Original Article

Analysis of the Prevalence and Species of *Anisakis* nematode in Sekisaba, *Scomber japonicus* Caught in Coastal Waters off Saganoseki, Oita in Japan

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SUMMARY: Anisakidosis is developed by ingesting *Anisakis* in marine fish, including the chub mackerel, *Scomber japonicus*, without proper pre-treatment such as cooking or freezing. Two sibling species of *Anisakis* are found in *S. japonicus* from Japanese waters, and the prevalence and species of *Anisakis* in the fish depend on the sea area. For example, *Anisakis simplex* sensu stricto (s.s.) is found in the Pacific stock of *S. japonicus*, whereas *A. pegreffii* is found in the Tsushima Warm Current stock. *S. japonicus* caught in the Bungo Channel, off the coast of Saganoseki in Oita Prefecture, which is branded as Sekisaba, inhabits a very limited area; however, the infection states of *Anisakis* found in Sekisaba remain unclear. In this study, we compared the infection states of *Anisakis* in Sekisaba with those in *S. japonicus* caught in the South Oita area and Nagasaki Prefecture. All *Anisakis* from the Nagasaki Prefecture were *A. pegreffii*, while most of them found in Sekisaba and fish from the South Oita area were *A. simplex* s.s. Interestingly, the prevalence of *Anisakis* in Sekisaba was significantly lower than that in the other two areas. This may reflect the fact that Sekisaba might belong to a distinct stock of *S. japonicus*, varying from other stocks.

INTRODUCTION

Anisakidosis is a fish-borne parasitic zoonosis caused by the accidental ingestion of larval nematodes, including *Anisakis* and *Pseudoterranova*, found in raw fish such as the chub mackerel (*Scomber japonicus*) (1,2). *Anisakis* type I larvae penetrate the gastric mucosa, resulting in acute epigastric pain, occasionally accompanied by nausea and vomiting (3). Currently, the most effective treatment is endoscopic removal of the nematode, although a recent report has shown that an over-the-counter medicine containing wood creosote (Seirogan) ameliorates the symptoms (4).

Anisakis simplex sensu lato (s.l.) is highly associated with anisakidosis and presently comprises three sibling species, *A. simplex* sensu stricto (s.s.), *A. pegreffii*, and *A. berlandi*. It has become possible to identify the species

of *A. simplex* s.l. using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) (5). PCR-RFLP analysis of the ribosomal DNA internal transcribed spacer (rDNA ITS) provides a useful approach for the specific identification of both distantly and closely related ascaridoid species, including *Anisakis* type I larvae, as these spacers show high levels of interspecific sequence differences with low-level intraspecific variation (5–7).

Scomber japonicus is one of the most common fish in Japanese coastal waters and forms two stocks, the Tsushima Warm Current (TWC) stock (found in the East China Sea and the Sea of Japan) and the Pacific stock (found off the Pacific coast of Japan). *S. japonicus* from the Nagasaki Prefecture on the Sea of Japan coast and the East China Sea coast belong to the TWC stock, while those from the southern area of the Oita Prefecture on the Pacific Ocean coast (South Oita area) belong to the Pacific stock (8). As previously reported, the species of *Anisakis* type I larvae found in *S. japonicus* differed between these two stocks. Umehara et al. revealed that *A. simplex* s.s. was found in the Pacific stock of *S. japonicus*, whereas *A. pegreffii* was predominantly found in the TWC stock (9).

Scomber japonicus caught in the Bungo Channel, off the coast of Saganoseki in the Oita Prefecture, is

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Fig. 1. The prevalence and mean intensity of *Anisakis* type I larvae in *S. japonicus* from Saganoseki. (A) A map showing the collection sites of *S. japonicus* in Japanese waters around Kyushu Island. (B) Bar graph shows prevalence (%) of *Anisakis* type I larvae in each group of *S. japonicus*. Statistical significance of differences between experimental groups of *S. japonicus* was determined using the Fisher's exact test. (C) Mean intensity of *Anisakis* type I larvae in *S. japonicus* from the three different areas in Kyushu, Japan. Data are presented as mean $\pm$ SE. Statistical significance of differences between groups was determined using the bootstrap t-test. *: $P < 0.005$. **: $P < 0.0001$. NS: not significant.

called Sekisaba, a brand of chub mackerel, which is frequently eaten raw in Japan. Sekisaba inhabits the Bungo Channel, which is a geomorphologically unique strait separating the Kyushu and Shikoku islands and connects the western part of the Seto Inland Sea to the Pacific Ocean (Fig. 1A). By far, the prevalence (the proportion of fish infected), mean intensity (the average number of parasites per infected fish), and the species of *Anisakis* type I larvae in Sekisaba have not been investigated.

In this study, we compared the prevalence of *Anisakis* type I larvae found in *S. japonicus* captured from three different areas of the Kyushu Island, including the Nagasaki Prefecture (representative of the TWC stock), the South Oita area (representative of the Pacific stock), and Saganoseki (Sekisaba). We found that the prevalence of *Anisakis* type I larvae in Sekisaba was

significantly lower than that in *S. japonicus* from the other two areas. A possible explanation for the low prevalence is that Sekisaba may form a unique stock that is distinguishable from the other stocks of *S. japonicus* in Japanese waters.

MATERIALS AND METHODS

Fish samples and method for detecting anisakid nematodes: *S. japonicus* was obtained in batches from fish markets in Oita, Japan, from October 2014 to April 2016. We purchased 40 fish (in a pool of $n = 4$ –13 whole bodies of fish) caught in coastal waters off the South Oita area and Nagasaki Prefecture. For *S. japonicus* from Saganoseki (Sekisaba), 10 whole bodies of fish and the viscera from 64 fish were provided by the Oita fishermen's cooperative association (Saganoseki branch)

(Fig. 1A). Each fish sample was weighed and measured. *Scomber japonicus* from the Nagasaki Prefecture ($n = 40$; mean fork length: 36.7 cm, range: 33–41 cm; mean body weight: 582.6 g, range: 416–835 g), the South Oita area ($n = 40$; mean fork length: 37.7 cm, range: 34–42 cm; mean body weight: 582.1 g, range: 435–775 g) and Saganoseki ($n = 10$; mean fork length: 38.9 cm, range: 32–46 cm; mean body weight: 604.8 g, range: 528–693 g) were used for examination. The fish samples were examined within 24 h of purchase. After separating the viscera and muscles from the body of the fish, anisakid larvae were detected by visual inspection and removed using forceps, and the morphology of the isolated larvae was observed using a stereomicroscope. In particular, anisakid larvae in the muscle were detected by gently pressing the muscle between two glass plates. *Anisakis* type I larvae were used for further investigations.

Epidemiological data of *Anisakis* type I larvae in *S. japonicus*: Epidemiological data, including the prevalence and mean intensity of *Anisakis* type I larvae in *S. japonicus* were analyzed using the method described by Bush et al. (1997) (10). Prevalence was defined as the proportion of fish hosts infected with the *Anisakis* species. The mean was determined as the average number of parasites found in the infected hosts. Differences in the prevalence and mean intensity values of *Anisakis* type I larvae infection between *S. japonicus* from the three areas were assessed with Fisher's exact test using the GraphPad Prism software version 8.42 (GraphPad Software Inc., San Diego, CA, USA), and the bootstrap t-test using QPweb software, respectively (11).

PCR-RFLP: Genomic DNA from *Anisakis* type I larvae were prepared using the alkaline extraction method. The larvae were boiled in 180 μ l of 50 mM NaOH for 60 min, following which 20 μ l of 1 M Tris-HCl (pH 8.0) was added. PCR-RFLP analysis was performed to identify the *A. simplex* species, as previously described (5). Briefly, PCR amplification of the rDNA ITS region (ITS-1 and ITS-2 plus their spanning regions) was performed in a 25 μ l reaction volume containing 10 ng template DNA, 0.25 units of KOD FX neo (TOYOBO, Osaka, Japan), 12.5 μ l of 2 $\times$ buffer (provided with the polymerase by the manufacturer), 5 μ l of deoxynucleoside triphosphates (2 mM), 0.75 μ l of forward primer (5'-GTAGGTGAACCTGCGGAAGGATCATT-3', 10 μ M), and 0.75 μ l of reverse primer (5'-TTAGTTTCTTTTCTCCGC-3', 10 μ M). The PCR conditions were as follows: 95°C for 2 min for 1 cycle, 95°C for 15 s, 60°C for 30 s, and 68°C for 1 min for 30 cycles, and a final extension of 5 min at 68°C. The PCR products were then digested by a restriction endonuclease, Hha I (Takara, Shiga, Japan) or Hinf I (Takara) according to the manufacturer's recommendations for RFLP analysis of rDNA ITS. The digested products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, visualized by illumination with short-wave ultraviolet (UV) light and photographed.

RESULTS

In this study, all anisakid nematodes were detected in

the viscera and no nematodes were found in the muscle tissues. *Anisakis* type I larvae were counted among the detected anisakid nematodes. The prevalence of *Anisakis* type I larvae in *S. japonicus* from the Nagasaki Prefecture was 75% (30/40) (Fig. 1B). Conversely, the prevalence of *Anisakis* type I larvae in *S. japonicus* from the South Oita area was 38% (15/40). Surprisingly, the prevalence of *Anisakis* type I larvae in Sekisaba was only 6.8% (5/74). This difference in prevalence was statistically significant between the groups; Nagasaki Prefecture vs. the South Oita area: $P < 0.005$, the South Oita area vs. Saganoseki: $P < 0.0001$, and Nagasaki Prefecture vs. Saganoseki: $P < 0.0001$.

Next, we compared the average number of *Anisakis* type I larvae found in the infected fish from the three groups. Although Sekisaba showed relatively low intensity, there was no significant difference in the mean intensity between the groups (Nagasaki Prefecture: 16.2 ± 5.97 [$n = 30$], the South Oita area: 5.9 ± 1.54 [$n = 15$], Saganoseki: 2.8 ± 1.61 [$n = 5$] [larvae per fish]) (Fig. 1C).

To identify the species of *Anisakis* type I larvae found in *S. japonicus* from these three areas, we performed the PCR-RFLP analysis. Amplification of the rDNA ITS resulted in an approximately 1,000-base pair (bp) fragment (Fig. 2A), which was followed by digestion with either Hha I (Fig. 2B) or Hinf I (Fig. 2C) to enable identification of the seven closely related *Anisakis* species.

Hha I digestion of a PCR product amplified from the ITS region of rDNA of *A. simplex* s.s. resulted in two fragments of 530 bp and 420 bp (Fig. 2B left) and Hinf I digestion yielded 620 bp, 250 bp, and 80 bp fragments, although the 80 bp band was too faint to be seen in the photograph (Fig. 2C left). Hha I digestion of *A. pegreffii* showed 530 bp and 420 bp fragments (Fig. 2B right) and 370 bp, 300 bp, and 250 bp fragments by Hinf I digestion (Fig. 2C right). As shown in Fig. 2D, all the *Anisakis* type I larvae collected from the Nagasaki Prefecture were identified as *A. pegreffii*, while 93% of the larvae collected from the South Oita area were *A. simplex* s.s. Identification of the *Anisakis* type I larvae in Sekisaba resulted in a trend similar to that of the South Oita area. Of the 14 larvae analyzed, 13 (93%) were *A. simplex* s.s., and the one other larva was that of *A. pegreffii*. Recently, Umehara et al. reported that hybrid genotypes possibly generated by natural interspecific hybridization between *A. simplex* s.s. and *A. pegreffii* have been identified in *S. japonicus* (6). However, no hybrid genotype of larvae was identified in this study.

DISCUSSION

According to the annual report of the National Institute of Infectious Diseases in Japan, it is estimated that more than 7,000 cases of anisakidosis occur annually in Japan due to the Japanese food habit of eating raw fish, such as sushi and sashimi (12).

In particular, the chub mackerel is frequently eaten raw in Kyushu Island, further highlighting the importance of investigating the prevalence of *Anisakis* type I larvae in *S. japonicus*.

In Kyushu, there is easy access to fish from both the Pacific and TWC stocks of *S. japonicus*. In this study,

Fig. 2. PCR-RFLP analysis for the identification of *Anisakis* type I larvae species in *S. japonicus*. (A) The ITS region of rDNA of either *A. simplex s.s.* or *A. pegreffii* was amplified by PCR and the products were separated by agarose gel electrophoresis. M indicates DNA size maker (GeneRuler DNA Ladder, Thermo Fisher Scientific [Waltham, MA, USA]). (B) PCR products in A were digested with Hha I and separated by agarose gel electrophoresis. (C) PCR products in A were digested with Hinf I and separated by agarose gel electrophoresis. (D) Percentage of genotypes of *Anisakis* type I larvae species determined by PCR-RFLP analysis.

we investigated the prevalence and species of *Anisakis* type I larvae in Sekisaba, a brand of chub mackerel from the Oita Prefecture in Kyushu.

First, the *Anisakis* type I larvae found in this study were most prevalent in the viscera of the *S. japonicus* we inspected; however, they were not found in the fish muscle in this surveillance study. In this study, we examined *Anisakis* type I larvae by visually inspecting the viscera under a stereomicroscope and the muscles between two glass plates. However, the inspection method had a limitation in terms of detecting anisakid larvae due to low sensitivity. To detect *Anisakis* type I larvae more accurately in *S. japonicus*, it is necessary to employ more sensitive methods, such as the artificial peptic digestion method and the UV-press method. We need to use the above methods in future studies to achieve more accurate results. Next, we found that

most of the *Anisakis* type I larvae found in Sekisaba were *A. simplex s.s.*, whereas all the *Anisakis* type I larvae collected from the Nagasaki Prefecture were *A. pegreffii*, suggesting that Sekisaba does not belong to the TWC stock of *S. japonicus*. This was also supported by the finding that no hybrid genotypes were detected in Sekisaba. Consistent with a previous report, the prevalence of *Anisakis* type I larvae in *S. japonicus* from the South Oita area was lower than that in the Nagasaki Prefecture (8). Notably, the prevalence of *Anisakis* type I larvae in Sekisaba (6.8%) was even lower than that in the South Oita area (38%), which may be due to the different habitats of the two populations of *S. japonicus*. Indeed, the main feeding grounds of the chub mackerel of the Pacific stock are thought to be located in the waters off northeastern Japan, and their spawning grounds are in the coastal waters around the Izu Islands

and off southwestern Japan (13). However, Sekisaba is thought to use the waters around the Bungo Channel for both feeding and spawning (14). In the Bungo Channel, there are two depressions in the seafloor of the strait, and the tides induce a strong upwelling followed by the blooming of phytoplankton with a high concentration of nutrients (15). Thus, the geomorphological factors of the Bungo Channel provide a unique marine ecological environment that may in turn create a unique local chub mackerel population which may partially explain the low prevalence of *Anisakis* type I larvae in fish from that area.

The habitat of the intermediate hosts as well as the final hosts is a critical factor influencing the prevalence of *Anisakis* type I larvae in paratenic hosts. The most important first intermediate hosts in the life cycle of *Anisakis* type I larvae species are Euphausiids (krill) (16) that transmit the infective larvae along the food chain into paratenic hosts such as *S. japonicus*. The prevalence of infection in Euphausiids is generally low (17); however, it is uncertain whether the prevalence of *Anisakis* type I larvae in the first intermediate hosts is relatively low in the Bungo Channel. In contrast, the adult stages of *Anisakis* type I larvae reside in the stomach of marine mammals such as dolphins and whales (16). It is known that dolphins (*Delphinus capensis*, *Tursiops truncatus*), finless porpoises (*Neophocaena phocaenoides*), and whales (*Megaptera novaeangliae*) inhabit the Bungo Channel (18); however, the prevalence of *Anisakis* type I larvae in the final hosts in that area is still unknown. Thus, the distribution and abundance of the intermediate and final hosts infected with *Anisakis* type I larvae in the Bungo Channel should be investigated. To fully elucidate this complex relationship, it is necessary to integrate the knowledge of various fields, including parasitology, gastroenterology, ecology, oceanography, and fisheries science.

In conclusion, we revealed that the prevalence of *Anisakis* type I larvae in Sekisaba was significantly lower than that observed in *S. japonicus* from the other two areas. Our findings suggest that Sekisaba forms a unique stock that is distinguishable from the other stocks of *S. japonicus* in Japanese waters.

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Conflict of interest None to declare.

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