COD (*Gadus* sp.) SOLD FOR HUMAN CONSUMPTION IN BRAZIL

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SUMMARY

Anisakiasis and Pseudoterranovosis are human diseases caused by the ingestion of live Anisakidae larvae in raw, undercooked or lightly marinated fish. Larvae were collected from one salted cod sold for human consumption in a Sao Paulo market in 2013. One section of one brownish larva was used for molecular analyses. The partial COX2 gene sequence from the larva had a nucleotide identity of 99.8 % with *Pseudoterranova azarasi*, which belongs to the *Pseudoterranova decipiens* species complex. The risk of allergy when consuming dead larvae in salted fish is not well known and should be considered.

KEYWORDS: Anisakidae; Pseudoterranovosis; Allergy.

INTRODUCTION

Anisakidosis are human diseases caused by the ingestion of live larvae of parasites of the Anisakidae family in raw, undercooked or lightly marinated fish. Anisakiasis and pseudoterranovosis are caused by the ingestion of parasites from the genus *Anisakis* and *Pseudoterranova*, respectively. The most common species reported in humans are from the *Anisakis simplex* and *Pseudoterranova decipiens* species complex. This type of disease is very common in Japan due to eating habits, but also has been reported in other countries. The increase of this kind of fish consumption can raise the prevalence and intensity of infection, a public health problem leading to economic losses to the fishing industry due to the decrease of fish consumption. (see HOCHBERG & HAMER, 2010, for a review).

Larvae can be found in the flesh of numerous species of fish that serve as intermediate or paratenic hosts of the cycle, and that is completed in marine mammals, the definitive hosts. The detailed investigation of morphological characteristics of small larval stages is very problematic and, therefore, there is a great difficulty in differentiating genera and species of the Anisakidae family (MATTIUCCI & NASCETTI, 2006, 2008).

In Brazil, Anisakidae larvae are found in marine and freshwater fish, including various species of commercial value, and human anisakidosis manifestations have already been registered (AMATO *et al*. 2007; KNOFF *et al.* 2007; CRUZ *et al.* 2010). In a survey performed by the Adolfo Lutz Institute between 1997 and 1998, 27% of the cod (*Gadus* spp.) samples, salted or fresh, presented Anisakidae larvae from the genera *Anisakis* and *Pseudoterranova* (PEREIRA *et al.* 2000). These fishes are considered contaminated by the presence of larvae and inappropriate for human consumption according to the Brazilian legislation (Brasil, Ministerio da Agricultura, 1980; PEREIRA *et al.* 2000). The main objective of this report was to confirm, by molecular data, the identification of the larvae found in cod sold in Sao Paulo.

CASE REPORT

Larvae from this study were found and collected from salted cod fillets sold for human consumption in a Sao Paulo market in 2013. One section of the body of one brownish larva was cut and used for molecular analysis. The larva section was washed in distilled water several times (one-two days, refrigerated) and fragmented with a scalpel inside a petri dish before DNA extraction, performed with a standard phenol-chloroform protocol (SAMBROOK *et al.* 1989). PCR was performed using primers directed to the ribosomal DNA subunit sequences (rDNA) 18S and 5.8S of the Anisakidae family in order to amplify the ITS region of the rDNA, and also primers to amplify the cytochrome oxidase subunit 2 (COX2) gene of the mitochondrial DNA (mtDNA). Primers utilized were: NC5 (forward; 5'-GTAGGTGAACCTGCGGAAGGATCATT-3', ZHU *et al*. 2000), ITSR/5.8 (reverse, 5'-TAGTGCTCAATGTGTCTGCAATTCGC-3', this study), COXF (forward, 5'-TTGRTTTCATAAYTTTAATTGTAG-3', this study), and 210R (reverse, 5'-CACCAA CTCTTAAAATTATC-3', NADLER & HUDSPETH 2000). Each reaction contained 1x PCR Buffer,

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1.5 mM $MgCl_2$, 200 µM of each dNTP, 500 nM of each primer, 1U Taq polymerase (Invitrogen by life Technologies, Carlsbad, CA, USA) and 3 µL of template DNA, in a final volume of 50 µL. The amplification was performed in a thermocycler (BioRad C1000, Hercules, CA, USA) under the following conditions: 95 ºC for 5 min, followed by 30 cycles of 95 ºC for 45 s, 46 ºC (COX2) or 56 ºC (ITS1) for 30 s, and 72 ºC for 45 s, and a final extension of 72 ºC for 5 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The PCR products were purified and submitted to bidirectional sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) and an ABI 3500 sequencer (Applied Biosystems). The quality of the sequences was determined using Phred Electropherogram Quality Analysis software (TOGAWA & BRIGIDO 2003), available at http://asparagin.cenargen.embrapa.br/phph. The sequences obtained from the larva (GenBank accession number KJ480816/ITS1 and KM853036/COX2) were aligned using the program ClustalW (DNA) implemented in the MEGA version 5.2 software package (TAMURA *et al*. 2011) with *Pseudoterranova decipiens* sibling species sequences available at GenBank under accession numbers: JX138341, AJ413968, JQ673262, AJ413973, AB576757, AJ413981, AJ413965, AJ413970, KF017610 for ITS1, and HM147281, AF179920, HM14727, JX500060, KC782949, JX500061, HM147279, HM147280, HM147282 for COX2 analysis. A COX2 sequence of *Anisakis nascettii* (GenBank accession number GQ118171) was used as an out-group. The identity values of the nucleotide sequences were calculated using the program Bioedit Sequence Alignment Editor version 7.0.5.3 (Hall 1999). Phylogenetic analysis were conducted in the MEGA version 5.2 software package (TAMURA *et al*. 2011), using Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (ML) and Neighbor-Joining method using the Maximum Composite Likelihood approach (NJ), both with 1,000 bootstrap replicates.

The amplified ITS1 region and COX2 gene from the larva generated PCR products with expected sizes of 472 and 568 bp, respectively. The analyses of the ITS1 fragment of 352 bp, when compared to the GenBank sequences, had 100% of nucleotide identity with sequences of *P. decipiens sensu stricto* (s.s.) and *P. azarasi* (data not shown). On the other hand, the 509 bp COX2 gene sequence had 99.8% of nucleotide identity with *P. azarasi* and 98.0% identity with *P. decipiens* (s.s). Phylogenetic analysis of COX2 gene sequence clustered with *P. azarasi* obtained from *Eumetopias jubatus* (Steller's or Northern sea lion, HM147281) from the Northern Pacific (Fig. 1). Therefore, we identified this larva as *P. azarasi*.

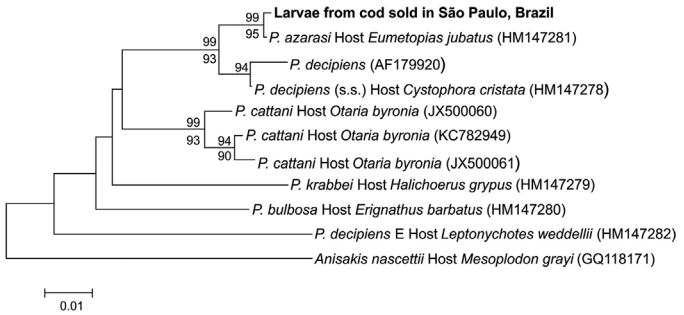

Fig. 1 - Phylogenetic tree of cytochrome oxidase subunit II (COX2) gene constructed by Neighbor-Joining method using the Maximum Composite Likelihood approach (superior bootstrap value) and Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (inferior bootstrap value). Only bootstrap values superior to 70 are shown at the nodes. *Anisakis nascettii* was used as an out-group, the sequence in bold indicates the one from this study.

DISCUSSION

Sealworms or codworms are nematodes belonging to the *P. decipiens* species complex (Anisakidae). The morphospecies *P. decipiens sensu lato* (s.l.) is a common anisakid nematode of many aquatic hosts and has global geographical distribution. Three of these sibling species, *P. decipiens* (s.s.), *P. krabbei* and *P. bulbosa*, have been reported in the North Atlantic, Norwegian and Barents Seas, while *P. decipiens* E has been identified in Antarctic waters and *P. azarasi* in northwestern Pacific (including Japan) (see McCLELLAND 2002 and MATTIUCCI & NASCETTI 2008 for reviews).

To our knowledge, this is the first molecular identification of *P. azarasi* in cod sold for human consumption in Brazil, and its role in human infections worldwide is still unknown. Furthermore, although there are morphological records, but no sequences available of *Pseudoterranova* species in Brazilian fish (KNOFF *et al.* 2007), and only *P. cattani* was molecularly confirmed in Argentina to date (TIMI *et al.* 2014), we consider that the DNA sequence data presented in this study confirmed the origin of the cod, probably caught in the Northern hemisphere, maybe in Japan. Additionally, as mentioned in the literature, the taxonomy of Anisakidae is still being defined with the aid of molecular tools and it is important to use multiple markers whenever possible since the ITS1 and ITS2 sequences, for instance, may show little difference among some species (ARIZONO *et al* 2011; TIMI *et al.* 2014).

In the anisakidosis infections, gastroallergic reactions are more frequently related to acute infections due to the ingestion of live larvae (SAKANARI & McKERROW 1989; AMATO *et al*. 2007; ARIZONO *et al*. 2011). Some Anisakidae allergens may resist cooking temperatures and enzymatic digestion, and sensitizing antigens can be preserved for months in frozen fish (CABALLERO & MONEO 2004; AUDICANA & KENNEDY 2008; RODRÍGUEZ-MAHILLO *et al.* 2010). Allergic symptoms have been also reported after exposure to small doses of Anisakidae proteins during occupational exposure, consumption of cooked or pickled fish (NIEUWENHUIZEN *et al* 2006; AUDICANA & KENNEDY 2008; JURADO-PALOMO *et al*. 2010; NIEUWENHUIZEN & LOPATA 2013), and even after consumption of meat from chicken that was probably fed with parasitized fish (ARMENTIA *et al.* 2006). Despite that, there is still controversy on whether allergy symptoms, such as anaphylaxis and rash, are induced exclusively by live larvae (DASCHNER *et al*. 2012). In Brazil, this aspect has been little explored. A recent study among volunteers (FIGUEIREDO JUNIOR *et al*. 2013) showed a link between the frequency of fish consumption and immunoreactivity to *Anisakis simplex* antigens in healthy adults, suggesting the previous immune sensitization without allergy symptoms. In this aspect, we point to a possible exposition to sensitizing Anisakidae antigens through the consumption of salted imported cod. Also, the intense exportation of fish associated with the growth in consumption has already increased the concern and interest in fish-transmitted diseases (McCLELLAND 2002; HOCHBERG & HAMER 2010; ARIZONO *et al*. 2011; NIEUWENHUIZEN & LOPATA 2013).

Finally, considering that different species of Anisakidae and its

proteins may cause human infections with distinct clinical courses, the proper identification of alive or dead larvae through molecular techniques could be used in food safety actions.

RESUMO

Identificação molecular de larva de *Pseudoterranova azarasi* em bacalhau (*Gadus* sp.) vendido para consumo humano no Brasil

Os termos Anisakiasis e Pseudoterranovosis são utilizados para doença em humanos causada pela ingestão de larvas vivas de parasitas da Família Anisakidae em peixes crus, mal cozidos ou levemente marinados. As larvas foram coletadas de bacalhau salgado vendido para consumo humano num mercado de São Paulo em 2013. Uma parte da larva de cor castanha foi utilizada em análises moleculares. A sequencia parcial do gene COX2 obtida da larva mostrou 99,8% de identidade de nucleotídeos com *Pseudoterranova azarasi*, que faz parte do complexo de espécies *Pseudoterranova decipiens*. O risco de reação alérgica envolvido no consumo de larvas mortas em peixe salgado não é bem conhecido e deve ser considerado.

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