Journal MVZ Cordoba
2020; 25(2):e1781.
https://doi.org/10.21897/rmvz.1781

Original

Anisakis physeteris and *Pseudoterranova decipiens* in the *Mugil curema* fish caught in Tumaco, Colombia

Jenniffer Castellanos G[1,2*] Ph.D; Rubén Mercado P[3] Ph.D; Sebastián Peña F[3] M.Sc; María Carolina Pustovrh R[1] Ph.D; Liliana Salazar M[1] M.Sc.

[1]Universidad del Valle, Facultad de Salud, Departamento de Morfología, Grupo de Investigación en Tejidos Blandos y Mineralizados, Cali – Colombia.
[2]Unidad Central del Valle, Facultad de Ingenieria, Tulua, Colombia.
[3]Universidad de Chile, Facultad de Medicina, Unidad Docente de Parasitología, Santiago, Chile.
Correspondencia: jennniffer.castellanos@correounivalle.edu.co

Received: October 2019; Accepted: February 2020; Published: May 2020.

ABSTRACT

Objective: Identification nematodes Anisakidae family in *Mugil curema* fish. **Materials and methods:** For this study, 16 Lisa fish (*Mugil curema*) were obtained from the port of Tumaco, a city on the Colombian Pacific coast. Morphological identification of larvae was made by classical taxonomy and the percentage of larval infestation was calculated. For molecular identification, a multiplex PCR was carried out with primers for six species, *Anisakis physeteris, Pseudoterranova decipiens, Anisakis simplex sensu stricto, Contracaecum osculatum, Hysterothylacium aduncum* and *Anisakis pegreffii*. **Results:** The taxonomic revision enabled the identification of type II larvae of the genus *Anisakis* and larvae of the genus *Pseudoterranova*. The larvae were isolated mainly from the intestine, where it was found that 94% of the fish were parasitized by anisakid nematodes. The multiplex PCR enabled the identification of the species *A. physeteris* (Larva type II), and *P. decipiens*. **Conclusions:** This study in the first report of nematode Anisakidae on Tumaco, Colombia. These results provide a compelling justification for further study into the Anisakidae family in Colombia, as a public health problem

Key words: Parasites, Human feeding, artisanal fishing, zoonoses. (*Sourse: Tesaurio agropecuario para Colombia*)

RESUMEN

Objetivo. Identificar nematodos de la familia Anisakidae en el pez de consumo *Mugil curema*. **Materiales y métodos.** Para este estudio, se recolectaron 16 peces Lisa (*M. curema*) del puerto de Tumaco, una ciudad en la costa colombiana del Pacífico. La identificación morfológica de las larvas se realizó mediante taxonomía clásica y se calculó el porcentaje de infestación de larvas. Para la identificación molecular, se realizó una PCR múltiple con cebadores para las especies *Anisakis*

How to cite (Vancouver).
Castellanos GJA, Mercado PR, Peña FS, Pustovrh RM, Salazar ML *Anisakis physeteris* and *Pseudoterranova decipiens* in the *Mugil curema* fish caught in Tumaco, Colombia. Rev MVZ Cordoba. 2020; 25(2):e1781. https://doi.org/10.21897/rmvz.1781

ISSNe: 1909-0544

physeteris, *Pseudoterranova decipiens*, *Anisakis simplex* sensu stricto, *Contracaecum osculatum*, *Hysterothylacium aduncum* y *Anisakis pegreffii*. **Resultados.** La revisión taxonómica permitió la identificación de larvas de tipo II del género *Anisakis* y larvas del género *Pseudoterranova*. Las larvas se aislaron principalmente del intestino, donde se encontró que el 94% de los peces estaban parasitados por nematodos anisakidos. La PCR multiplex permitió la identificación de la especie *A. physeteris* (Larva tipo II) y *P. decipiens*. **Conclusiones.** Este estudio en el primer reporte de nematodo Anisakidae en Tumaco, Colombia. Estos resultados proporcionan una justificación convincente para un estudio adicional sobre la familia Anisakidae en Colombia, como un problema de salud pública.

Palabras clave: Parásitos, Alimentación humana, pesca artesanal, zoonosis. (*Fuente: Tesaurio ambiental para Colombia*)

INTRODUCTION

The Colombian Pacific coast is mainland territory that runs from the border limit with Panama in the north to the border with Ecuador in the south and is located between the Pacific Ocean and the Western Cordillera (mountain range) of the Andes, covering an approximate area of 71.000km² (1). In the coastal zone of this region the departments of Nariño, Chocó, Cauca and Valle del Cauca have jurisdiction (1). More than 90% of the 650.000 inhabitants are Afro-Colombian and populations are concentrated in the urban centres of the municipalities of Buenaventura, Tumaco and Bahía Solano, alongside the main ports and commercial activities of the region (1). Tumaco is a port city located on the west coast of the department of Nariño – a department which borders Ecuador – and it has a population of 199.659 inhabitants (DANE, Colombia, 2015). The principal economic activities in Tumaco are focused on agriculture, fishing and tourism.

The port of Tumaco is considered the second most important on the Pacific coast in Colombia after the port of Buenaventura: for the year 2013, exports of whole frozen tuna out of Tumaco were worth US$ 8.045.375 (2). Seafood is a principal protein source for the Pacific coast population, a foodstuff that has been promoted due to the protective health benefits that studies have identified, such as in the prevention of cardiovascular diseases (3–6). However, the consumption of minimally processed fish, (either raw, semi-raw, in sushi, salted, or marinated), has been associated with various health issues primarily caused by the presence of parasites in fish (3,7). The nematodes of the Anisakidae family are a priority in public health studies, as they are responsible for anisakidosis, a zoonotic disease reported in the coastal populations of five continents (7,8,9,10,11,12,13,14) , caused mainly by the species *Anisakis simplex* and the genus *Pseudoterranova*, and responsible for causing gastric, allergic or gastro-allergic symptoms (15).

Of the species that make up the Anisakidae family, *A. simplex* is the species that has the highest number of records of human anisakiasis with allergic symptoms (16). However, since 2015, several investigations have confirmed that *A. simplex* is a complex of species, finding *A. simplex* sensu stricto, *A. simplex* C and *A. pegreffii*, which do not present morphological differences, making a differential DNA diagnosis necessary for individual identification (17).

In Colombia, these pathogens are unknown by the majority of health personnel which is why there is likely to be an underreporting of the disease (3,18).

In the Americas, the only known reports of anisakidosis are from Chile and Peru (10,19), while, in Colombia, although there have been no reported cases of anisakidosis in humans, research conducted both along the Caribbean coast and in Buenaventura have recorded the presence of *Contracaecum* sp. and *Anisakis* sp. in fish for human consumption (14,20,21). It is important to point out, however, that taxonomic identification has been carried out only at a genus level. A deeper characterization down to species level is necessary to provide a more detailed picture of the anisakid species that circulate in Colombian marine waters and establish potential relationships with any diseases specific species may cause.

With the above in mind, the objective of this study was to carry out a species level identification of the anisakid larvae found in the *M. curema* fish from Tumaco, a species of economic importance for the region, one of the principal sources of

protein for the local population and a species that, in recent years, has seen high rates of capture, as reported by artisanal fisheries.

MATERIALS AND METHODS

Area of study. The samples were collected in the fishing port of Tumaco, in the department of Nariño Colombia.

Sampling. All fish were supplied by fishermen from the region. After initial storage and labelling, they were packed in a refrigerator with ice for transportation. The parasitological examination was carried out in the Histology laboratory at the Morphology Department in the Universidad del Valle in Cali, Colombia.

The samples obtained were examined in both the muscular and the visceral components for the presence of any anisakid nematodes. Any parasites found were placed in Petri dishes with distilled water then subsequently fixed in 4% (v/v) hot formalin and immediately transferred to 96% (v/v) alcohol until their identification by taxonomy and molecular biology.

Morphological identification of nematodes. The nematodes were clarified in gradual solutions of glycerine. An observation of the nematodes' internal structures was carried out under an optical microscope with a built-in clear camera (Leica DM750). The nematodes of the Anisakidae family were identified to a genus level. They were photographed with magnifications of 40x, 100x and 400x (Application Suite LAS V 3.8). After, the anisakids were separated for characteristic morphologic, counted, and the percentage of infestation was calculated.

DNA extraction. The larvae were cut into three sections. Individual extraction of each section was performed using the PureLink ™ Genomic DNA Mini Kit (Invitrogen, USA), following the manufacturer's instructions. The sample of DNA was eluted in elution buffer which was maintained at a temperature of -20°C until use.

Identification by conventional PCR for larvae of the anisakidae family. For conventional PCR, specific forward primers designed for six anisakid species were used (22): *Anisakis physeteris* (Baylis, 1923) APY (5'-GGCTGGTTGATGAACTGTTG-3'), *Pseudoterranova decipiens* (Krabbe,1878) PD (5'-CGAGTACTTTTTATGGTCGTGAAGT-3'), *Anisakis simplex sensu stricto* (Rudolphi, 1809) AC (5'-GACATTGTTATTTCATTGTATGTGTTGAAAATG-3'), *Contracaecum osculatum* (Rudolphi, 1802) COS (5'-TGATATGCTTGAAAGGCAGG-3'), *Hysterothylacium aduncum* (Rudolphi, 1802) HAD (5'-GCCTTCCATATGCGCGTATA-3') and for *Anisakis pegreffii* (Campana-Rouget & Biocca,1955) two primers were used: APE1 (5'-GAGCAGCAGCTTAAGGCAGAGGC-3') and APE2 (5'-GAGCAGCAGCTTAA GGCAGATGC-3'). A universal type B (5'-GCCGGATCCGAATC CTGGTTAGTTTCTTTTCCT-3') (Integrated DNA Technologies, USA) was used as a reverse primer.

Identification by multiplex PCR. PCRs were performed, using a final reaction volume 25µl: 9.38 µl of PCR-grade water; 2.5 µl PCR buffer (10X HotMaster™ Taq 10X Reaction Buffer, 5PRIME, USA); 0.63 µl of dNTPs mixture (0.2 mM each 10 mM dNTPs RBC Bioscience, USA), 1 µl for each specific primer (2 ng/µl), 0.5 µl of polymerase enzyme (5 U/µl HotMaster™ Taq DNA Polymerase, 5PRIME, USA) and genomic DNA (5 µl). The thermocycler programme (2720 Thermal Cycler, Applied Biosystems, USA) 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 (s), hybridization at 52°C for 30s, extension at 72°C for 45s, and a final extension at 72°C for seven minutes. All products were subjected to electrophoresis in 1% (w/v) agarose gel (UltraPure ™ Agarose, Invitrogen, USA) visualized with Gel-Red® staining (Biotium Inc., USA). As a positive control, genomic DNA from L3 larvae of *A. simplex, A. pegreffii* and *P. decipiens,* supplied by Dr. Hiroshi Yamasaki of the National Institute of Infectious Diseases of Tokyo, Japan, was used.

This study was aprobad for "Comité Institucional de Revisión de Ética con Animales en Experimentación" of the Universidad del Valle. Reference number 004-015

RESULTS

A total of 16 Lisa fish (*M. curema*) were collected, of which 15 were parasitized by anisakid nematodes, an infestation percentage of 94%. The morphological characteristics observed enabled the identification of the genera *Anisakis* and *Pseudoterranova* both of the Anisakidae family, but the *Anisakis* genus was the most common, present in 90% of cases.

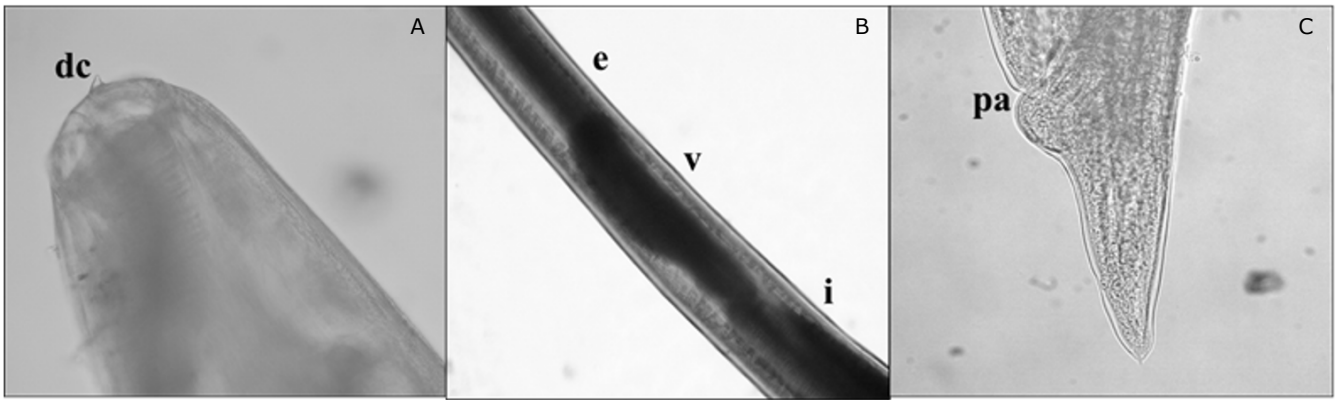

Figure 1. Larva (L3) type II of the *Anisakidae* family.
A. Anterior end, dc: cuticular tooth. (40x) **B.** Central section, e: oesophagus, v: ventricle, i: intestine (20x). **C**. Posterior end with conical termination, pa: anal pore (40x).

Using an optical microscope, they were identified as members of the genus *Anisakis* with morphology of Type II larvae (L3), i.e. whitish colour with cuticular transverse striae along the whole body, pronounced at the posterior section, with a previous section with a mouth composed of three lips surrounding the cuticular tooth (Figure 1A). In addition, the ventricle was observed as elongated with a direct union to the intestine and straight along the longitudinal axis of the nematode (Figure 1B), with a posterior end tapering in a conical form with no mucron (Figure 1C).

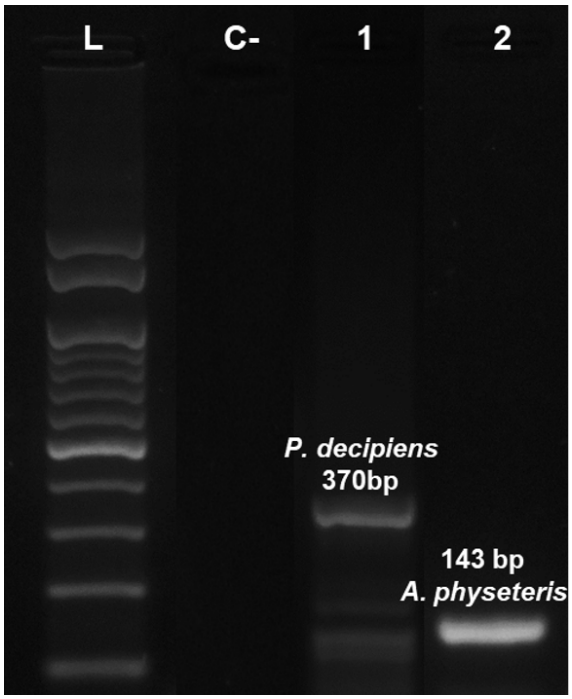

Figure 2. Molecular identification of anisakid nematodes by Multiplex PCR. Line L: molecular weight marker of 100 bp. Line 1: 370 bp, *P. decipiens* (*M. curema*). Line 2: 143 bp, *A. physeteris* (*M. curema*).

Multiplex PCR identification enabled the identification of the *A. physeteris* and *P. decipiens* species parasitizing *M. curema* fish. The specific primers were able to detect *P. decipiens* with an expected amplification product of 370 bp, and *A. physeteris* with a weight of 143 bp (Figure 2).

DISCUSSION

The Lisa fish (*M. curema*), of the Mugilidae family is widely distributed in the Pacific Ocean, from the southern Chile to coast of north in the United States (22). This species lives in coastal and estuarine waters. Its diet has led researchers to classify it as detritivore, iliophage, herbivore, omnivore and phytophagous (23), dietary behaviours that favour parasitic infections. It is for this reason the Mugilids are the fish family with the highest reported incidence of anisakids (17,21,22,23,24,25,26).

In this investigation we have confirmed the presence of third-stage (L3) larvae of anisakid nematodes in this type of fish caught in the waters of the Colombian Pacific; the fish presented a high percentage of infestation by nematodes anisakids. The larvae L3 of *Anisakis* are the etiological agents of human anisakiasis caused by consumption of raw or undercooked seafood infected with anisakid nematodes (27).

The identification of larvae L3 was made based on the structural characteristics of the ventricle and the characteristic shape of the end portion of the larvae (28). These same morphological characteristics were observed in others studies using from light microscopy, and scanning electron microscopy (SEM) (27,29,30).

In recent reports of anisakids in fish of the genus *Mugil* in Colombia, our group registered parasites of the genus *Anisakis* in fish for human consumption in the Pacific Ocean 2017 (14,20,21). In addition, on Colombia's Caribbean coast, the genera *Contracaecum* and *Pseudoterranova* were found in the fish *M. incilis* caught for commercial sale from the waters of the Bay of Cartagena in the Atlantic Ocean (23,26,31). In these investigations, however, anisakid larvae were identified taxonomically merely at the genus level, so this study constitutes the first identification to species level of the anisakid nematodes present in fish consumed from the Pacific coast of Colombia.

For the identification of anisakid species molecular biology methods such as PCR-RFLP and the sequencing of the rRNA gene of mitochondrial DNA have been developed. These techniques are used mainly for the identification of sister species of *A. simplex* and require highly specialized equipment and high-cost materials and reagents. Therefore, to achieve the objective of this study, the multiplex PCR proposed by Umehara et al (22) was adapted for the identification differential of larvae anisakid, an alternative to traditional methods. This technique can be used in regions that have a basic laboratory of molecular biology and thus make a rapid diagnosis of high sensitivity and specificity for the recognition of anisakid species. It is mainly used on samples where the larvae have been cut or damaged and identification down to genus level based on morphology is not possible. This method enabled the identification of nematodes *A. physeteris*, and *P. decipiens* parasitizing the *M. curema* fish. However, it must be acknowledged that the use of other molecular techniques such as sequencing may serve to confirm or vary these results, given that these parasites are a complex of species and their taxonomy changes constantly.

Identifying not simply the genus but the specific species of anisakid, in this case, *A. physeteris* and *P. decipiens*, allows for a better diagnostic approach in cases of gastrointestinal infections. Previous studies reported in Chile, Venezuela and Peru, documented the presence of *P. decipiens* in *M. curema* fish, associated with cases of anisakidosis and the same species (*P. decipiens)* in fish *Mugil* sp. was related to cases of gastric infection in Chile (3,8,10,19,24,32,33).

The geographical distribution of anisakids is well documented being, with the *Anisakis* genus the most prevalent in the Mediterranean and the *Pseudoterranova* in the north-eastern Atlantic. However, there are no known records for the northern Pacific Ocean (34,35). Therefore, these results constitute the first report of the species *A. physeteris* and *P. decipiens* in Colombia, thus contributing to the study of the geographical distribution of these nematodes worldwide (34,35).

With the confirmation of anisakids in Tumaco fish destined for human consumption, it is important to draw attention to the associated risk factors for public health. This parasitic disease is little recognized by local health personnel yet potentially widespread, given that artisanal fishing provides both a staple food locally and occupies third place among the principal income sources in the local economy. Factors such as the conditions of critical poverty with deficits in the health system, an illiteracy rate of 16%, the lack of running water and sewerage systems in the homes of fishermen all contribute to the low levels of hygiene in these populations, affecting both their own health as well as hygiene levels in fish handling (36). When discussing larger-scale production, sealed, hygienic processing facilities with adequate dock services and cooling systems must be guaranteed in the port of Tumaco in order to ensure the hygienic shipment of fishery products to the interior of the country. Failure to provide this infrastructure favours the survival of pathogenic parasites such as anisakid larvae (14). It is probable that unhygienic practices are reflected in the registry of diseases for the Valle del Cauca department in 2016, where documented foodborne diseases specifically associated with the consumption of fish and shellfish were 3.5% (1). However, there is certainly significant underreporting in relation to the specific causes of many gastrointestinal diseases.

Both the results of this study and previous reports for Colombia highlight the evident need for public health entities and the fishing industry to develop rigorous training, diagnosis and prevention plans for the control of anisakids-related illnessesin the Colombian Pacific coastal region.

Conflict of interests

The authors declare no conflicts of interest.

Acknowledgements

The authors of this study would like to thank the COLCIENCIAS (Departamento de Ciencia, Tecnologia e Innovacion - Convocatoria 567), the fishermen of the port of Tumaco, the Histology laboratory of the Faculty of Health at the Universidad del Valle for permission to carry out the taxonomic identification of the samples. We also thank the International Cooperation Agency of Chile and the Alianza del Pacífico Scholarship which provided the funds for Jenniffer Castellanos to carry out molecular biology studies at the University of Chile. We thank Dr. Hiroshi Yamasaki of the National Institute of Infectious Diseases of Tokyo, Japan for donating controls for molecular biology and to Dr. Shozo Ozaki of Virginia Commonwealth University for sharing their expertise in parasitology.

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