

Extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi* manifesting as strangulated inguinal hernia



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ABSTRACT

The incidence of anisakidosis continues to increase worldwide due to increased consumption of undercooked fish. Although anisakidosis can be categorized into four clinical phenotypes (gastric, intestinal, ectopic, and allergic), ectopic forms of the condition are much less common than gastric or intestinal forms when caused by *Pseudoterranova* spp. We report the case of a 5-year-old patient who presented with a rapidly growing left inguinal mass and was subsequently diagnosed with extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi*.

1. Introduction

Anisakidosis is a foodborne disease caused by the accidental ingestion of larval nematodes (commonly *Anisakis* spp. or *Pseudoterranova* spp.) in undercooked fish [1,2]. Although the highest number of human cases has historically been reported in Japan [3], the incidence of anisakidosis continues to increase worldwide due to increased consumption of undercooked fish [4–8].

To the best of our knowledge, no reports to date have documented cases of ectopic anisakidosis caused by *Pseudoterranova* spp. in humans. In Japan, only *Pseudoterranova azarasi*—a sibling species of *Pseudoterranova decipiens* sensu lato—has been reported as a causal agent of human gastric anisakidosis [9]. In the present report, we discuss the case of a 5-year-old girl who presented with an acute, rapidly growing left inguinal mass and received a final diagnosis of extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi*.

2. Case report

A previously healthy 5-year-old Japanese girl presented with a rapidly growing left inguinal mass. On the previous day, she had developed itchiness in the left inguinal region. At that time, her mother first noticed the left inguinal mass and tenderness of the affected area. The patient had no significant past medical or family history. Her mother

also denied any allergic symptoms. She experienced no loss of appetite, nausea, vomiting, or diarrhea.

Upon admission, her vital signs were stable, and she was in generally good condition. Physical examination revealed a firm mass of approximately 5 cm accompanied by redness, swelling, and tenderness in her left inguinal region. Laboratory tests revealed a white blood cell count of 7400/μl with 6.5% eosinophils, a hemoglobin level of 13.6 g/dl, and platelet level of 235,000/μl. Serum chemistry revealed no abnormalities. Abdominal ultrasonography revealed a mass in the left inguinal region, though the origin of the eviscerated structure remained unknown. Following consultation with a surgeon, the patient underwent emergency surgery for the treatment of suspected strangulated inguinal hernia. Intraoperatively, her inguinal canal was markedly edematous and adhered. However, no eviscerated structure was observed in the left hernia sac. Therefore, we suspected that the mass represented inflammation of the hernia sac itself, and the mass was dissected (Fig. 1(a)).

A hematoxylin-and-eosin-stained section of the mass demonstrated a presumed single nematode, which was surrounded by granulomatous inflammation and numerous eosinophils (Fig. 1(b)). Although helminth infection was pathologically suspected, we were unable to make a final diagnosis from the broken pathological specimen. Therefore, we measured levels of parasite-specific immunoglobulin G (IgG) antibodies including those for *Dirofilaria immitis*, *Toxocara canis*, *Ascaris suum*,

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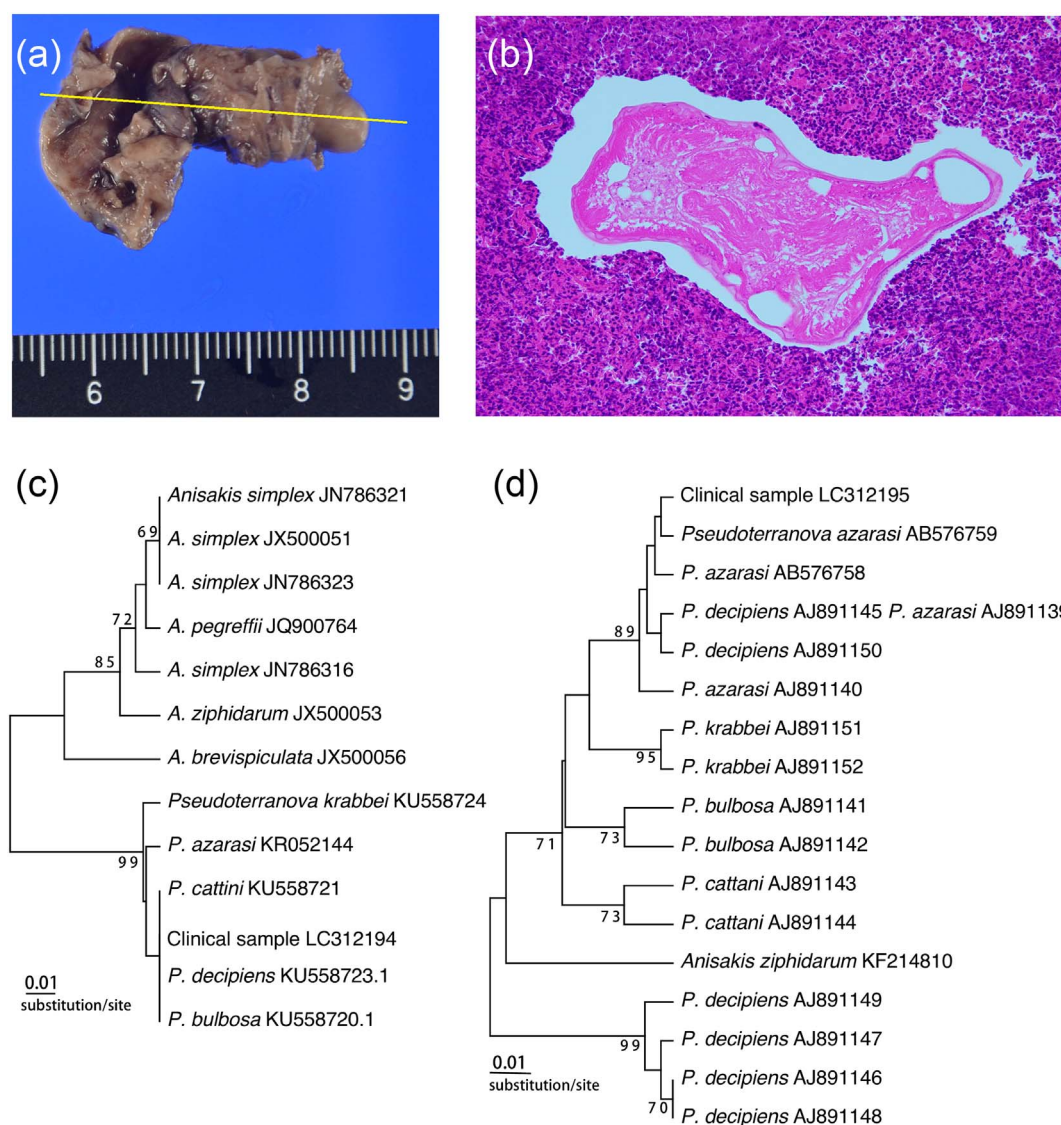


Fig. 1. (a). The resected specimen of the inguinal sac. (b). Transverse section of *Pseudoterranova* spp. larvae surrounded by inflammatory cells (hematoxylin-and-eosin staining, $\times 100$ magnification). (c), (d). Reconstructed phylogenetic trees of partial sequences of 12S rRNA (C) and COX1 (D) gene loci. Only the top 60% of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Anisakis, *Gnathostoma*, *Strongyloides stercoralis*, *Paragonimus westermani*, *Paragonimus miyazakii*, *Fasciola hepatica*, *Clonorchis sinensis*, *Spirometra erinaceieuropaei*, and *Cysticercus cellulosae*. However, all such test results were negative.

To identify the parasite species detected in the tissue sample (Fig. 1(a), (b)), total DNA was extracted from the sliced paraffin embedded-tissue using TaKaRa DEXPAT® (TaKaRa Bio inc, Shiga, Japan), and a 202-bp short fragment of 12S ribosomal RNA (12S rRNA) gene locus was amplified using a set of in-house designed primers for universal PCR, which is theoretically able to detect all causal organisms of toxocarasis, anisakidosis, filarialiasis, and gnathostomiasis. The PCR was performed in a final volume of 10 μ l using LA Taq® PCR with GC buffer I including 0.5 U of LA Taq® polymerase (TaKaRa Bio inc., Shiga, Japan), 1 μ l volume of extracted total DNA as a template, 0.4 μ M each of forward MT761 primer 5'-GTTCCAGAATAATCGGCTA-3' and reverse MT766 primer 5'-TTAAACAAAACCTTTACTC-3', and 5.0 μ M of each deoxynucleoside triphosphate (dNTP) with 0.1% dimethyl sulfoxide. The cycle parameters for the PCR were as follows: initial denaturation at 94.0 °C for 1 min, followed by 35 cycles of 94.0 °C for 30 s, 50.0 °C (annealing temperature) for 30 s, 72.0 °C for 15 s, and a final extension at 72.0 °C for 2 min. The target amplicon was excised from the

electrophoresed gel and purified using the FastGene® gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan), in accordance with the manufacturer's instructions. Direct sequencing of the purified PCR product was conducted using the forward and reverse primers and the ABI Prism BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Japan, Tokyo, Japan) on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies).

The confirmed 165-bp DNA sequence from the 202-bp partial sequence of 12S rRNA gene exhibited 100% similarity to the position of 12,662 to 12,826 on the reference mitochondrial genome sequence of *Pseudoterranova decipiens* sensu lato from the German repository (KU558723). Fig. 1(c) shows all reference sequences of the 12S rRNA gene locus of family Anisakidae (*Anisakis* spp. and *Pseudoterranova* spp.) obtained from the DNA Data Bank of Japan (DDBJ). After aligning the confirmed sample sequence with the reference samples, the unweighted pair group method with arithmetic mean (UPGMA) was performed for phylogenetic reconstruction using MEGA6 [10]. All positions containing gaps and missing data were eliminated. There were a total of 107 positions in the final dataset. The monophyletic cluster formation of the sample sequence and all reference sequences of *Pseudoterranova* spp. (Fig. 1(c)) was significantly supported by the bootstrap value

(99%), and thus the genus-level identification was confirmed as *Pseudoterranova*. To confirm the species-level identification, the cytochrome c oxidase subunit I (COX1) gene targeting PCR was employed using the MZ11 forward primer (5'-TTTGTGGGCATCTGAGGTTTAT-3') [11]/MZ12 primer (5'-GTAGCAGCAGTAAATAAGCAGC-3') [12]. The PCR condition and cycle parameters were the same as those listed above, except that the annealing temperature was 54.0 °C, and the same PCR amplification was repeated using 0.2 µl of the first PCR products as the template. The 219-bp partial sequence of the COX1 gene was amplified from the sample DNA, and direct sequencing confirmed the 171-bp DNA sequence.

In the UPGMA tree of the 171-bp partial sequence of COX1 (Fig. 1(d)), the confirmed DNA sequence of the clinical sample formed a monophyletic cluster with the reference sequences of *P. azarasi* (AB576758, AB576759, AJ891139, AJ891140) and *P. decipiens* (AJ891145, AJ891150). All DNA substitutions among the reference and sample DNA sequences in the monophyletic cluster converged on a deduced amino acid sequence (57 aa), indicating that all substitutions among the DNA sequences represented silent mutations: Clinical sample: [Gly8(ggt) Ser11(agt) Lys20(aag) Try44(tga) Val51(gta)], *P. azarasi* (AB576758): [Gly8(ggc) Ser11(agt) Lys20(aaa) Try44(tga) Val51(gta)], *P. azarasi* (AB576759): [Gly8(ggt) Ser11(agt) Lys20(aaa) Try44(tga) Val51(gta)], *P. decipiens* (AJ891145) and *P. azarasi* (AJ891139): [Gly8(ggt) Ser11(agt) Lys20(aaa) Try44(tgg) Val51(gta)], *P. decipiens* (AJ891150): [Gly8(ggt) Ser11(agt) Lys20(aaa) Try44(tga) Val51(gtg)], and *P. azarasi* (AJ891140): [Gly8(ggc) Ser11(agt) Lys20(aaa) Try44(tga) Val51(gta)]. All deduced amino acid sequences from the other references including the *P. decipiens* (AJ891146–9) cluster were unmatched to the convergent sequence. The sample sequence was categorized into the *P. azarasi* cluster with significant bootstrap value (89%), and the deduced amino acid was a perfect match to the converged amino acid sequence of the cluster. Therefore, the detected parasite was considered to be *P. azarasi*. The confirmed partial sequences of 12SrRNA and COX1 gene loci were registered to DDBJ as accession numbers LC312194 and LC312195, respectively.

The patient received a final diagnosis of extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi*. During a subsequent interview, the patient's mother reported that she enjoys eating raw fish and sushi at a Japanese restaurant twice per month. She was discharged without complications on the following day.

3. Discussion

This present report is the first to discuss the case of a patient with acute extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi*. Anisakidosis can be categorized into four clinical phenotypes, including gastric, intestinal, ectopic (or extra-gastrointestinal), and allergic [1,2]. Patients infected with *Pseudoterranova* spp. often experience sub-acute to acute abdominal pain [8,9]. As these specimens can usually be extracted from the stomach via endoscopy [9], the invasive capacity of *Pseudoterranova* spp. larvae is very low relative to that of *Anisakis* spp., and the ectopic form of anisakidosis is much less common than the gastric and intestinal forms [13]. In the present case, our patient developed itchiness and tenderness at the left inguinal region, and her mother noticed that the mass had increased in size 1 day prior to admission, suggesting that the size of the mass increases rapidly following the prompt migration of larvae to extra-gastrointestinal areas in patients with ectopic anisakidosis. This pathophysiology is rare among cases involving *Pseudoterranova* spp.

Although ectopic anisakidosis is difficult to diagnose using pathological specimens, accurate diagnosis is possible using PCR. However, such PCR detection using extracted DNA from formalin-fixed and paraffin-embedded tissue remains a challenge. To manage known difficulties such as cross-linked protein-DNA complexes and DNA fragmentation [14], we employed the TaKaRa DEXPAT® (TaKaRa Bio inc.) kit for the DNA extraction, and successfully amplified the 202-bp partial

12SrRNA gene fragment and the 219-bp partial fragment of COX1 gene. Even with such short DNA sequences (final sequence lengths after analysis were 107 and 171 positions, respectively), the method of phylogenetic reconstruction enabled us to determine genus and species levels of identification (Fig. 1(c) and (d)). To avoid confusion in future studies regarding *Pseudoterranova* spp., it should be noted that although the definitions of AJ891145 and AJ891150 are both "*P. decipiens*" in GenBank, the sibling species name is considered *P. azarashi* (Fig. 1(d)). The presence of the *P. azarashi* (AJ891139) reference, which is a 100% match to the COX1 gene locus (393 bp) of the AJ891145, also supports this estimation.

Although previous studies have indicated that more than 90% of anisakidosis cases occur in Japan, recent reports have documented cases in North and South America, Europe, and other Asian countries [4,5,7,8]. One explanation for this finding is the increased consumption of undercooked fish worldwide, as Japanese and sushi restaurants have become more popular throughout the world. Therefore, physicians and surgeons should remain aware of the signs, symptoms, and methods for identifying causal agents of anisakidosis.

In conclusion, the findings of the present report emphasize the rarity of acute extra-gastrointestinal anisakidosis caused by *Pseudoterranova azarasi* as well as the potential for accurate diagnosis via PCR. Furthermore, increased consumption of undercooked fish requires pediatricians and pediatric surgeons to remain aware of the potential for parasitic infection. Anisakidosis should be included in the differential diagnosis when patients present with unusual extra-gastrointestinal hernia symptoms.

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Declaration of conflict of interests

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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