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Molecular Identification of Parasites Isolated from the Stomach of Patients with Anisakis Food Poisoning in Toyama Prefecture, Japan, in 2018

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As consumption of raw seafood such as sashimi and sushi, is commonplace in Japan, Anisakis food poisoning is frequently observed throughout the country. This type of food poisoning is caused by parasitic larvae of the members of family Anisakidae; these larvae frequently enter gastric walls and in rare instances, intestinal walls, to induce acute gastroenteritis. The Manual of Food Poisoning Statistics lists Anisakis and Pseudoterranova species among all anisakid nematodes as distinct causative agents of Anisakis 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, regarded as a species complex or A. simplex sensu lato, has been considered as the main causative agent of Anisakis food poisoning, and has been categorized into three sibling species: A. simplex sensu stricto, A. pegreffii, and A. berlandi (2).

Studies based on these 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 yet. Among the fish and cephalopods that possibly act 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 East China Sea often harbor A. pegreffii. Additionally, post-capture migration of larvae from the viscera of fish to their muscle tissues occurs much more frequently for A. simplex sensu stricto than for A. pegreffii. This 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. Therefore, Toyama Prefecture, together with the Toyama Institute of Health, has begun analyzing cases of Anisakis food poisoning to establish preventive countermeasures by obtaining 29 anisakid larvae from all 29 cases of food poisoning that occurred in this prefecture between January 2018 and June 2018. The fish species reported to be consumed by the patients were as follows: 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 as the causative food, though chub mackerel was recorded in 7 of these cases.

The larvae used in this study were isolated from the patients in medical settings, and were 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 using PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. The ITS region of nuclear ribosomal DNA, spanning the ITS1, ITS2, and 5.8 S subunit, was amplified by PCR using the following primer pair: NC5 forward, 5′-TTAGTTTCTTTTCCTCCGCT-3′ (4). PCR amplicons of approximately 950 bp were generated using all DNA samples prepared from the isolated anisakid larvae.

The amplicons were first digested with the restriction enzyme HinfI 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 and latter patterns were identical to those of A. pegreffii and of A. simplex sensu stricto or A. berlandi, respectively.
The species identified using RFLP analyses were verified by sequencing using the undigested amplicons; the sequences obtained were identical to those of Anisakis berlandi (3). These findings confirmed that there was no contamination with A. berlandi (3).

The amplicons were then digested with the restriction enzyme Hhal (lanes 1 and 2) or HhaI (lanes 3 and 4) and electrophoresed on 3% agarose (w/v) gels. Location of the bands after digestion with HinfI and HhaI are indicated on the right-hand side (underlined).

**Fig. 1.** RFLP patterns of PCR products amplified from the DNA samples of A. pegreffii (lanes 1 and 3) or A. simplex sensu stricto (lanes 2 and 4) larvae isolated from Anisakis food poisoning patients. The ITS PCR products were treated with the restriction enzymes HinfI (lanes 1 and 2) or HhaI (lanes 3 and 4) and electrophoresed on 3% agarose (w/v) gels. Location of the bands after digestion with HinfI and HhaI 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).

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

Molecular identification of the anisakid larvae isolated from Anisakis food poisoning patients revealed that 27 and 2 larvae corresponded to A. simplex sensu stricto and A. pegreffii, respectively. A. pegreffii has been identified as the causative agent in very few cases of Anisakis food poisoning (5–8). Here, A. pegreffii contamination was presumed to originate 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 in order to explore the possibility of food poisoning by A. pegreffii being more frequent than that estimated previously.

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**Conflict of interest** None to declare.

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