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Received: May 1, 2020. Accepted: June 12, 2020. Published online: June 30, 2020. DOI: 10.7883/yoken.JJID.2020.289

Advance Publication articles have been accepted by JJID but have not been copyedited or formatted for publication.
Molecular identification of parasites isolated from the stomach of patients with *Anisakis* food poisoning in Toyama Prefecture, Japan, in 2018

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Keywords: *Anisakis pegreffii, Anisakis simplex* sensu stricto, horse mackerel, chub mackerel, food poisoning

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As the consumption of raw seafood, such as sashimi and sushi, is commonplace in Japan, *Anisakis* food poisoning frequently occurs throughout the country. This type of food poisoning is caused by the parasitic larvae of members of the Anisakidae family, which frequently and rarely enter the gastric and intestinal walls, respectively, promoting acute gastroenteritis. The Manual of Food Poisoning Statistics lists *Anisakis* and *Pseudoterranova* species among all anisakid nematodes as distinct causative agents of this type of food poisoning (1). The definitive hosts of these nematodes are marine mammals, namely, cetaceans for *Anisakis* spp. and pinnipeds for *Pseudoterranova* spp. Among these nematodes, *Anisakis simplex*, which is regarded as a species complex or *A. simplex* sensu lato, was considered the main causative agent of this type of food poisoning and has been categorized into three sibling species: *A. simplex* sensu stricto, *A. pegreffii*, and *A. berlandi* (2).

Studies based on this systematics have shown that *A. simplex* sensu stricto accounts for almost all cases of *Anisakis* food poisoning in Japan. *A. pegreffii* also causes food poisoning, but in very few cases, and no case of human food poisoning by *A. berlandi* has been recorded. Among the fish and cephalopods possibly acting as paratenic hosts of these *Anisakis* species, chub
mackerel is the most common, and, therefore, the prevalence and intensity of *Anisakis* larvae at the sibling species level have been closely investigated in this fish species. Chub mackerel caught in the Pacific Ocean is commonly infested with *A. simplex* sensu stricto, but those from the Sea of Japan and the East China Sea often harbor *A. pegreffii*. Additionally, the post-capture migration of larvae from the viscera of the fish to the muscle tissues occurs much more frequently for *A. simplex* sensu stricto than for *A. pegreffii*. This type of behavior explains why *A. simplex* sensu stricto is responsible for almost all cases of *Anisakis* food poisoning (3).

The number of *Anisakis* food poisoning cases has been increasing annually throughout Japan. In response, Toyama Prefecture, together with the Toyama Institute of Health, began analyzing *Anisakis* food poisoning cases to establish preventive countermeasures by obtaining 29 anisakid larvae from all 29 food poisoning cases that occurred in this prefecture between January 2018 and June 2018. The fish species that the patients reported having consumed were chub mackerel in 10 cases, horse mackerel and skipjack tuna in 2 cases each, and Japanese amberjack and Japanese pilchard in 1 case each; the causative fish species was unknown in 13 cases (Table 1). In these 13 cases,
multiple fish species were suspected to be the causative food, although chub mackerel was recorded in 7 of these 13 cases.

The larvae used in this study were isolated from patients in medical settings and delivered alive in physiological saline to the Toyama Institute of Health. This delivery procedure was suggested by the institute to prepare integrated DNA samples for molecular identification by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. The ITS region of the nuclear ribosomal DNA, spanning the ITS1, ITS2, and 5.8S subunit, was amplified by PCR using the following primer pair: NC5 forward, 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′; NC2 reverse, 5′-TTAGTTTCTTTCCTCCGCT-3′ (4). PCR amplicons of approximately 950 bp were generated from all DNA samples prepared from isolated anisakid larvae.

The amplicons were first digested with the restriction enzyme Hinfl and showed the following two RFLP patterns (Fig. 1): five fragments of approximately 330, 280, 240, 70, and 30 bp, or four fragments of approximately 610, 240, 70, and 30 bp. The former pattern was identical to that of A. pegreffii and the latter to that of A. simplex sensu stricto or A. berlandi (3). The amplicons were then digested with the restriction enzyme HhaI, which generated two fragments of
approximately 530 and 420 bp for all DNA samples (Fig. 1). These findings confirmed no contamination with *A. berlandi* (3).

The species identified by the RFLP analyses were verified by sequencing using the undigested amplicons; the sequences obtained were identical to those of *A. simplex* sensu strict or *A. pegreffii* (4). The nucleotide sequences have been deposited in the DDBJ/EMBL/GenBank database as *A. simplex* sensu stricto and *A. pegreffii* at the larval stage under the accession numbers LC536534 and LC536532, respectively.

The molecular identification of the anisakid larvae isolated from *Anisakis* food poisoning patients revealed that 27 corresponded to *A. simplex* sensu stricto and 2 to *A. pegreffii*. *A. pegreffii* has been identified as the causative agent in very few cases of *Anisakis* food poisoning (5–8). Here, the *A. pegreffii* contaminations are presumed to be derived from horse mackerel sashimi sold in late May or early June by two separate fishmongers operating in the same city of eastern Toyama Prefecture (Table 1). Further studies are ongoing to detect *A. pegreffii* in the muscle tissues of horse mackerel from various parts of Japan to explore the possibility of *A. pegreffii* causing food poisoning more frequently than previously estimated.
This article appeared in the Infectious Agents Surveillance Report (IASR), vol. 41, p. 34, 2020, in Japanese.

Acknowledgments:
This study was supported by grants from the Japan Agency for Medical Research and Development, AMED (16fk0108309j0203) and the Food Safety Commission, Cabinet Office, Government of Japan (Research Program for Risk Assessment Study on Food Safety, No. 1909).

Conflict of interest None to declare.

References:


**Figure legend**

Fig. 1. RFLP patterns of PCR products amplified from the DNA samples of *Anisakis pegreffii* (lanes 1 and 3) or *Anisakis simplex* sensu stricto (lanes 2 and 4) larvae isolated from *Anisakis* food poisoning patients. The ITS PCR products were treated with the restriction enzymes Hinfl (lanes 1 and 2) or Hhal (lanes 3 and 4) and electrophoresed on 3% agarose (w/v) gels. Location of the bands after digestion with Hinfl and Hhal are indicated on the left- and right-hand sides, respectively, as base pairs. The 100 bp DNA ladder was used to estimate the size of bands (lanes M), and the location of the 1,000-bp and 100-bp bands are indicated on the right-hand side (underlined).
Table 1. Suspected dishes of fish species consumed, and anisakid larvae isolated from patients with *Anisakis* food poisoning¹)

<table>
<thead>
<tr>
<th>Consumed fish species</th>
<th>No. of cases caused by</th>
<th>No. of suspected dishes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As²)</td>
<td>Ap³)</td>
</tr>
<tr>
<td>Chub mackerel</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Horse mackerel</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Skipjack tuna</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Japanese amberjack</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Japanese pilchard</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>27</strong></td>
<td><strong>2</strong></td>
</tr>
</tbody>
</table>

1) Based on molecular identification; regulatory examination performed in Toyama Prefectural Institute of Health between January 2018 and June 2018

2) As: *Anisakis simplex* sensu stricto

3) Ap: *Anisakis pegreffii*

4) N/A: not applicable
Fig. 1