

Molecular identification of the etiological agent of the human anisakiasis in Japan

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Abstract

Anisakis simplex complex presently comprises three sibling species, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C. *A. simplex* is a common parasite in fishes and cephalopods and capable of causing anisakiasis in humans. Therefore, identification of sibling species of *A. simplex* was important for human health. In this study, one hundred *Anisakis* type I larvae isolated from eighty five patients with anisakiasis in Hokkaido and Kyushu in Japan were analyzed by adapting the new molecular method that can identify the sibling species of *A. simplex* complex. Based on the restriction fragment length polymorphism (RFLP) pattern of ITS regions including 5.8 subunit rRNA gene, we identified two sibling species, *A. simplex* s. str. and *A. pegreffii*. However, the infection rate of *A. simplex* s. str. was significantly higher than that of *A. pegreffii*. Eighty four (98.8%) out of the eighty five patients were infected with *A. simplex* s. str. On the contrary, one patients (1.2%) in Kyushu infected with *A. pegreffii*. This study provided basic information about human infection with *A. simplex* complex. Furthermore, we suggested that *A. simplex* s. str. is the most important etiological agent in Japan.

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Keywords: *Anisakis simplex* sensu stricto; *Anisakis pegreffii*; Anisakiasis; PCR-RFLP; Japan

1. Introduction

Anisakiasis is the disease caused by infection with anisakid nematodes, particularly *Anisakis simplex* and *Pseudoterranova decipiens*. Humans become infected by eating raw or undercooked infected marine fish. The first case was reported in the Netherlands in 1960 [1] and since then 33,747 of similar cases have been reported throughout twenty seven countries including Japan until 1998 [2]. In particular, many cases have been reported from Japan, anisakiasis is public health problem due to the consumption of raw fish. Although the annual incidence of anisakiasis in Japan cannot be accurately documented, it has been estimated to be over 2000–3000.

Studies employing data from multilocus enzyme electrophoresis have revealed that *A. simplex* sensu lato consists of

genetically differentiated sibling species with different geographic and host distributions [3–8]. Such studies have facilitated the development of other molecular diagnostic tools for these species, in particular, those based on PCR-RFLP [7,9–17]. These DNA-based diagnostic techniques are advantageous because individual larva which lacks morphological features for identification can be identified. Although the PCR-RFLP approaches have proved to be useful as tools for identification of *Anisakis* larvae recovered from infected humans [7,9,16], there are few studies that reveal the human infection with *A. simplex* complex. In this study, *Anisakis* type I larvae obtained from patients in Japan were analyzed using PCR-RFLP of the ITS region including 5.8 subunit rRNA gene.

2. Materials and methods

2.1. Parasite materials

A total of one hundred larvae from Japanese patients were used in this study. Larvae were recovered from eighty five

Abbreviations: rRNA, ribosomal RNA; ITS, internal transcribed spacer; RFLP, restriction fragment length polymorphism.

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Table 1
Collecting data of Anisakis type I larvae

Locality	No. of patients (Sex)	No. of samples
Hokkaido	4 (F ^a)	4
	1 (M ^b)	1
Kyushu	30 (F)	38
	50 (M)	57

* Parasite samples were collected from December 1986 to October 2005.

^a Female.

^b Male.

patients, including thirty four female patients and fifty one male patients in the hospital in Hokkaido (Asahikawa city) and Kyushu (Kurume city, Fukuoka Prefecture) (Table 1, Fig. 1) and preserved in alcohol solution until use. For morphological examination, larvae were cleared in Gehter's solution and observed by the microscope.

2.2. DNA extraction

Larvae were washed in distilled water to eliminate Gehter's solution. Each larva was soaked in high concentration urea

buffer (8 M urea; 10 mM Tris–HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS) [18] for a few hours at room temperature with several buffer changes. Then the larva was digested overnight at 37 °C in digestion buffer (50 µl high concentration urea buffer and 20 µl of Proteinase K). DNA was subsequently extracted with phenol: chloroform: isoamylalcohol (25:24:1, v/v/v), then precipitated on ice with 2.5 × volume of 99.5% cold ethanol and 0.1 × volume of 3 M sodium acetate, and finally resuspended in Tris–EDTA buffer (10 mM Tris–HCl (pH8.0), 1 mM EDTA (pH8.0)).

2.3. PCR amplification

Amplification of the ITS region including 5.8 subunit rRNA gene was carried out using the above described method [10]. Two universal primers, the forward primer A (5'-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3') and the reverse primer B (5'-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3') were used for amplification. PCR was carried out in a 30 µl final volume containing extracted DNA, PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 170 µM dNTP mixture, 0.3 µM each primer and 0.75

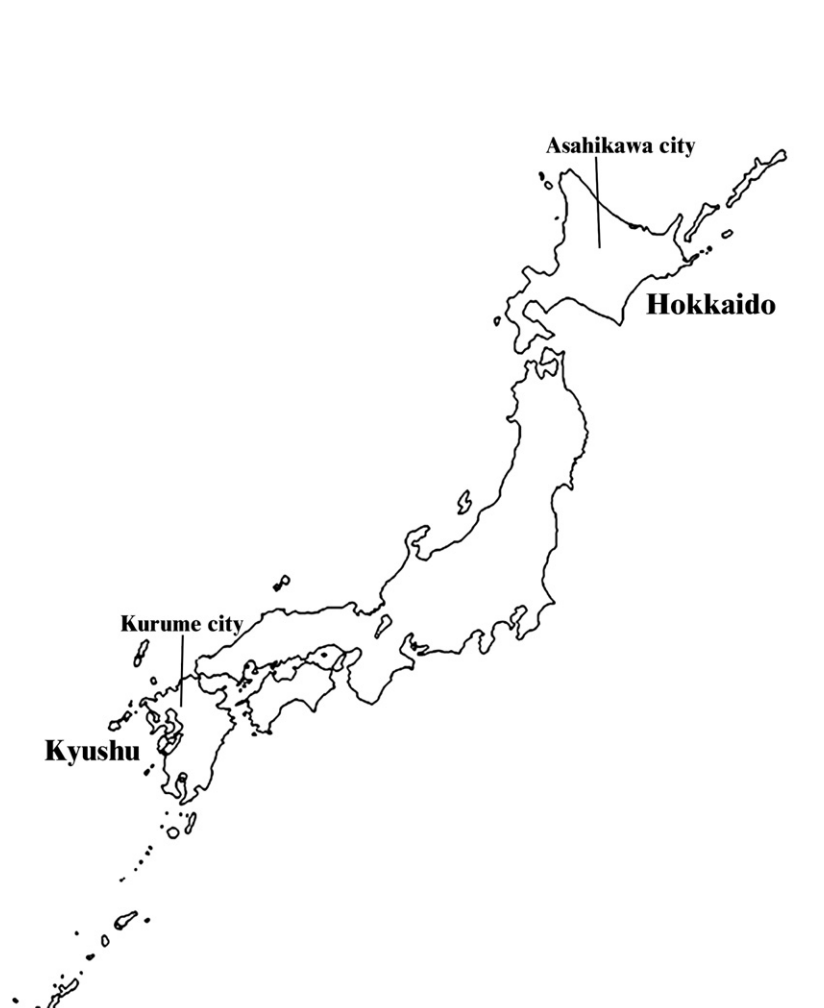


Fig. 1. Collection sites of samples from the patient in Japan.

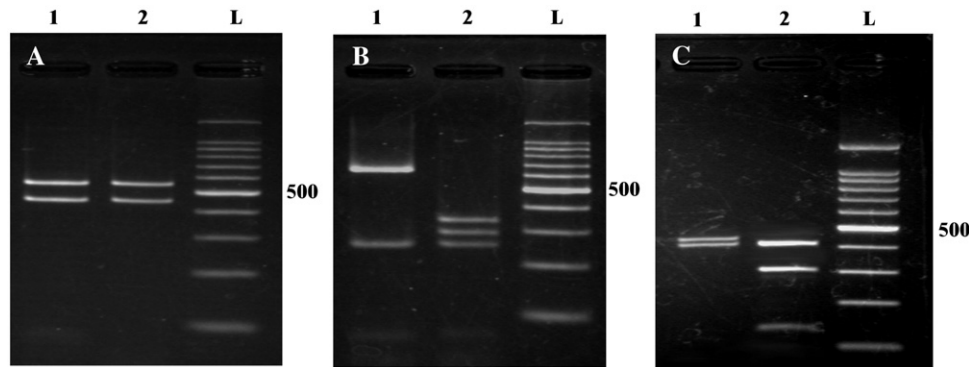


Fig. 2. RFLP patterns of the ITS region including 5.8 subunit rRNA gene using *HhaI* (A), *HinfI* (B) and *TaqI* (C). Lanes: 1 *A. simplex* s. str., 2 *A. pegreffii*, L 100 bp ladder.

units of *TaKaRa EX Taq* (Takara Bio Inc., Shiga, Japan). Twenty six cycles of amplification were performed in a RoboCycler Gradient 96 with Hot Top (Stratagene Japan K.K., Tokyo, Japan) after initial denaturation at 95 °C for 10 min. Each cycle consisted of a denaturation step at 95 °C for 1 min, an annealing step at 50 °C for 1 min, and an extension step at 72 °C for 1 min, with a final extension at 72 °C for 7 min following the last cycle. PCR products were separated by electrophoresis in a 2.0% seakem GTG agarose gel (Cambrex Bio Science Rockland, Inc., Rockland, Maine) in Tris–Borate–EDTA buffer and visualized by illumination with UV light after ethidium bromide staining.

2.4. RFLP analysis

Each PCR product was digested separately with three restriction enzymes, *HhaI*, *HinfI* and *TaqI* for the identification of the members of *A. simplex* complex according to genetic key of D'Amelio *et al.* [10]. Restriction fragments were separated by electrophoresis in a 3.0% seakem GTG agarose gel in Tris–Borate–EDTA buffer and visualized by illumination with UV light after ethidium bromide staining.

3. Results

3.1. Identification of sibling species by PCR-RFLP analysis

In morphological analysis, all specimens were identified as *Anisakis* type I larvae based on the presence of a boring tooth, a long ventriculus with an oblique ventricular-intestinal junction and a rounded tail possessing a mucron [19]. In molecular

analysis, universal primer pairs were able to successfully amplify the ITS region of all larvae tested, providing a single PCR product of approximately 1000 bp. Analysis using three restriction enzymes, *HhaI*, *HinfI* and *TaqI* produced species-specific RFLP pattern reported previously. Therefore, we identified *A. simplex* sensu stricto and *A. pegreffii* based on the combination of RFLP patterns. Digestion of ITS region with *HhaI* produced same pattern between two sibling species. Two bands corresponding to 550 and 430 bp were detected. On the other hand, digestion with *HinfI* and *TaqI* produced different patterns between two sibling species. The digestion of ITS region of *A. simplex* s. str. with *HinfI* produced two strong bands and one weak band (620, 250 and 80 bp, respectively), while the *TaqI* digestion produced two bands of 440 and 410 bp. The digestion of ITS region of *A. pegreffii* with *HinfI* produced three bands of 370, 300 and 250 bp, while the *TaqI* digestion produced three bands of 420, 310 and 140 bp (Fig. 2).

3.2. The infection rates of sibling species of *A. simplex* in the Japanese patients

There were significant difference in infection rate between *A. simplex* s. str. and *A. pegreffii*. Eighty four (98.8%) out of eighty five patients in Hokkaido and Kyushu infected with only *A. simplex* s. str. On the other hand, one patient (1.2%) in Kyushu infected with only *A. pegreffii* (Table 2). Many patients were infected with multiple larvae. However, the patient infected with *A. pegreffii* was infested by only one larva.

We report here for the first time the identification of *A. pegreffii* as a human pathogen in Japan. Considering that numerous larvae of *A. pegreffii* is detected in the southern Sea of Japan in our previously report [17], these suggests transmission risk for this sibling species in Kyushu area.

4. Discussion

Up to now, the cases of larva migrans by *A. simplex* described in the entire world constitute most of all anisakiasis reported. Recently, it is recognized that the morphospecies *A. simplex* is not a single species but a complex of three sibling species, *A. simplex* s. str., *A. pegreffii* and *A. simplex* C [3–5].

Table 2
Numbers of *A. simplex* complex isolated from patients in Hokkaido and Kyushu

Species	Patients			
	Hokkaido male	Hokkaido female	Kyushu male	Kyushu female
<i>A. simplex</i> s. str.	1	4	56	38
<i>A. pegreffii</i>	0	0	1	0

This work therefore identified the etiological agent of anisakiasis at the sibling species level. The ITS region sequences including 5.8 subunit rRNA gene have been used as targets for the identification because they display sequence variation between three sibling species, but only minor variation within the ITS 1 and 2 regions [12,13,16]. However, in this study, the presence of minor sequence variation within the ITS 1 region allowed us to distinguish between *A. simplex* s. str. and *A. pegreffii*. We therefore supported the usefulness of PCR-RFLP analysis of ITS region for sibling species level identification.

The molecular approach to identify sibling species of *A. simplex* in the diagnosis of anisakiasis was reported for the first time in 1999 [9]. Although diagnostic molecular identification for anisakiasis is currently available, there are only two reports that identified at the sibling species level [9,16]. In this study, we successfully identified one hundred larvae of *A. simplex* recovered from eighty five patients with anisakiasis at the sibling species level. Therefore, this study will serve to provide basic information about human infection.

Recently, *A. simplex* s. str. and *A. pegreffii* were detected from Pacific cod landed in Hokkaido in Japan [13]. We also detected *A. simplex* s. str. and *A. pegreffii* from fishes in Japanese waters. In our previous study, sixty five larvae (100%) collected from Hokkaido were identified as *A. simplex* s. str., thirty seven larvae (97%) collected from Kyushu were identified as *A. pegreffii*. On the other hand, one larva (3%) collected from Kyushu were hybrid genotypes [17]. However, the difference in infection rate of two sibling species in humans was significant, 98.8% of Japanese patients with anisakiasis infected with *A. simplex* s. str. in this study. Although the reason for difference in results within this study is still unclear, it is evident that the infection rate of sibling species of *A. simplex* complex differs in human. Therefore, further geographical and seasonal examination of *A. simplex* complex in fish is needed to reveal etiological background. This study suggested that identification of *A. simplex* at the sibling species level will have clinical importance in the future. On the other hand, *A. simplex* C and hybrid genotype were not detected in patients with anisakiasis in this study. *A. simplex* C has been found in the Canadian Pacific and in regions south of 35° S [5] and there has been no report of distribution of this species in Japanese waters. Although we detected hybrid genotype between *A. simplex* s. str. and *A. pegreffii* in Japanese waters [17], this genotype was not yet recognized as common genotype. Thus, we consider that there is little opportunity to infect with both sibling species in human in Japan.

In other parasitic nematodes, remarkable variability of infectivity in hosts between genotypes were observed in *Trichinella* genotypes [20]. Therefore, experimental data concerning infectivity of sibling species of *A. simplex* is needed.

We identified etiological agents from eighty five cases in Hokkaido and Kyushu in this study. Although anisakiasis in Japan is widely distributed from Hokkaido to Okinawa, the incidence is high on Hokkaido and Kyushu [21]. Based on the results in this study, it was thought that *A. simplex* s. str. is major etiological agent on anisakiasis in Japan.

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