Molecular Genotyping of Anisakis Larvae in Middle Eastern Japan and Endoscopic Evidence for Preferential Penetration of Normal over Atrophic Mucosa

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Abstract

Background: Anisakiasis is a parasitic disease caused primarily by Anisakis spp. larvae in Asia and in Western countries. The aim of this study was to investigate the genotype of Anisakis larvae endoscopically removed from Middle Eastern Japanese patients and to determine whether mucosal atrophy affects the risk of penetration in gastric anisakiasis.

Methods: In this study, 57 larvae collected from 44 patients with anisakiasis (42 gastric and 2 colonic anisakiasis) were analyzed retrospectively. Genotyping was confirmed by restriction fragment length polymorphism (RFLP) analysis of ITS regions and by sequencing the mitochondrial small subunit (SSU) region. In the cases of gastric anisakiasis, correlation analyses were conducted between the frequency of larval penetration in normal/atrophic areas and the manifestation of clinical symptoms.

Results: Nearly all larvae were A. simplex seusu stricto (s.s.) (99%), and one larva displayed a hybrid genotype. The A. simplex larvae penetrated normal mucosa more frequently than atrophic area (p = 0.005). Finally, patients with normal mucosa infection were more likely to exhibit clinical symptoms than those with atrophic mucosa infection (odds ratio, 6.96; 95% confidence interval, 1.52–31.8).

Conclusions: In Japan, A. simplex s.s. is the main etiological agent of human anisakiasis and tends to penetrate normal gastric mucosa. Careful endoscopic examination of normal gastric mucosa, particularly in the greater curvature of the stomach will improve the detection of Anisakis larvae.

Introduction

Anisakiasis is a parasitic infection caused by nematodes, particularly Anisakis simplex, A. physeteris or Pseudoterranova decipiens [1]. A total of 2,000 cases of Anisakiasis are reported every year worldwide, with more than 90% of the cases in Japan. The disease is contracted by eating raw or undercooked fish contaminated with the parasites. The first cases of anisakiasis were reported in the Netherlands, followed by widespread infection in Japan [2]. In the past decade, the incidence of anisakiasis has been increasing in several countries, including Austria and Italy [1–5]. Most patients are infected by A. simplex worldwide. Isolated cases of A. physeteris infection were identified in Japan and Spain [1,5]. Pseudoterranova novasis has been reported primarily in the United States and Canada, with a few reports in south Japan and Europe [1,5–7].

Recent molecular studies using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing of the internal transcribed spacer (ITS) region revealed that A. simplex consists of 3 major sibling species: A. simplex seusu stricto (s.s.), A. pegreffii, and A. simplex C [8–10]. In North and South Japan, A. simplex s.s. is the most common species. However, the source of infection has not been identified, and no study has investigated regional differences in Anisakis spp. in Central Japan [10].

Currently, the only effective treatment is the endoscopic removal of the live larvae from the gastric mucosa. Therefore, it is important to find the larvae quickly and remove them...
thoroughly. Only few studies examined endoscopic findings of gastric anisakiasis [11,12]. The Anisakis larvae are usually found in the greater curvature [11]. However, they are often hidden in the gastric folds. Furthermore, the mucosa undergoes a series of morphological changes from the time of contaminated food ingestion to the endoscopic examination, including edema and tumor formation, which may hide the larvae [12]. Furthermore, it is often hidden among the gastric folds with mucosal edema. For these reasons, the endoscopic detection and removal of all live larvae remains a challenge, and more information is required on the penetration sites.

To the best of our knowledge, no study has investigated whether the health status of the mucosa affects Anisakis penetration in the human stomach. A recent in vitro study showed that Anisakis penetrates agar gel more easily under acidic conditions [13]. In the stomach, the pH of normal mucosa is lower than that of atrophic area [14]. Therefore, we hypothesized that Anisakis may preferentially invade normal gastric mucosa. In this report, we genotyped Anisakis larvae removed from patients in Middle Eastern part of Japan and tested the impact of mucosal properties on larval penetration.

Materials and Methods

Patients

From January 2011 to December 2012, 44 patients with anisakiasis visited three hospitals of Middle Eastern Japan (Kameda General Hospital, Kameda Clinic and Toukatsu Hospital). A total of 55 larvae were removed by upper gastrointestinal endoscopic examination and 2 larvae were removed by colonoscopy.

Morphological examination

The collected larvae were sent to our laboratory and morphological observation was carried out under a light microscope. We identified the genus and stage of the larvae, as previously described [6,15,16].

Table 1. Characteristics of the patients.

| Characteristics               | Value |
|-------------------------------|-------|
| Number of patients            | 44    |
| Gender (men/women)            | 16/28 |
| Age, mean (SD)                | 54.5 (± 15.6) |
| Antacid medicines (cases)     | 9     |
| Source of infection (%)       |       |
| Sardines                      | 33    |
| Mackerel                      | 28    |
| Yellow tail                   | 21    |
| Horse mackerel                | 5     |
| Flatfish                      | 5     |
| Rockfish                      | 2     |
| Trout                         | 2     |
| Squid                         | 2     |
| Tuna                          | 2     |
| Symptomatic/Asymptomatic (cases) | 33/11 |
| Onset of symptoms after eating, mean (SD) (day) | 1.4 (± 1.1) |

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Table 2. Characteristics of Anisakis larvae.

| Characteristics               | Value |
|-------------------------------|-------|
| Stage of larvae (cases)       | 57    |
| Stage III                     | 56    |
| Stage IV                      | 1     |
| Species (cases)               | 57    |
| A. simplex s. s.              | 56    |
| Hybrid genotype               | 1     |
| Location of larvae (cases)    | 57    |
| Stomach*                      | 54    |
| Duodenum                      | 1     |
| Colon                         | 2     |
| Number of cases in multiple infection (2–4 larvae) | 7     |
| Stomach                       | 7     |
| Colon                         | none  |

*M52% was in the gastric body, and most of them in the greater curvature.

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Molecular identification

Total DNA was extracted with the Get pure DNA Kit-Cell, Tissue (Dojindo, Japan) according to the manufacturer’s instructions. The ITS region (ITS1, 5.8S and ITS2) of ribosomal DNA (rDNA) was amplified with the forward primer NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and the reverse primer NC2 (5′-TTAGTTTTCTTTTCCTCCGCT-3′) [8–10]. Mitochondrial small subunit (SSU) of ribosomal RNA (rRNA) was amplified with the forward primer MH3 (5′-TTGTCTCCA-GAATAATCGGCTAGACTT-3′) and the reverse primer MH4 (5′-TCTACTTTACTAGACTTGG-3′). The PCR reaction was performed as previously described [17]. Each PCR product was digested by two restriction enzyme HhaI and HinfI (Takara, Japan) and evaluated based on previous reports [8–10,15–17]. To identify the hybrid genotype, the PCR products of the ITS and mitochondrial SSU regions were sequenced using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer’s instructions,
Statistical analysis

Statistical analyses were performed using test for population proportion and Fisher’s exact test (Statcel 3 software, OMS Publishing Inc., Saitama, Japan); p value of less than 0.05 was considered to be statistically significant.

Results

Characteristics of the patients

A total of 42 patients diagnosed with gastric anisakiasis and 2 patients with colonic anisakiasis were admitted in the three Middle Eastern Japan hospitals during the study period. The onset of symptoms was 1.4 ± 1.1 days (range: 0–5 days) before admission to a hospital (Table 1). The sources of infection were raw or undercooked edible fish, including sardine (33%), mackerel (28%), and yellowtail snapper (21%). Interestingly, 11 cases were asymptomatic for anisakiasis. Four patients were admitted for a follow-up examination on gastric or colon cancer after endoscopic treatment, five patients were screened for gastric cancer by detailed endoscopic examination, and two patients were admitted for a follow-up examination on gastroesophageal reflux disease.

Morphological and genotyping of Anisakis larvae

In total, 55 larvae were removed by gastrointestinal endoscopy and 2 larvae by colonoscopy. Macroscopic examination revealed that all larvae belonged to the genus Anisakis (Table 2). No Pseudoterranova type was found. Fifty-six larvae were identified at the 3rd larval stage, and one larva exhibited the characteristics of the 4th larval stage, with shedding of part of its outer cuticle and absence of mucron and boring tooth, as reported previously [6,15]. There were no morphological differences between the symptomatic isolates and asymptomatic isolates. The RFLP profiles obtained by digestion of the ITS region (Hha I and Hinf I) showed that 99% of the larvae were A. simplex s.s. (550–430 and 620–250 bp), and one larva had a hybrid genotype (550–430 bp and 620–370–300–250 bp) (Figure 1). This observation was consistent with a hybrid genotype as reported previously [17]. To confirm the diagnosis, the ITS region and mitochondrial SSU regions were further examined by sequence analysis. The sequencing of the ITS region supported the presence of heterozygotes (C/T) at position 240 and 256. Mitochondrial SSU alignment revealed that the sequence of the hybrid was consistent with that of A. simplex s.s., but different from that of A. pegreffii at positions 30, 32, and 429 (see Table 3), which indicated the female parent of hybrid was A. simplex s.s.
### Discussion

Anisakiasis is a major issue in Japan due to the nutritional habits of the general population. The medical record of the patients included in this study revealed that they consume a large variety of raw or undercooked edible fishes as sashimi or sushi. Because this habit is deeply rooted in the culture of the country, the current focus is to improve the efficiency of endoscopic removal of the larvae implanted in the gastrointestinal wall. The present study identified the genotype of the larvae affecting the Middle Eastern Japanese population, and offers invaluable information on the distribution of the larvae in the gastric mucosa.

In Southern and Northern Japan, the major etiological agent of human anisakiasis was identified as *A. simplex* s.s. [10]. The present study identified the same parasite in 99% of the larvae isolated from Middle Eastern Japanese patients with gastric anisakiasis. Interestingly, 1 larva exhibited a hybrid genotype, which is the first report for human anisakiasis. The larva with the hybrid genotype was collected from a patient who ate trout and was infected with three larvae by gastrointestinal endoscopy; this patient had abdominal pain. The existence of a hybrid genotype was explained as the result of natural interspecies hybridization between *A. simplex* s.s. and *A. pegreffii* [17,21,22]. In the previous study, hybrid genotypes were detected in *Blue whiting*, *Scomber japonicas*, and *Scomber scombrus* [21,22]. We hypothesized that the hybrid genotype could parasite in various marine fish. There was no morphological difference between *A. simplex* s.s. and the hybrid genotype. Moreover, we did not find the pathogenic characteristics between *A. simplex* s.s. and the hybrid genotype. Further studies are required to characterize the hybrid genotype.

These data suggest that *A. simplex* s.s. is the causative agent of anisakiasis everywhere in Japan. Incidentally, *A. simplex* s.s. larvae was reported to penetrate muscle tissue more easily than *A. pegreffii* larvae in edible fish [23,24]. Therefore, the predominance of gastric infection by *A. simplex* s.s. does not necessarily reflect the relative abundance of these parasites in the ocean.

### Correlation between mucosal status on larval penetration

For this analysis, the larvae from patients who were prescribed antacid medicines were excluded because they may affect the morphology of the gastric mucosa. Accordingly, a total of 45 larvae from 35 patients were included. First, we identified a statistically significant correlation between clinical symptoms and penetration site (*p* = 0.014; Table 4). Patients with infected normal mucosa were more likely to exhibit symptoms (odds ratio, 6.96; 95% confidence interval, 1.52–31.8) than those with infected atrophic mucosa. Also, we tested whether the larvae preferentially infected normal or atrophic mucosa. Statistically significant correlation was found between the penetration sites and normal mucosa by test for population proportion (*p* = 0.005) (Table 5). Thus, we suggested that *A. simplex* s.s. tends to penetrate normal mucosa than atrophic area, regardless of the extent of background atrophic gastritis.

### Table 4. Correlation between penetration site and symptoms.

| Penetration site in stomach | Atrophic mucosa | Normal mucosa |
|-----------------------------|-----------------|---------------|
| Symptomatic                 | 5               | 29            |
| Asymptomatic                | 6               | 5             |

### Correlation between penetrating site and mucosa background in the stomach.

| Atrophic border | Normal | Atrophic | Observation number of larvae | Total | Theoretical number of larvae | Normal | Atrophic | Total |
|-----------------|--------|----------|-------------------------------|-------|-----------------------------|--------|----------|-------|
| C1              | 0.83   | 0.17     | 5                             | 0     | 5                           | 4.15   | 0.85     | 5     |
| C2              | 0.65   | 0.35     | 5                             | 0     | 5                           | 3.25   | 1.75     | 5     |
| C3              | 0.44   | 0.56     | 3                             | 1     | 4                           | 1.76   | 2.24     | 4     |
| O1              | 0.17   | 0.83     | 2                             | 3     | 5                           | 0.85   | 4.15     | 5     |
| O2              | 0.1    | 0.9      | 2                             | 2     | 4                           | 0.4    | 3.6      | 4     |

### Table 5. Correlation between penetrating site and mucosa background in the stomach.

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mentioned that symptomatic anisakiasis is caused by an allergic response [28,29]. Accordingly, clinicians noted the efficacy of corticosteroids therapy for gastric and intestinal anisakiasis [30]. Since this is a retrospective study, anti-Anisakis IgE antibody was not determined. Future studies comparing serum IgE levels between symptomatic and asymptomatic patients may shed some light on the mechanisms of anisakiasis.

Thus, after treatment of anisakiasis, it may be necessary to follow-up when the patients take NSAIDs. In conclusion, the present study showed that *A. simplex* s.s. is the predominant etiological agent for anisakiasis in Japan, and the larvae preferentially penetrate normal gastric mucosa. Careful endoscopic examination of normal mucosa, particularly in the greater curvature of the gastric body, will improve detection of the larvae and treatment efficiency. Finally, since patients with widespread atrophic mucosa may not manifest the clinical symptoms of anisakiasis, routine endoscopic screening could potentially avoid unexplainable complications and adverse reactions to common medications.

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**Author Contributions**

Conceived and designed the experiments: TA NA NO. Performed the experiments: TA NA. Analyzed the data: TA NA HI NO T. Seki NH. Contributed reagents/materials/analysis tools: TA NA TK NH SN KY MHT. Shiraori MK HF EI MN SS TY NS MS TT. Arranged the paper: TA NA NO.

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