Molecular identification and epidemiological data of *Anisakis* spp. (Nematoda: Anisakidae) larvae from Southeastern Pacific Ocean off Peru

Rosa Martínez-Rojas, Aarón Mondragón-Martínez, Estrellita Rojas De-Los-Santos, Lidia Cruz-Neyra, Enrique García-Candela, Abraham Delgado-Escalante, J. R. Sanchez-Venegas

**Keywords:** Anisakiasis, Teleost fish, Peruvian sea, *Anisakis pegreffii*, Zoonosis

**ABSTRACT**

The objective of this study is to determine the infection status of nematode larvae and record epidemiological molecular data in commercial fish from the Southeast Pacific off the central coast of Peru. Anisakiasis is a fish-borne zoonosis caused by *Anisakis* larvae, parasites of relevance in the fishery resources that have negative impact on public health. Between January 2012 to December 2014, 345 specimens of four fish species (*Trachurus symmetricus*, *Scomber japonicus peruanus*, *Merluccius gayi peruanus* and *Sertolella violacea*) were examined for *Anisakis* sp. larvae. A total of 997 *Anisakis* sp. larvae were found in the body cavity of 196 fish (total prevalence 53.7%, total mean intensity 5.08). After morphological analysis, 958 (96.08%) larvae were identified as Type I and 39 (3.92%) as Type II. Specimens were identified by molecular analysis of the mitochondrial cytochrome c oxidase subunit II (COX2) gene, confirming that *A. pegreffii* is the predominant species and the most important agent of human anisakiasis off the Peru Central Coast. In addition, we revealed the occurrence of *A. physeteris* (s.l.) in *S. japonicus peruanus* (*P* = 18.0%; MI = 2.17).

Therefore, the results obtained in the present study improve the knowledge of the occurrence of *Anisakis* species in the commercial fish from the Southeastern Pacific Ocean, highlighting the importance of considering a potential hazard for humans and the necessity of further research in other fishes of greater preference by the Peruvian population.

**1. Introduction**

Infections by fish-borne nematode larvae (L3) affect human health worldwide, particularly in some countries such as Peru, Chile, Ecuador, or Colombia has been associated to the consumption of traditional raw fish-based dishes, such as ceviche, or insufficiently undercooked marine fish (Cabrera and Suárez-Ogino, 2000; Cabrera and Trillo-Altamirano, 2004; Eiras et al., 2018; Martínez-Rojas et al., 2020). Despite the high consumption of raw fish in South America to date, few human cases have been reported, especially in Peru and Chile (Tantaleán and Huiza, 1993; Mercado et al., 1997, 2001, 2006; Barriga et al., 1999; Cabrera et al., 2003; Patiño and Olivera, 2019). Anisakiasis is a serious zoonosis produced by nematode larvae of the genus *Anisakis* that are widespread in fish populations worldwide acquiring a high social relevance for causing digestive disorders or initiating hypersensitivity states and allergies (Mattiucci et al., 2013, 2018). Dead worms can also cause allergic reactions and in the worst case leading to anaphylactic shock (Audicana et al., 2002). Fishes and cephalopods are paratenic hosts for the *Anisakis* larvae, while adult’s parasites are found in marine mammals and seabirds, however, humans can become part of the cycle as accidental hosts (Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). The European Food Safety Authority recommends that it is necessary to continue with the investigation of parasites present in fishery products implicated in public health (European Food Safety Authority EFSA, 2010). The “Organismo Nacional de Sanidad Pesquera” (SANIPES) is implementing normative for the protection against pathogens in fishery products to

* Corresponding author. Laboratory of Parasitology in Wildlife and Zoonoses, Faculty of Biological Sciences, National University of San Marcos, Peru.

E-mail address: aaron72.mondragon@gmail.com (A. Mondragón-Martínez).

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prevent the spread of diseases in Peru, being one of its priorities the control of zoosanitary parasites in hidrobiologic resources (SANIPES, 2020). For this reason, it is necessary to take preventive measures due to the increase of Peruvian fishery exports that represent about 336,942.98 tons of frozen hidrobiologic resources to countries like Spain (23%), China (14%), South Korea (12%), United States (8%), Thailand (6%), Italy (4%) and others (33%) (PRODUCE, 2018).

Larvae of Anisakis attached to the gastric mucosa extracted by endoscopy and expelled orally have been reported (Salazar and Barriga, 1999). During the El Niño Phenomenon 1997–1998, possible cases of anisakid infestation were recorded identifying the etiological agent of the larval stages. Preventive measures are necessary to avoid the spread of diseases in Peru, being one of its priorities the epidemiology of anisakid species distributed along the Southeastern Pacific Ocean (Cabrera and Suarez-Ognio, 2000; Mattiucci et al., 2018).

In the present study we provided epidemiology data, taxonomic and molecular identification of Anisakis spp. collected from four commercially important fish species according to the Encuesta Nacional de Hogares (Encuesta Nacional de Hogares ENAHO, 2018), where the consumption of fish by Peruvian families increased steadily in the last five years, going from 12.9 kilos per inhabitant in 2013 to 14.5 kilos in 2017. This increase could be related to an increase in Peruvian fishery exports that represent about 336,942.98 tons of frozen hidrobiologic resources to countries like Spain (23%), China (14%), South Korea (12%), United States (8%), Thailand (6%), Italy (4%) and others (33%) (PRODUCE, 2018).

2. Materials and methods

2.1. Sample collection

Between January 2012 and December 2014, 365 specimens of four commercial fish species were examined, Trachurus symmetricus murphyi (length 34.83 ± 2.32 cm; weight: 325.78 ± 54.7 g), Merluccius gayi peruanus (length 39.17 ± 2.6 cm; weight 459.2 ± 45.2 g), Scomber japonicus peruanus (length: 33 ± 2.7 cm; 315.5 ± 34.15 g) and Seriolella violacea (length 41 ± 3.9 cm; weight 3895 ± 85.4 g) off the coast of the Peruvian Sea (Lima and constitutional province of Callao) (Table 1).

Specimens were examined in fresh conditions in the laboratory of Zoonosis of “Universidad Nacional Mayor de San Marcos”, fishes were identified according to Chirichigno and Cornejo (2001).

2.2. Parasitological examination

A total of 997 larvae were located in the body cavity, removed and repeatedly washed in 0.9% saline solution and morphologically identified at genus level by optical microscope (Leica EZ4, Germany). Anisakis larvae were grouped into Type I and II (sensu Berland, 1961), were identified to genus level by optical microscope (Leica EZ4, Germany). Larvae were grouped into Type I and II (sensu Berland, 1961), were repeatedly washed in 0.9% saline solution and morphologically identified at genus level by optical microscope (Leica EZ4, Germany).

The locus was amplified by PCR in a Veriti™ 96-well thermocycler (Applied Biosystems, California, USA) with a final volume of 50 μL, including 5 μL of genomic DNA. The reaction mixture contained 2.5 U/μL Taq polymerase (Hot Star Taq DNA Polymerase Qiagen Kit, Hilden, Germany), and 0.5 μM of each primer (Macrogen, South Korea).

Table 1

| Species                  | Parameter | Total     | 2012      | 2013      | 2014      |
|-------------------------|-----------|-----------|-----------|-----------|-----------|
| Trachurus symmetricus murphyi | N         | 105       | 40        | 30        | 35        |
|                         | Length ±SD| 34.83 ± 2.32 | 34.5 ± 2.29 | 35.5 ± 2.3 | 34.5 ± 2.2 |
|                         | Weight ±SD| 325.78 ± 54.7 | 318.5 ± 52.71 | 341.93 ± 56.28 | 319.98 ± 52.08 |
|                         | Prevalence | 64.76     | 62.5      | 70        | 62.86     |
|                         | CI 95%     | 55.74–74.05 | 46.82–78.18 | 52.60–87.40 | 46.02–79.70 |
|                         | Mean intensity (range) | 4.77 (1–10) | 4.72 (1–10) | 4.67 (1–9) | 4.95 (2–10) |
|                         | CI 95%     | 4.14–5.42 | 3.67–5.77 | 3.43–5.91 | 3.70–6.21 |
|                         | Mean abundance | 3.1       | 2.95      | 3.27      | 3.11      |
|                         | CI 95%     | 2.49–3.70 | 1.97–3.93 | 2.09–4.44 | 1.98–4.25 |
| Merluccius gayi peruanus | N         | 85        | 28        | 32        | 25        |
|                         | Length ±SD| 39.17 ± 2.64 | 38.5 ± 2.17 | 39.5 ± 1.87 | 39.3 ± 2.59 |
|                         | Weight ±SD| 459.17 ± 95.28 | 431.02 ± 73.98 | 465.09 ± 67.51 | 459.17 ± 92.59 |
|                         | Prevalence | 77.65     | 78.57     | 75        | 80        |
|                         | CI 95%     | 68.61–86.69 | 62.37–94.77 | 59.14–90.86 | 63.15–96.85 |
|                         | Mean intensity (range) | 3.7 (1–7) | 3.86 (1–7) | 3.83 (1–7) | 3.35 (2–7) |
|                         | CI 95%     | 3.27–4.12 | 3.03–4.70 | 3.10–4.57 | 2.62–4.08 |
|                         | Mean abundance | 2.87       | 3.04      | 2.88      | 2.68      |
|                         | CI 95%     | 2.40–3.34 | 2.14–3.93 | 2.06–3.69 | 1.87–3.49 |
| Seriolella violacea     | N         | 75        | 26        | 24        | 25        |
|                         | Length ±SD| 41 ± 3.89  | 39 ± 2.58 | 41.5 ± 2.87 | 42.5 ± 2.67 |
|                         | Weight ±SD| 3895 ± 369.97 | 3705 ± 245.29 | 3942.5 ± 272.87 | 4037.5 ± 271.96 |
|                         | Prevalence | 22.67     | 26.92     | 16.67     | 24        |
|                         | CI 95%     | 12.97–32.36 | 8.65–45.10 | 0.59–32.74 | 6.41–99.99 |
|                         | Mean intensity (range) | 9.88 (1–48) | 6 (1–10) | 8.5 (7–10) | 15.33 (7–48) |
|                         | CI 95%     | 4.66–15.11 | 2.89–9.11 | 6.45–10.55 | (1.31–32.17) |
|                         | Mean abundance | 2.24       | 1.62      | 1.42      | 0        |
|                         | CI 95%     | 0.79–3.69 | 0.33–2.90 | 0.04–2.80 | (0.41–7.78) |
| Scomber japonicus peruanus | N         | 100       | 37        | 33        | 30        |
|                         | Length ±SD| 33 ± 2.74  | 32.5 ± 2.31 | 32.5 ± 1.71 | 33.5 ± 2.05 |
|                         | Weight ±SD| 315.58 ± 84.15 | 309.18 ± 62.42 | 308.68 ± 46.46 | 336.98 ± 64.81 |
|                         | Prevalence | 45        | 48.65     | 45.45     | 40        |
|                         | CI 95%     | 35.08–54.92 | 31.75–65.54 | 27.52–63.38 | 21.39–58.61 |
|                         | Mean intensity (range) | 4.91 (1–9) | 4.72 (1–9) | 4.87 (2–8) | 5.25 (2–9) |
|                         | CI 95%     | 4.26–5.56 | 3.53–5.91 | 3.96–5.78 | 3.64–6.86 |
|                         | Mean abundance | 2.21       | 2.3       | 2.21      | 2.1       |
|                         | CI 95%     | 1.64–2.78 | 1.33–3.27 | 1.26–3.17 | 0.96–3.24 |
Korea). The amplification condition was optimized as follows: one cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 46 °C for 60 s, 70 °C for 90 s, and a final cycle of 70 °C for 10 min; storage at 4 °C. The amplified fragments were visualized on 1% agarose gel.

The nucleotide sequences obtained by PCR were subjected to known sequences by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the *Anisakis* spp. identified have been deposited in GenBank databases (accession numbers are highlighted in black, see Fig. 2), likewise, we use reference sequences obtained in other previous studies for the same gene and deposited in GenBank: *A. pegreffii* (JQ900759; JQ900760; JQ900761; MW074865; MW074866), *A. physeteris* (LC538424; LCS43844; LCS43849; MW691145; MW691146; MW074867; MW074868; AB592798; DQ116432), *A. brevispiculata* (KC342901; KC342899), *A. paggi* (KC821730; KC342896), *A. berlandi* (KC809999; KC810000), *A. simplex* s.str. (KC810003), *A. typical* (KF356650; KF356649), *A. siphidarum* (KC821732; KC821736).

2.4. Phylogenetic analysis

Phylogenetic relationships were evaluated with maximum likelihood (ML) in the MEGA version X program (Kumar et al., 2018) using the Kimura’s 2-parameter substitution model and the nodal support values were calculated by running 1000 bootstrap replicates (Kimura, 1980). Bayesian inference criteria (BIC) were analyzed in the Bayesian Evolutionary Analysis program by Sampling Trees (BEAST) version 1.7 (Drummond et al., 2012). The BIC model selected was HKY + G + I running a chain of 10 million generations and sampling tree topologies every 10,000 generations and the burning fraction were set at 10%.

2.5. Statistical analysis

The epidemiological parameters were determined following Rózsa et al. (2000). Prevalence (P%), mean intensity (MI) and mean abundance (MA) were calculated using the software Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005). Sterne’s exact test was used at 95% confidence limits for prevalence. To compare MI and MA, the bootstrap procedure was applied with 1000 replications at the 95% confidence interval. Comparison between the levels of infection of *Anisakis* spp. larvae were calculated by Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005) using Fisher’s exact test or exact unconditional test (prevalence-depending on sample size) (Reiczigel et al., 2008) and bootstrap two-sample t-test (mean abundance). Differences were considered significant when p < 0.05.

3. Results

3.1. Morphological analysis

All the larvae collected from *S. violacea, T. symmetricus murphyi* and *M. gayi peruanus* were morphologically characterized as *Anisakis* Type I, and in *S. japonicus peruanus* co-infected by larvae of *Anisakis* Type I and II (sensu Berland, 1961). SEM showed the presence of an irregularly distributed cuticle with shallow transverse striation and very fine longitudinal striations distributed parallel to the body in both types of larvae. A small mouth with an oral opening was observed, surrounded by three rudimentary labial protuberances: one dorsal and two subventral, including a penetrating triangular tooth that is ventral with respect to the mouth and behind the excretory pore. In type I, the tail end has a slightly curved cone-shaped mucron and in type II it ends in tip (Fig. 1).

![Fig. 1. Scanning electron micrographs of *Anisakis* type I and II.1a and 2a. Cephalic end. Detail of the structures: oral cavity (oc), tooth (t), excretory pore (ep), subventral lip bulge (s). 1b. caudal end of *Anisakis pegreffii*. 2b. caudal end of *Anisakis physeteris*. Detail of the structures: anal pore (ap), mucron (m).](image-url)
3.2. Molecular identification and phylogenetic analysis

Amplification of the mitochondrial cytochrome c oxidase subunit II (cox2) gene of 19 larvae produced a fragment of about 600 bp. The results showed that *Anisakis* type I (N = 12) belongs to *A. pegreffii* (identity values of 99–100%) when comparing with the reference sequences deposited in GenBank (i.e., JQ900759; JQ900760; JQ900761; MW074865; MW074866). The two phylogenetic methods yielded the same results in terms of clades with high support values. However, the sequence obtained in the *cox2* gene locus of *Anisakis* type II (N = 7) mtDNA is highly similar (identity values of 99.81–98.87%) from reference sequences recently deposited in GenBank (i.e., LC543849; LC543844; MW074868; MW074867) of *A. physeteris*, but compared to other *cox2* mtDNA genetic sequences (e.g., AB592798; DQ116432; MW691145; MW691145) the genetic similarity was quite different (96.62%). The phylogenetic tree of the mtDNA *cox2* gene was constructed using BI and ML confirming the assignment of type II larvae clustered with *A. physeteris* (s.l.) (Fig. 2).

3.3. Epidemiological parameters

A total of 997 *Anisakis* sp. larvae were found in 196 fish (total prevalence 53.7%, total mean intensity 5.08). After morphological analysis, 958 (96.08%) larvae were identified as type I and 39 (3.92%)
Table 2

| Parameter                  | Total | 2012     | 2013     | 2014     |
|----------------------------|-------|----------|----------|----------|
| *Scomber japonicus peruanus* |       |          |          |          |
| Prevalence                 | 18    | 13.51    | 18.18    | 23.33    |
| CI 95%                     | 10.34-25.66 | 1.96-25.07 | 4.29-32.07 | 7.27-39.40 |
| Mean intensity (range)     | 2.17 (1-3) | 2.4 (2-3) | 2.17 (1-3) | 2 (1-3) |
| CI 95%                     | 1.78-2.56 | 1.72-3.08 | 1.38-2.96 | 1.08-2.92 |
| Mean abundance             | 0.39   | 0.32     | 0.39     | 0.47     |
| CI 95%                     | 0.21-0.57 | 0.04-0.61 | 0.08-0.71 | 0.10-0.83 |
central coast Peruvian. Additionally, the five sequences obtained have 99% identity to the sequences previously deposited in GenBank by Aco Alburqueque et al., 2020. This would represent a new gene pool in the A. physeteris (s.l.) species complex, and needs further genetic investigation of other gene loci to clarify their phylogenetic status and taxonomic position as also suggested in the findings by Aco Alburqueque et al., 2020. A. pegreffii is common in fish from the Mediterranean and Adriatic Sea and has also been reported as a common and dominant species in different fish species (Mladineo et al., 2014; Molina-Fernández et al., 2018; Cipriani et al., 2018; Debenedetti et al., 2019).

Furthermore, the levels of infection by A. pegreffii can oscillate significantly according to the geographical area, as recorded in Merluccius merluccius from the Tyrrhenian Sea and the Spanish Atlantic coast (Cipriani et al., 2015). In the Southeastern Pacific Ocean off the Peru coast, a high prevalence of A. pegreffii has been reported (Aco Alburqueque et al., 2020). Similarly, we reported a significantly high incidence and prevalence of A. pegreffii in fish hosts sampled in the central Peruvian Sea. In addition, a study revealed pronounced differences in the level and pattern of infection with the Anisakis species among mackerel populations, where A. pegreffii was the dominant species in Mediterranean waters sample locations, while A. simplex (s.s.) was the most species prevalent in mackerel samples from Atlantic catching areas (Levens et al., 2018). We reported mixed infections with species of A. pegreffii and A. physeteris (s.l.) in samples of Peruvian mackerel, being A. pegreffii highly predominant, these results agree with the study by Aco Alburqueque et al., 2020 for two capture areas of mackerel samples difficult to detect by visual inspection (Levsen and Lunestad, 2010; Levsen et al., 2014), where they recommend endoscopy for the diagnosis of suspected cases.

Anisakiasis has been considered an emerging zoonosis less than a decade ago in Italy (Mattucci et al., 2013), Korea (Lim et al., 2015) and Croatia (Broglj and Kapel, 2011), being A. pegreffii, the most important ethological agent of human anisakiasis. In Japan, reported 158 patients that manifested acute gastrointestinal discomfort caused by anisakiasis, where they recommend endoscopy for the diagnosis of suspected cases (Furuya et al., 2018).

In Poland, the first case of gastric anisakiasis due to A. simplex (s.s.) was reported, the larvae were removed alive from an adult woman who manifesting persistent stomach pain (Kołodziejczyk et al., 2020). To date, in several clinical case reports, the molecular diagnostic of the etiologic agent has been shown that only A. simplex (s.s.) and A. pegreffii have the ability to cause “invasive anisakiasis” in humans (Mattucci et al., 2018). As consumption of fishery resources increases in Peruvian, reports of anisakiasis are likely to be more frequent, for this reason, it is necessary for health centers record cases of patients that present as symptoms gastrointestinal ailments after the consumption of fish and shellfish as possible clinical reports of anisakiasis. Therefore, prevention and protection against zoonotic parasites in fishery products intended for human consumption have become a priority (Levens and Lunestad, 2010).

In the present study, the viscera and musculature were thoroughly examined, however, all the larvae were isolated from the coelomic cavity of teleost fish. Aco Alburqueque et al., 2020 reported the presence of A. pegreffii larval in fish flesh isolated from T. murphyi and S. japonicus with very low prevalence for Northern and Central coast of Peru. So far, the behavior of Anisakis larvae is unknown, they generally remain in the visceral cavity or within the visceral organs of the fish, while, in other cases, they can migrate and penetrate deep into the muscle and are difficult to detect by visual inspection (Levens and Lunestad, 2010; Cipriani et al., 2016; Levens et al., 2017).

The risk management measures for Anisakis must be adapted to commercial species, considering all ecological and phylogenetic traits of the host-parasite found in a specific fishing ground will be encompassed, resulting in satisfactory control conditions (Mladineo and Poljak, 2014). These measures are being adapted and regulated under the standards of the European Community (CE) and the US Food and Drug Administration (FDA).

In conclusion, the reported results provide valuable information on the occurrence of Anisakis in the study area, suggesting that the dominant species on the central coast of the Peruvian Sea is A. pegreffii, it can be used to guide public policies in the fishing sector.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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