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Usefulness of PCR-Restriction Fragment Length Polymorphism Analysis of the Internal Transcribed Spacer Region of rDNA for Identification of *Anisakis simplex* Complex

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Anisakiasis is a disease caused by infection with an anisakid larva, primarily *Anisakis simplex*, through the ingestion of raw marine fish, known worldwide as “sashimi”, or of a wide variety of undercooked marine fish. In Japan, anisakiasis is quite prevalent, with more than 1,000 new cases estimated to be diagnosed annually (1). At the end of 1999, “The Food Sanitation Law Enforcement Regulation” was amended, and anisakid larvae were newly added to the causative agents of food poisoning (2).

Recent molecular analyses of the internal transcribed spacer (ITS) regions of rDNA, spanning the ITS-1, ITS-2 and the 5.8S subunit, demonstrated that the morphological species *A. simplex* is a complex of three or more sibling species, including *A. simplex sensu strict, A. pegreffii* and *A. simplex C;* the latter species has wider intraspecific genetic diversity than the other two (3). The marked differences in their respective ecological niches and their low proportion of hosts shared (3) may lead to different transmission pathways for the parasites as well as potential pathogenicity to humans. However, until now, the identification of the anisakid larvae recovered from gastric anisakiasis patients in Japan has been based exclusively on their morphological features.

In the present study, a clinical isolate of anisakid larva obtained by an endoscopic operation from a 65-year-old Japanese female with epigastralgia (Fig. 1) was analyzed by both sequencing and PCR-restriction fragment length polymorphism (RFLP) mapping of the ITS regions of the rDNA. The clinical isolate was fixed in 70% ethanol and cut into two parts. The anterior half of the larva where the esophagus and ventriculus are located was cleared by immersion in the lactophenol solution for microscopic observations and was confirmed to be *A. simplex* complex by its morphological features. The remaining half of the larva was simultaneously

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Fig. 1. *Anisakis simplex*-like nematode isolated from the stomach of patient by gastroendoscopy. The ruler is graduated in mm.
The larva was cut into small pieces in 200 processes for sequencing and RFLP mapping of the PCR sequence of one of the sibling species of (Fig. 2) and was identified at the species level as A. simplex 950 bps in size, was sequenced, and its RFLP patterns for applied for PCR amplification with the primer pair, NC5 and boiling for 10 min. DNA extracted by the use of QIAamp subjected three times to freezing and thawing, followed by the combination of RFLP banding patterns of the ITS pattern of approximately 620, 250 bps) is distinguishable from that of A. pegreffii (AB196670) (5). According to the above-mentioned criteria, the clinical isolate is quite identical to that of A. simplex s. str. The sequences of both primer regions are omitted.

The PCR product of the clinical isolate had the identical sequence of one of the sibling species of A. simplex complex (Fig. 2) and was identified at the species level as A. simplex s. str. for the first time in Japan. The genetic differences among A. simplex sibling species can be demonstrated by comparing the combination of RFLP banding patterns of the ITS regions for HinII and HhaI. Namely, the ITS RFLP banding pattern of A. simplex s. str. for HinII (Fig. 3, lane 2: approximately 620, 250 bps) is distinguishable from that of A. pegreffii by the differences in fragment sizes (Fig. 3, lane 1: approximately 370, 300, 250 bps) while A. simplex C is not. Similarly, the RFLP pattern of A. simplex s. str. for HhaI (Fig. 3, lane 5) is distinguishable from that of A. simplex C, whose RFLP fragments are approximately 550, 300 and 130 bps (6). There is no visible difference between A. simplex s. str. and A. pegreffii in the RFLP banding patterns for HhaI (Fig. 3, lanes 4 and 5), but there are 2 bp substitutions between the two (4,5). According to the above-mentioned criteria, the clinical isolate (Fig. 3, lanes 3 and 6) was again identified as A. simplex s. str. by the combination of restriction profiles for HinII and HhaI. PCR-RFLP mapping is a rapid and cost-effective tool for the identification and molecular epidemiology of anisakid larvae of both human isolates and those from other sources.

REFERENCES


