ARTICULO

Detection of anisakids (Nematoda, Anisakidae) in food and human clinical samples in Argentina

Detección de anisákidos (Nematoda, Anisakidae) en alimentos y muestras clínicas humanas en Argentina

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ABSTRACT: Human anisakidosis is caused by the ingestion of anisakid third-stage larvae of the genera *Anisakis, Pseudoterranova, Contracaecum,* and *Hysterothylacium,* present in raw or undercooked fish and shellfish. The members of the complex *Anisakis simplex*, followed by those of the *Pseudoterranova decipiens* complex, are the most common anisakids infecting humans. In Argentina, infective larvae of these nematode species, have been identified in marine and freshwater fish. However, there are few human cases of anisakidosis from which the involved species, has not been documented yet. In this study, we reported the detection of anisakids in food and clinical samples from patients of Argentina. Nine samples of larvae were analyzed: two from clinical samples removed orally by patients, and seven found in raw, undercooked and cooked fish. The larvae from the two clinical samples were identified as *Pseudoteranova cattani* whereas larvae of *Anisakis pegreffii*, *Anisakis* sp., and *P. cattani* were found in food.

Keywords: molecular identification, diagnosis, nematode.

RESUMEN: La anisakidosis humana es causada por la ingestión de larvas de tercer estadío de los géneros *Anisakis*, *Pseudoterranova*, *Contracaecum* e *Hysterothylacium* a través del consumo de pescado y mariscos crudos o poco cocidos. El complejo de especies *Anisakis simplex*, seguidos por los nematodes del complejo *Pseudoterranova decipiens,* son los anisákidos más comunes que infectan humanos. En Argentina, se han identificado larvas infectivas de estas especies de nematodes en peces marinos y de agua dulce, sin embargo, existen muy pocos casos descriptos de anisakidosis humana, en los cuales las especies involucradas no han sido documentadas. En este estudio, informamos la detección de anisákidos en alimentos y en muestras clínicas humanas de Argentina. Se analizaron nueve muestras: dos muestras clínicas de larvas eliminadas por pacientes por vía oral y siete muestras de alimentos con larvas encontradas en pescado crudo, poco cocido o cocido. Las larvas de las dos muestras clínicas fueron identificadas como *Pseudoteranova cattani* mientras que en las de alimentos, se hallaron larvas de *Anisakis pegreffii*, *Anisakis* sp. y *P. cattani*.

Palabras clave: identificación molecular, diagnóstico, nematode.

INTRODUCTION

Anisakids occur worldwide, showing a species-specific geographical distribution (Navone *et al*., 1998; Torres *et al*., 1998; Mattiucci and Nascetti, 2007; Garbin, 2009; Mattiucci *et al*., 2009, 2013; Rossin *et al*., 2011; Knoff *et al*., 2012; D'Amelio *et al*., 2013; Garbin *et al.,* 2013; Hernández-Orts *et al*., 2013; Timi *et al*., 2014). Some species of the genera *Anisakis* Dujardin, 1845 and *Pseudoterranova* Mozgovoi, 1951 are the main etiological agents of human anisakidosis, although a few cases of *Contracaecum* Raillet *et* Henry, 1912 and a case by *Hysterothylacium aduncum* Rudolphi, 1802 have been reported (Yagi *et al*., 1996). All these genera use fish, crustaceans or squids as intermediate/paratenic hosts and marine mammals (*Anisakis*, *Pseudoterranova*, *Contracaecum*), birds (*Contracaecum*) or fish (*Hysterothylacium*) as definitive hosts.

Humans can accidentally be infected by the third-stage larvae consuming raw or undercooked fish and shellfish (D'Amelio *et al*., 2013). Most human cases have been reported from countries where raw or undercooked fish is traditionally consumed. Despite the larvae do not develop in humans, they can live and cause severe symptoms because they penetrate the stomach wall causing an acute abdominal pain, nausea and vomit within a few hours after ingestion. When they invade the gastric or intestinal mucosa,

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they often produce an ulcer or an eosinophilic granuloma, and usually cause allergic reactions ranging from urticaria to anaphylactic shock (D'Amelio *et al*., 2013).

In Argentina, only a few cases of human infection have been reported, one of them was considered non-autochthonous, since it involved a patient infected with *Anisakis* sp., who was a crew member of a foreign ship (Tanzola, 2011). Besides, the other one, was an autochthonous case of a girl who eliminated an anisakid third-stage larva in her feces (Menghi *et al*., 2011). *Anisakis pegreffii* Campana-Rouget *et* Biocca, 1955, was the only anisakid species genetically identified in a sample of 91 larvae found in hakes, *Merluccius hubbsi* Marini, 1933, from a fish-market in Buenos Aires City and off the coast of Mar del Plata, in the southwestern Atlantic Ocean (Hutler Wolkowicz, pers. com.). Adults of *Pseudoterranova cattani* George-Nascimento *et* Urrutia, 2000, have been reported parasitizing Patagonian sea lions (*Otaria flavescens,*Shaw 1800) from the southeastern Pacific Ocean, and the southwestern Atlantic Ocean. These larvae were detected in muscles of edible fish collected in Argentine and Chilean waters (Hernández-Orts *et al*., 2013; Timi *et al*., 2014), and isolated from patients in Chile (Mercado *et al*., 1997, 2001; Weitzel *et al*., 2015). Finally, larvae of *P. decipiens sensu latum* were recovered from human patients in Perú (Tantaleán, 1972; Cabrera and Trillo-Altamirano, 2004).

Diagnosis of anisakidosis may be difficult due to its nonspecific symptomatology, so inquires, to the patients, about chronology of previous ingest of raw or undercooked fish or squid, are always necessary. The presence of larvae and lesions caused by them are often revealed by ultrasonography, x-rays or gastroscopy. The definitive diagnosis relies on the identification of larvae removed by endoscopy and surgery, found in vomit and stools, or by the histopathological examination of biopsies (Zuloaga *et al*., 2004). Larvae are identified up to the genus level based on the morphology of the digestive tract and excretory system under light microscopy. However, it is very difficult to identify worms which have been partly destroyed or lack key morphological features. Thus, DNA differential diagnosis is considered a key procedure for the accurate identification of clinically obtained worms.

The objective of this study is detection and molecular identification of anisakids in food and clinical samples from Argentina during 2012-2017.

MATERIALS AND METHODS

Nine samples sent to Department of Parasitology of INEI ANLIS "Carlos G. Malbrán" for diagnosis, between 2012-2017, were analyzed. Two of these were from clinical samples, and the remaining ones were collected from food.

The two infected humans were adult females and lived in Buenos Aires city. One patient from Colombia expectorated a roundworm, and she reported that had eaten ceviche in Perú 15 days before the incident (Table 1). One larva was found between teeth in the other patient. She refered to have been eating sushi every 15 days, and travelled five months previously, to Dubai and Spain, where she also ate some dishes based on raw fish (Table 1). She was treated with mebendazole.

All food samples were collected from fish sold in the local market of Buenos Aires city, except one from which no source data were available (Table 2). Six samples out of seven were fixed with ethanol 70%, and the other one was preserved in formaldehyde. Parasites were analized by morphological criteria and PCR amplification of the internal transcribed spacer (ITS1) of nuclear ribosomal DNA followed by nucleotide sequencing (Degese *et al*., 2016). A 3-mm long piece from the mid-body region of each specimen was cut off for molecular study. DNA was isolated according to the "Rapid isolation of Mammalian DNA" protocol (Sambrook and Russell, 2001). The PCR mix was made up to a volume of 25 µl, containing 0.5 U of Taq polymerase (Invitrogen), 1X Taq buffer, 1.5 mM MgCl2, 0.8 µM of each dNTP, 0.25 uM of each primer (Ani-9F: CCGCCTTAATCGCAGTGG and Ani-552R: CAATTCGCACTATTTATCGCAGC) and 5 ng of parasite DNA. The cycling conditions were: 94ºC for 3 min, 40 cycles of 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min, and a final extension at 72ºC for 10 min. Amplification was carried out in a Px2 Thermal Cycler/Electron Corporation. Double-distilled water was used as negative control. The amplified fragments were separated by electrophoresis on a

Table 1. Characteristics of the two patients infected by *Pseudoterranova cattani* in Argentina.

Date	Patient	Origin of infection	Larvae collection	Symptoms	Suspected source of infection	*Period ingestion / expulsion	State of preservation	Larvae	Molecular identification
Jan-12	1	Perú	coughed up from the throat	coughing	ceviche	15 days	poor	1	*Pseudoterranova cattani*
Oct-16	2	CABA**	between the teeth	no	sushi	n/a	poor	1	*Pseudoteranova cattani*

*Period between fish ingestion to expulsion of larvae; ** CABA: Ciudad Autónoma de Buenos Aires. n/a: not available data

1.5% agarose gel, stained with GelRed® (Biotium) and compared to a 100-bp DNA ladder molecular weight marker (fermentas). Amplification fragments of the expected size were purified from the agarose gel using an AccuPrep Gel Purification Kit (Bioneer). Sequences were determined by an ABI 3500 Genetic Analyzer (Applied Biosystems). Chromatograms were viewed with Chromas Lite 2.01 and sequences were compared with those in GenBank database using BLASTn program (https://blast.ncbi.nlm.nih.gov). The anterior and posterior portions of each larva, were cleared in lactophenol for morphological examination.

RESULTS

All the worms were yellowish, and 2-4 cm long. Specimens were identified at genus level by morphological criteria only in three samples (Figs. 1, 2). The specimens in the remaining samples could not be identified by their poor state of preservation. Five out of seven fish samples were identified as *P. cattani* by molecular methods. From the other two, one was identified as *A. pegreffii*, and the other could not be analyzed since it was preserved in formaldehyde, a PCR inhibitor.

Only *P. cattani* was present in the two clinical samples (Table 1). Larvae of this species were also found in food samples from Argentine Sea Bass *Acanthistius brasilianus* (Cuvier, 1828), Brazilian codling *Urophycis brasiliensis* (Kaup, 1858), and *M. hubssi*. *Anisakis* sp., and *A. pegreffii* larvae were found in the white salmon *Stenodus leucichthys* (Güldenstädt, 1772), and hake, respectively (Table 2).

The DNA sequences obtained were deposited in the GenBank database under accession numbers MK492705 and MK492706 for *A. pegreffii* and *P. cattani,* respectively. The sequences showed 100-99% identity with available sequences in GenBank

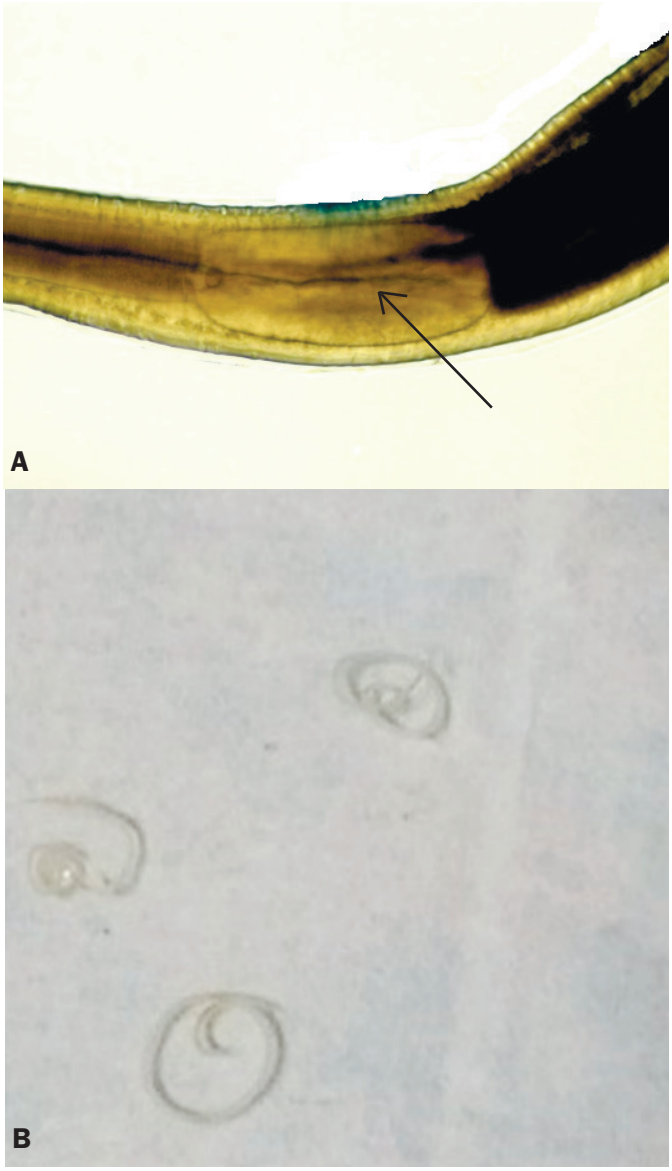

Figura 1. A. L3 larva of an anisakid worm, B. L3 larvae of *Anisakis* sp. Optical microscopy, arrow indicates the ventriculus.

Table 2. Characteristics of the seven samples of fish food with anisakids collected in Buenos Aires city, Argentina.

Date	Fish Species	Origin	Larvae collection	State of preservation	Larvae	Diagnostic method	Result	Food preparation
Feb-12	*Urophycis brasiliensis*	CABA*	during consumption	poor	3	molecular	*Pseudoterranova cattani*	undercooked
Apr-12	*Acanthistius brasilianus*	CABA	newly purchased fillets	poor	1	molecular	*Pseudoterranova cattani*	raw
Feb-15	*Stenodus leucichthys*	CABA	rolled salmon purchased fact	formaldehyde	4	morphological	*Anisakis sp.*	cooked
Mar-15	*Fish***	n/a	cleaning fish	poor	1	molecular	*Pseudoterranova cattani*	raw
Apr-15	*Urophycis brasiliensis*	CABA	before cooking	good	3	morphological and molecular	*Pseudoterranova cattani*	raw
Oct-16	*Merluccius hubbsi*	CABA	before cooking	poor	1	molecular	*Pseudoterranova cattani*	raw
Aug-17	*Merluccius hubbsi*	CABA	before cooking	good	4	morphological and molecular	*Anisakis pegreffii*	raw

*CABA: Ciudad Autónoma de Buenos Aires. **Unknown fish species. n/a: not available data

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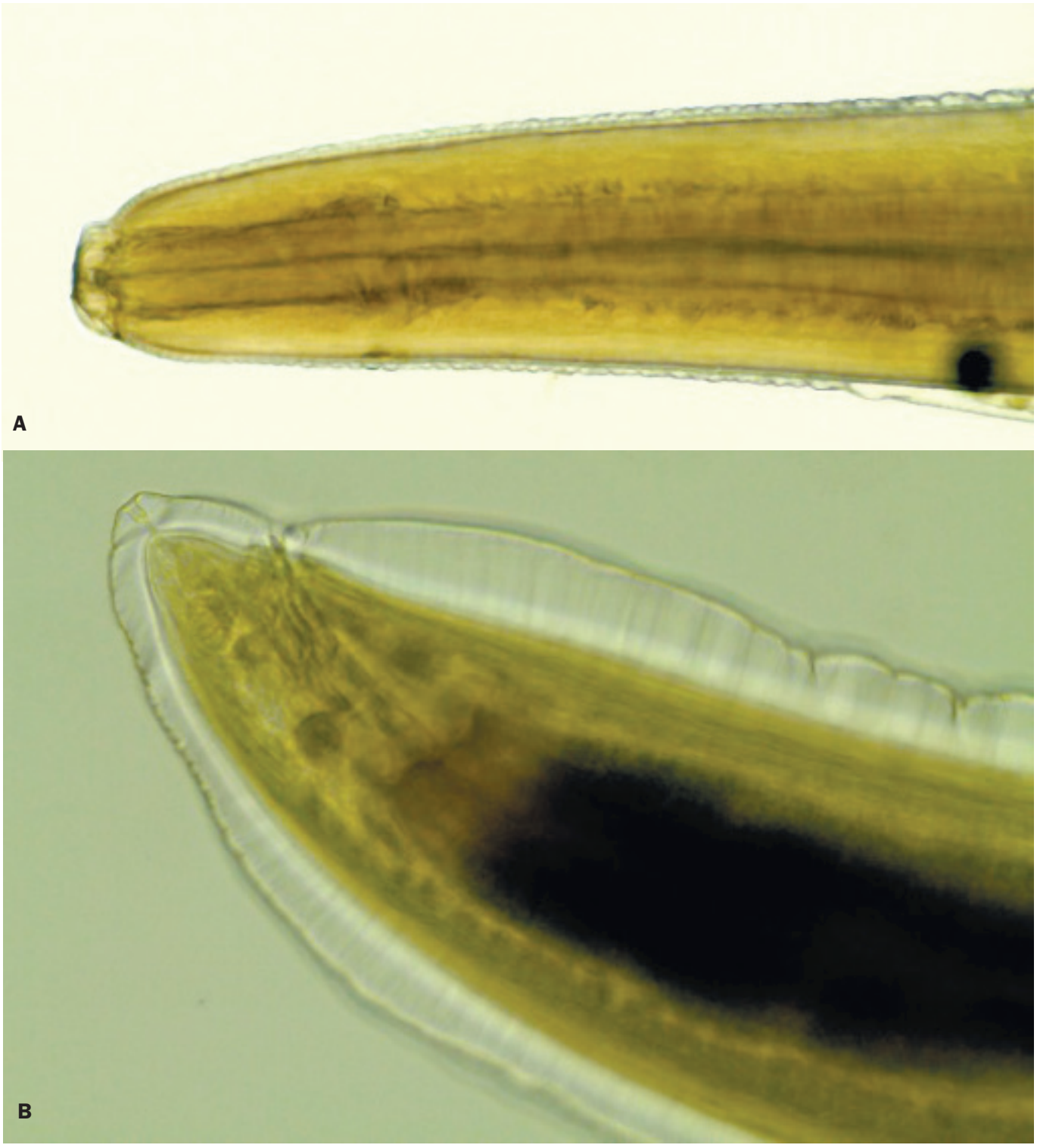

Figure 2. L3 larva of *Pseudoterranova* sp. A. Anterior end, B. Posterior end. Optical microscopy.

(Accession no KU991879, AB277823, HE997159, KF781285, KC970078, KF781284). Sequence variation was not observed between 2 larvae of *A. pegreffii* and in 7 isolates of *P. cattani* analyzed.

DISCUSSION

Our study is the first report of human anisakidosis caused by the genus *Pseudoterranova* in Argentina, and involves a non-native case belonging to a patient who had consumed ceviche 15 days before in Perú. The other one was a patient who often consumed sushi in Buenos Aires city, although she had also consumed raw fish dishes during a previous trip around Europe and Asia. The fact that, the trip was several months before the larvae elimination, and the species involved has not been related to these geographical areas, let us suspect this case is an autochthonous one. Clinical cases of human pseudoterranovosis by *Pseudoterranova* sp., herein described are similar to those reported from Chile, Perú, North America, and Europe, which have been classified as “transient luminal” with worms expelled by coughing (Tantaleán,

1972; Mercado *et al*., 1997; Cabrera and Trillo-Altamirano, 2004; Mattiucci *et al*., 2013; Weitzel *et al*., 2015). Most pseudoterranovosis usually cause severe pathology due to the penetration of the alimentary tract by larvae, as for example *Pseudoterranova azarasi* (Yamaguti *et* Arima, 1942) in Japan (Arizono *et al*., 2011). *Pseudoterranova cattani* uses the sea lion as definitive host in the southeastern Pacific and southwestern Atlantic Oceans. Also, larvae of this species were found in muscles of different edible fish species from Argentinean and Chilean waters (Hernández-Orts *et al*., 2013; Timi *et al*., 2014). In Chile, *P. cattani* has been involved in autochthonous human anisakidosis (Torres *et al*., 2007; Weitzel *et al*., 2015). Larvae of *P. decipiens sensu latum* were recovered from human cases in Perú (Mercado *et al*., 1997; Cabrera *et* Trillo-Altamirano, 2004), but taking into account the geographical distribution of its definitive host, the species involved probably belongs to *P. cattani*. On the other hand, no human cases have been yet reported in Argentina, probably because fish products are usually well-cooked before consumption or due to no recognition of symptons by physicians. It is very important to perform the differential diagnosis with other gastrointestinal pathologies, allowing in this way, an effective treatment of the patient.

The chosen procedure for larvae extraction was endoscopy or surgery. Gastrointestinal lesions refer back within two or three weeks after the parasite elimination. The diagnosis of intestinal anisakiasis is more difficult; however, it can usually be controlled without extracting worms since they eventually die in about three weeks. Surgery may be necessary for intestinal or extraintestinal infections when intestinal obstruction, appendicitis or peritonitis occur (Hochberg and Hamer, 2010). Limited evidence suggests that albendazole is an effective therapy, but the antihelmintic use of peppermint essential oil (*Mentha piperita,* Lineo 1758) in assays with experimental animals probe to be more effective than treatment with albendazole (Hochberg and Hamer, 2010; Romero *et al*., 2014).

In food samples, larvae of *P. cattani* were found in *A. brasilianus*, *U. brasiliensis*, and *M. hubssi* whereas those of *Anisakis* sp. and *A. pegreffii* were found in *S. leucichthys*, and *M. hubssi*, respectively. It is unknown if such larvae belonged to this fish or if a cross food contamination took place in the sale place.

The presence of anisakid larvae in the muscle of edible fish from Argentine waters supposes a risk to public health, and emphasizes the necessity of implementing adequate sanitary control measures to prevent anisakidosis in Argentina. Preventive measures include adequately freezing or cooking of fish (Adams *et al*., 1997).

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