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Presence of anisakid larvae in commercial fishes landed in the Pacific coast of Ecuador and Colombia

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Abstract

Introduction: Anisakidosis is a zoonotic disease caused by the consumption of raw or undercooked fish or crustaceans parasitized by nematode larvae of the Anisakidae family. In this study, the presence of anisakid larvae was identified in fish species of consumer of the Pacific coast in Ecuador and Colombia.

Methods: We obtained 438 samples grouped into twenty species of fish caught in the fishing ports of Manta, Santa Rosa, Buenaventura and Tumaco. The morphological identification of the larvae was made by taxonomy and the percentage of infection, were calculated. For the identification of species, a multiplex PCR was carried.

Results: The taxonomic review identified eight species of fish as hosts of the genders *Anisakis* and *Pseudoterranova*. The larvae were isolated mainly from the intestine with a percentage of infection between 18 and 100%. The percentage of infection and identification of anisakids in these fish will aid in the prevention and control of anisakiasis as a possible emerging disease for this area of the Pacific. With the multiplex PCR, *A. pegreffii*, *A. physeteris*, and *P. decipiens* were identified.

Conclusion: The identification of these species is reported for the first time in this geographical area, providing the basis for future research into the Anisakidae family.

Key words: Anisakid nematodes, molecular identification, commercial fishing.

Presencia de larvas de anisakidos en peces comercializados y desembarcados en la costa del Pacífico de Ecuador y Colombia

Resumen

Introducción: La anisakidosis es una enfermedad zoonótica causada por el consumo de pescado o crustáceos crudos o poco cocinados parasitados por las larvas de nematodos de la familia Anisakidae. En este estudio, se identificó la presencia de larvas de anisakidos en especies de peces de consumo de la costa del Pacífico en Ecuador y Colombia.

Métodos: Obtuvimos 438 muestras agrupadas en veinte especies de peces capturados en los puertos pesqueros de Manta, Santa Rosa, Buenaventura y Tumaco. La identificación morfológica de las larvas se realizó por taxonomía y se calculó el porcentaje de infección. Para la identificación de las especies, se llevó a cabo una PCR múltiplex.

Resultados: La revisión taxonómica identificó ocho especies de peces como huéspedes de los géneros *Anisakis* y *Pseudoterranova*. Las larvas se aislaron principalmente del intestino con un porcentaje de infección entre 18 y 100%. El porcentaje de infección e identificación de anisakidos en estos peces ayudará a prevenir y controlar la anisakiasis como una posible enfermedad emergente en esta área del Pacífico. Con la PCR múltiplex, se identifico *A. pegreffii*, *A. physeteris* y *P. decipiens*.

Conclusión: La identificación de estas especies se informa por primera vez en esta área geográfica, proporcionando la base para futuras investigaciones sobre la familia Anisakidae.

Palabras clave: nematodos anisákidos, identificación molecular, pesca commercial.

Introduction

Fish and fish products are an important source of animal protein, easily digestible, as well as rich in minerals and vitamins. The Pacific region straddling Colombia and Ecuador has a number of important fishing ports that have enabled the development of industrial and artisanal fishing activities[1] According to the Food and Agriculture Organization of the United Nations (FAO), average annual fish consumption per capita in Colombia and Ecuador is 4.73 kg and 5.6 kg, respectively[1,2]. Compared to countries such as Spain (38 kg / year), and Japan (54 kg / year), and even average consumption in Latin América in general (18 kg / year), consumption in Colombia and Ecuador is notably low. In recent years, with the introduction

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of the Mediterranean diet and the benefits of fish consumption for the prevention of cardiovascular diseases now well-documented, fish consumption has been increasing. However, the intake of these products has been connected to various infections in humans when fish is consumed that is minimally processed (i.e. raw, semi-raw, in sushi, salted or marinated)[3]. In some Latin American countries such as Brazil, Chile, México and Perú, most of the infections related to these types of fish preparation are associated with the presence of parasites in fish, a widespread phenomenon and particularly difficult to eliminate in raw and unprocessed fishery products[4].

The nematodes of the family Anisakidae are of particular concern to public health. These parasites cause anisakiasis, a zoonosis caused mainly by the genera *Anisakis* (Dujardin, 1845) and *Pseudoterranova*[5]. In humans, Anisakid larvae can cause gastric, allergic or gastro-allergic symptoms. Allergic reactions, (hypersensitivity type I), are the most common and caused mainly by the genus *Anisakis*, while gastric symptoms are more commonly associated with the genus *Pseudoterranova*, causing pseudoterranovosis[6].

Human anisakidosis has been reported in more than 26 countries on five continents including, Holland, Italy, Egypt, New Zealand, Canada, the United States, Brazil, Peru, and Chile[7–14]. The majority of reported cases have occurred in Spain, Japan and other Asian countries, where epidemiological studies have indicated that anisakiasis (caused by the genus *Anisakis*) is more frequent in coastal populations. In countries such as Brazil, Chile, Peru and Colombia, anisakidosis is considered an emerging public health risk and one that could increase with the introduction of a Mediterranean or Japanese diet[15].

The World Health Organization (WHO) and the FAO have established regulations, recommendations and guidelines to prevent and minimize the negative effect of anisakid nematodes on human health[16]. For its part, the European Community has specific sanitary regulations for foods of animal origin which are applicable to fishery products in relation to minimizing infection by anisakid parasites[17]. In Latin America, countries such as Nicaragua, Mexico and Ecuador follow some of these measures for the prevention and control of fish parasites that affect human health[18,19]. However, Colombia does not have clear legislation in this regard since these pathogens have not been fully identified.

Given that the process of globalization has popularized the consumption of raw or undercooked fish across various regions of the world and that fish parasitized with anisakids have been recorded in countries such as Chile, Peru, Argentina, Brazil, Colombia and Venezuela. Thus, the objective of this study was to determine the presence of anisakid nematodes in fish species that are sold commercially in the fishing ports of Colombia and Ecuador.

In addition, it is important to note that, in Colombia and Ecuador, no cases of anisakidosis in humans have been described to date, though this is most likely due to a lack of clinical and epidemiological information, and consequently an underreporting of the parasitosis as an emerging disease.

Materials and methods

Study area

Commercial fish species of particular economic importance were collected from the following fishing ports (Figure 1):

Ecuadorian Pacific: Manta (0 ° 57’00 “S 80 ° 42’58” W) and Santa Rosa (3 ° 27’08 “S 79 ° 57’42” W). These ports concentrate a catch of 23,000 tons of fish per year (1).

Colombian Pacific: Buenaventura (3 ° 52’38 “N 77 ° 01’36” W) and Tumaco (1 ° 48’24 “N 78 ° 45’53” W), the main ports of the Colombian Pacific coast where 53.89% of the catch comes from artisanal fishing and 46.11% from industrial fishing, which contributed a combined total of 1,099,568 tons of fish between 1998 and 2013[1].

Sampling

The samples were gathered from collection centers, points of sale, or supplied by fishermen in the region according to availability and the species’ economic importance. The samples were stored individually in labeled bags, kept in a refrigerator with ice and then transferred to the laboratory.

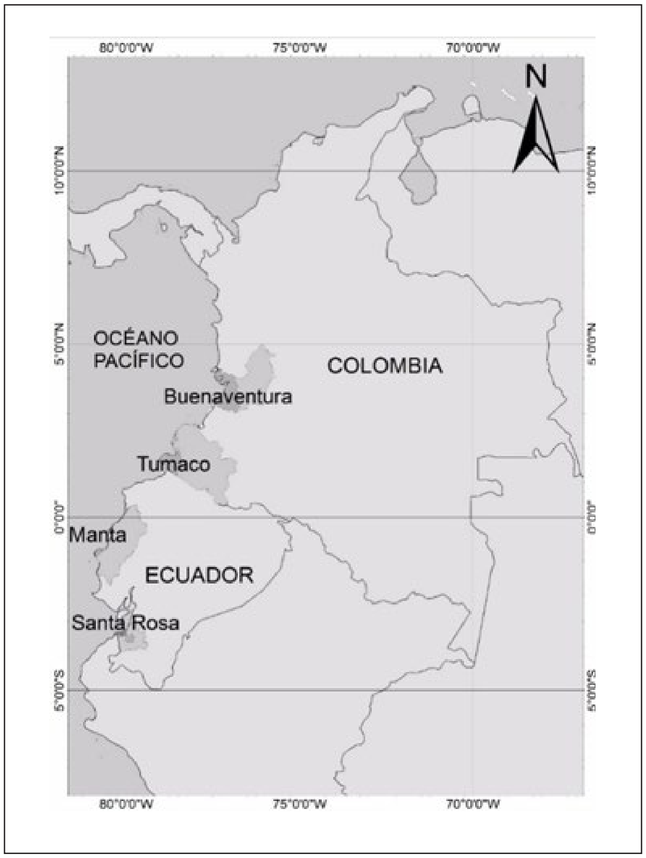

Figure 1. Location of the sampling sites in the Pacific coast of Colombia (Buenaventura and Tumaco) and Ecuador (Manta and Santa Rosa).

The study focused on the identification of anisakids in the viscera of the fish. In most cases, there was not access to the musculature of the fish due to its greater economic value. The nematodes were fixed in warm formalin at 4% (v / v) and immediately transferred to alcohol at 70% (v / v) until their identification by taxonomy. A subsample was stored directly in 96% alcohol (v / v) for subsequent identification by molecular biology.

Morphological identification of nematodes

The nematodes were clarified in gradual solutions of glycerin (20). The observation of their internal structures was carried out under an optical microscope with a built-in clear camera (Leica DM750). Nematodes of the Anisakidae family were identified up to a generic level, taking into account the characteristics described by Shiraki (1974). Images were taken with magnifications of 40x, 100x and 400x (Application Suite LAS v 3.8).

The morphologically identified anisakids were separated, counted and grouped by host. Finally, the percentage of infection for each host was calculated.

Molecular identification

DNA extraction: Thirty-eight anisakid nematodes were processed in the parasitology laboratory in the Faculty of Medicine at the Universidad de Chile. The larvae were sectioned into three and the individual extraction of each section of the nematodes was performed using the Invitrogen kit (Genomic DNA Mini Kit), following manufacturer instructions. The DNA was eluted in elution buffer and kept at -20°C until use.

Primers: Specific primers were used for six anisakid species: *Anisakis physeteris* (Baylis, 1923); *Pseudoterranova decipiens* (Krabbe, 1878); *Anisakis simplex* sensu stricto (Rudolphi, 1809); *Contracaecum osculatum* (Rudolphi, 1802); *Hysterothylacium aduncum* (Rudolphi, 1802) and *Anisakis pegreffii* (Bell-Rouget & Biocca, 1955), (Integrated DNA Technologies)[33].

Identification by multiplex PCR: A multiplex PCR was performed, using six specific primers and a universal primer[21]. The final reaction volume was 25µl: water (9.38µl); buffer 10x (2.5 µl) (HotMaster ™ Taq); dNTPs (0.63 L) (0.2 mM), for each specific primer (1 µl), universal primer (1 µl), HotMaster Taq DNA Polymerase (0.5 µl) (5PRIME) and genomic DNA (5 µl). The thermocycler program (Applied biosystems 2720) consisted of 30 cycles of initial denaturation at 95°C for three minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, hybridization at 52°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for seven minutes. All products were subjected to electrophoresis in 2% (w / v) agarose gel. As a positive control, genomic DNA of L3 larvae of *A. simplex, A. pegreffii* and *P. decipiens* was used, supplied by Dr. Hiroshi Yamasaki of the National Institute of Infectious Diseases in Tokyo, Japan.

Results

A total of 438 commercial fish destined for human consumption were collected and grouped into 20 species. Of those twenty, eight were found to be parasitized by anisakid nematodes with a percentage of infection of between 18 and 100%, and an average abundance between 0.4 and 45.5 (Table 1).

The nematodes were isolated mainly from the intestine (Table 2) and their morphological characteristics examined, resulting in the identification of the *Anisakis* and *Pseudoterranova* genera, both of the Anisakidae family (Table 3), with 90% identified as from the *Anisakis* genus.

The type I (Figure 2) and type II (Figure 3) larvae of the *Anisakis* genus were identified under an optical microscope. Type I larvae are characterized by a rounded posterior and the presence of a mucron (Figures 2c and 2d), while type II larvae present a conical termination with the absence of a mucron (Figures 3c and 3d).

The larvae present characteristics of the *Anisakis* genus; a whitish color, transverse grooves along the entire length of the body that become more pronounced towards its posterior end, (Figures 2b, 3c and 3d), a well-formed mouth composed of three lips surrounding the cuticular tooth, (Figures 2a and 3a), an elongated ventricle with a direct connection to the intestine and well-aligned along the longitudinal axis of the nematode (Figures 2b and 3b).

Multiplex PCR identification confirmed at the species level 26 of the 38 larvae analyzed. *Anisakis physeteris* species were identified in eight hosts and *P. decipiens* and *A. pegreffii* were each identified in one host. *Anisakis physeteris* was also reported in hosts already parasitized by *P. decipiens* and *A. pegreffii* (Table 3).

The specific primers were able to detect *P. decipiens* isolated from the *M. curema* (White mullet), using an amplification product with an expected weight of 370 bp*, A. pegreffii* with 672 bp, isolated from *M. gayi* (Merluza) and *A. physeteris* with 143 bp, isolated from *A. rochei* (Bullet tuna), *C. armatus* (Armed snook), *C. hippurus* (Common dolphinfish), *K. pelamis* (Skipjack tuna), *L. argenteus* (Silver drum), *M. gayi* (Merluza), *M. cephalus* (Flathead grey mullet) and *M. curema* (White mullet) (Figure 4).

Discussion

In the present study we have verified the presence of anisakid nematodes in fish for human consumption that inhabit the waters of the Ecuadorian and Colombian Pacific. Results found a high percentage of infection of *Anisakis* and *Pseudoterranova* parasites, the main causative agents of allergic and gastric anisakidosis respectively[6]. We identified third instar larvae (L3) based on the structural characteristics of the ventricle and the shape of the posterior end of the larvae[22]. In

Table 1: Percentage of infection of anisakid nematodes isolated from fish for human consumption from fishing ports in Colombia and Ecuador.

No	Scientific name	Common name	Country	Location	n	Percentage of infection	Abundance
1	*Auxis rochei*	Bullet tuna	Ecuador	Manta	6	50	0.5
			Ecuador	Santa Rosa	29	28	0.4
2	*Bagre pinnimaculatus*	Red sea catfish	Colombia	Buenaventura	6	0	0.0
3	*Brotula clarkae*	Pacific bearded brotula	Ecuador	Manta	5	0	0.0
4	*Caranx caballus*	Green jack	Ecuador	Manta	7	0	0.0
5	*Centropomus armatus*	Armed snook	Colombia	Buenaventura	12	42	2.8
6	*Centropomus medius*	Blackfin snook	Colombia	Buenaventura	4	0	0.0
7	*Coryphaena hippurus*	Common dolphinfish	Ecuador	Manta	69	30	5.6
8	*Cynoscion phoxocephalus*	Cachema weakfish	Colombia	Buenaventura	9	0	0.0
9	*Cynoscion* sp.		Ecuador	Manta	3	0	0.0
10	*Diplectrum maximun*	Torpedo sand perch	Ecuador	Manta	8	0	0.0
11	*Epinephelu sacanthistius*	Rooster hind	Ecuador	Manta	1	0	0.0
12	*Fistularia corneta*	Pacific cornetfish	Ecuador	Manta	8	0	0.0
13	Katsuwonus pelamis	Skipjack tuna	Ecuador	Manta	100	31	0.7
14	*Larimus argenteus*	Silver drum	Colombia	Buenaventura	2	100	45.5
15	*Lutjanus guttatus*	Spotted rose snapper	Colombia	Buenaventura	3	0	0.0
16	*Macrodon mordax*	Dogteeth weakfish	Colombia	Buenaventura	7	0	0.0
17	*Merluccius gayi*	Merluza	Ecuador	Manta	62	92	9.1
			Ecuador	Santa Rosa	11	18	0.2
18	*Mugil cephalus*	Flathead grey mullet	Colombia	Buenaventura	12	33	0.4
19	*Mugil curema*	White mullet	Colombia	Tumaco	16	94	21.8
20	*Thunnus albacares*	Yellowfin tuna	Ecuador	Manta	58	0	0.0
				Total	**438**		

fish such as *Merluccius* sp., *Centropomus armatus* and *Mugil* sp. - species that are widespread across the Pacific Ocean from the coast of California in the United States to southern Chile[23] - infection with anisakidos could be related to feeding habits. These species inhabit coastal and estuarine waters and they have been classified as detritivores, iliophages, herbivores, omnivores, phytophages and zooplanktons[24], feeding behaviors that favor parasitic infections[4].

In recent years, these fish species have recorded high rates of capture by artisanal fisheries and constitute one of the main sources of protein as an economically important species on the Pacific coast[25]. The identification of these fish species parasitized by *Anisakis* and *Pseudoterranova* can be used to develop measures for the prevention and control of human anisakidosis in Ecuador and Colombia; a disease that can be classified as emergent and easy to manage but whose spread must be addressed.

The location of the larvae is reported in this study, recorded as parasitizing the mesentery, stomach, intestine and liver. Our interpretation is that these results are related to the time that elapsed between the capture of the fish and the parasitological review. Most of the fish were reviewed fresh and it is known that the larvae of the anisakids require a minimum time of thirty minutes to migrate from the digestive system of the fish to the muscle[26]. Our results are similar to those of studies conducted in European countries, where hake (merluza) has a high commercial value and where *A. pegreffii* and *A. simplex* s.s. have been identified in viscera and muscle without significant differences in the values of prevalence between the two species of *Anisakis*[26]. These findings may be of economic interest for Ecuador and Colombia, providing information about the likely locations of larvae in order to eliminate them and therefore develop more effective and sanitary processing of the fish caught in the maritime waters of these countries.

Table 2: Location of anisakid larvae in each of the parasitized hosts.

No	Host species	Location of larvae (%)			
		Mesentery	Stomach	Intestine	Liver
1	*Auxis rochei*	45	27	27	27
2	*Centropomus armatus*	0	0	100	0
3	*Coryphaena hippurus*	14	67	19	5
4	*Katsuwonus pelamis*	16	71	19	0
5	*Larimus argenteus*	0	0	100	0
6	*Merluccius gayi*	32	54	29	31
7	*Mugil cephalus*	25	0	75	0
8	*Mugil curema*	21	7	86	7

The molecular characterization by PCR multiplex enabled the identification of the nematode *A. physeteris* in the fish species *A. rochei*, *C. armatus*, *C. hippurus*, *K. pelamis*, *L. argenteus*, *M. gayi* and *M. cephalus,* as well as *A. pegreffii* in *M. gayi* and *P. decipiens* in *M. curema*. However, it must be taken into account that the use of other molecular methods such as sequencing could vary these results given that these parasites are a complex of species and their taxonomy changes constantly. Nevertheless, these results concur with comparable studies by different authors who previously reported *A. physeteris* parasitizing *C. hippurus* fish in Peru with a prevalence of 33.33%[27–29] and in the *Mugil* sp. fish in Chile where *P. decipiens* was isolated and identified, a parasite associated with cases of gastric infection in that country[5,30]. However, in other investigations, *A. typica* has been recorded in the same hosts examined in our study, as in the *A. rochei* and *K. pelamis* fish in Indonesia[31,32] and the *C. hippurus* fish in the Indian Ocean[33,34].

Hake (merluza) is considered a potential host for all *Anisakis* species[31]. We report *A. pegreffii* and *A. physeteris* for this species, while in Chile it is associated with cases of pseudoterranovosis and in Colombia there is the report of *Contracaecum* sp. parasitizing the fish *Merluccius* sp. from the Caribbean Sea[5,9,30,32].

Additionally, the mugilidae constitute the family of fish with the highest number of anisakidos reports. We identified *P. decipiens* in Lisa fish, as in Chile, Venezuela and Peru, where it has been reported as the causative agent of several cases of anisakidosis[4,27,30,35]. Other authors report this species as a host for *Contracaecum* sp. in Colombia[24,25,36–38] and *A. pegreffii* in the Yellow Sea and in the Mediterranean Sea[31,39]. However, our bibliographic research did not find any reports of anisakids for the *C. armatus* and *Larimus argenteus* fish suggesting our study may be the first report of parasitosis in these species by *A. physeteris*. Similarly, we did not find reports of anisakidos in Ecuador.

Although in the majority of human cases of anisakidosis where molecular biology has been performed *A. simplex s.s.* has been identified as the main etiologic agent[40], in Italy *A. pegreffii* is recognized as the main cause of gastric anisakiasis with the molecular larvae isolated from three clinical cases being identified by molecular techniques[41]. It is important to note that in Japan, where parasitized fish species are widely studied, it is recognized that there are maritime areas of sympatry between *A. simplex* s.s. and *A. pegreffii*, and hybrid individuals have been recorded, while in the western Mediterranean *A. simplex* s.s. is not present, and *A. pegreffii* has been found to parasitize in a broader range of fish species[40]. Such variations underline the importance of conducting further research into fish from the Pacific coast to ascertain whether there are fish parasitized by *A. simplex* in addition to the *A. pegreffii* nematodes identified by this study.

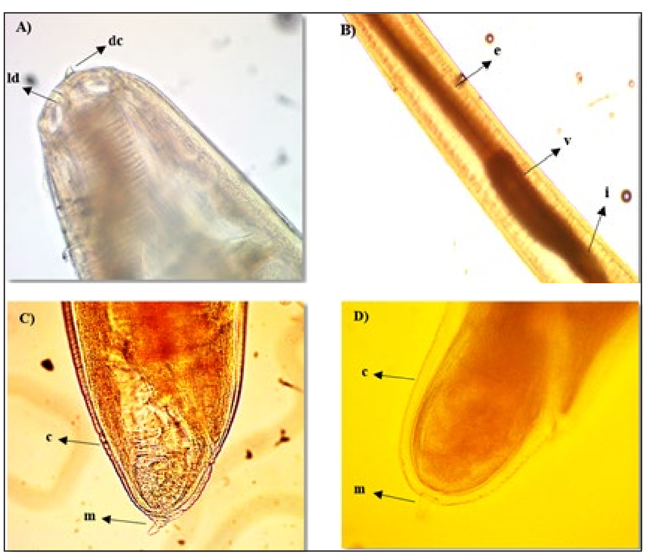

Figure 2. Larva (L3) type I of the Anisakidae family. A. Front end, dc: cuticular tooth, ld: dorsal lip (40x). B. Middle section, e: esophagus, v: ventricle, i: intestine (20x). C and D. Posterior end, m: mucron, c: cuticle (40x).

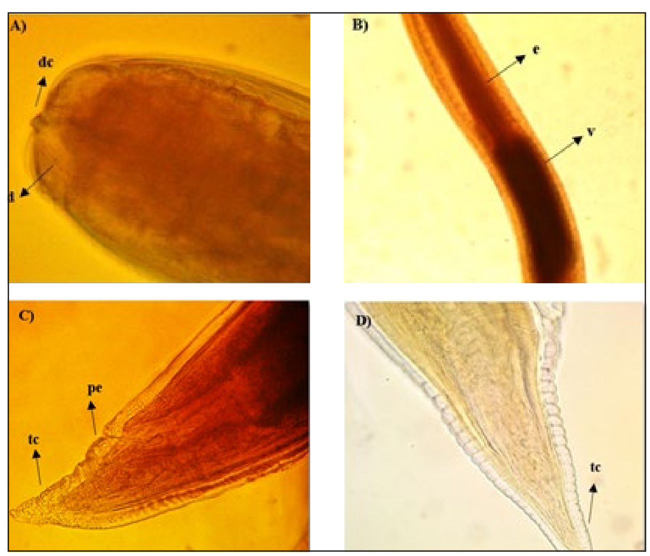

Figure 3. Larva (L3) type II of the Anisakidae family. A. Front end, dc: cuticular tooth, ld: dorsal lip (40x). B. Middle section, e: esophagus, v: ventricle (20x). C and D. Posterior end, tc: conical termination, pa: anal pore (40x).

Table 3: Morphological and molecular identification of larvae of the Anisakidae family and their hosts in the Colombian and Ecuadorian Pacific.

Host	Origin		Morphological Identification	PCR Multiplex Identification
Scientific name	Country	Location		
Auxis rochei	Ecuador	Santa Rosa	*Anisakis* spp.	*Anisakis physeteris*
Centropomus armatus	Colombia	Buenaventura	Familia Anisakidae	*Anisakis physeteris*
Coryphaena hippurus	Ecuador	Manta	*Anisakis* spp.	*Anisakis physeteris*
Katsuwonus pelamis	Ecuador	Manta	*Anisakis* spp.	*Anisakis physeteris*
Larimus argenteus	Colombia	Buenaventura	Familia Anisakidae	*Anisakis physeteris*
Merluccius gayi	Ecuador	Manta	Familia Anisakidae	*Anisakis pegreffii* *Anisakis physeteris*
Mugil cephalus	Colombia	Buenaventura	*Anisakis* spp.	*Anisakis physeteris*
Mugil curema	Colombia	Tumaco	*Pseudoterranova* sp.	*Pseudoterranova decipiens* *Anisakis physeteris*

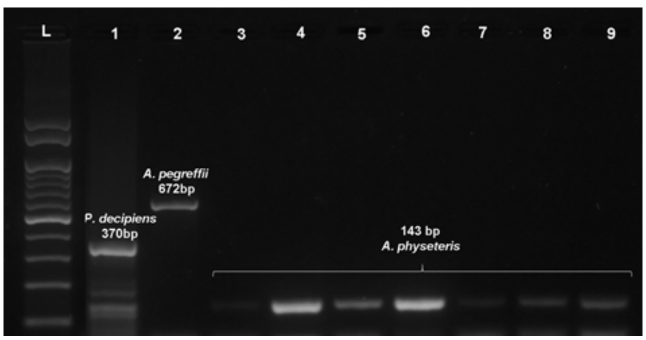

Figure 4. Molecular identification of anisakid nematodes by Multiplex PCR. Line 1: 370 bp, *P. decipiens* (*M. curema*). Line 2: 672 bp, *A. pegreffii* (*M. gayi*) and Lines 3 to 9: 143 bp, *A. physeteris* (*A. rochei, C. armatus, C. hippurus, K. pelamis, L. argenteus, M. gayi, M. cephalus and M. curema*). Line L: molecular weight marker of 100 bp.

We can not rule out that the absence of clinical cases diagnosed in Ecuador and Colombia may be related to a lack of knowledge on the part of physicians about anisakiasis and parasitic allergies, and its confusion with other diseases that present similar clinical pictures.

Our research constitutes the first report of the species *A. pegreffii*, *A.physeteris* and *P. decipiens* in Colombia and Ecuador, complementing the studies of geographical distribution worldwide where the *Anisakis* genus is the most reported in the Mediterranean while *Anisakis simplex* sensu stricto and *H. aduncum* are more frequent in the North Atlantic, and *Pseudoterranova* in the northeastern Atlantic, with no distribution of a particular species apparent in the north of the Pacific Ocean as we have reported.

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Declaration of conflict of interest

The authors declare no conflicts of interest.

Ethical considerations

This study was carried out in accordance with the permit granted by the National Environmental Licensing Authority of the Ministry of Environment & Sustainable Development, (resolution 1070 of August 28, 2015) to the Universidad del Valle and with the approval (Code 004-015) of the Institutional Review of Animal Ethics Committee at the Universidad del Valle.

Protection of human and animal subjects. This research do not used human material.

Confidentiality of data. Not applicable

Right to privacy and informed consent. No applicable

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