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Note

Survival of *Anisakis* species larvae of chub mackerel (*Scomber japonicus*) in different kinds of condiments

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Anisakiasis is a well-known zoonosis caused by ingestion of raw or thermally undercooked seafood product contaminated with live *Anisakis* nematode third stage larvae (L3). Several traditional processing techniques have been used to kill or remove the *Anisakis* larvae worldwide, but thermal processing or deep freezing are the most effective treatments to kill the *Anisakis* larvae. In this study, we investigated the survival of *Anisakis* larvae in several condiments (soy bean sauce, wasabi, vinegar, red pepper paste) commonly consumed when eating raw fish in Korea. We also examined several different media (NaCl solution, absolute alcohol, soju) to investigate their larvicidal effect. When directly exposed to various condiments, the most effective larvicidal effect was observed in the mixture of wasabi and soy bean sauce. When exposed to different NaCl solutions, the larvicidal ability became more effective as the concentration increased, but did not show 100% killing effect. In soju, the L3 were killed under less than 4 hr. We observed the larvicidal effects of several condiments in this study, but these results are thought to be carefully interpreted for actual use because all the condiments in this study showed the effect in hours and in general, the L3 are exposed to these condiments only for seconds before ingested in real situation.

Key words: *Anisakis* spp, Viability, *Scomber japonicus*, Wasabi, Soy bean sauce, Larvicidal Effect

Introduction

Anisakid nematodes are cosmopolitan parasites in marine ecosystems and cause one of the well-known food-borne zoonoses, anisakiasis. They use fish and squids as intermediate or paratenic hosts, and marine mammals and fish-eating birds as definitive hosts. Humans can be accidentally infected with the live third stage larvae (L3) by ingesting raw, undercooked or thermally unprocessed (e.g., smoked, salted, marinated) fish or cephalopods harboring them, and the clinical symptoms are mainly characterized by gastrointestinal symptoms or allergic reactions (Audicana and Kennedy, 2008). Since its first record in the Netherlands in 1960s, more than 20,000 cases have been reported worldwide and the number of reports has become increasing due to changes of dietary habits, globalization and improvement of diagnostic technology (van Thiel and van Houten, 1967; Audicana and Kennedy, 2008). Currently 9 species exist in the genus *Anisakis* and of them, 2 species (*Anisakis simplex* (sensu stricto) and *Anisakis pegreffii*) are known as the etiological agent of anisakiasis (Mattiucci et al., 2018).

From the food hygienic point of view, the anisakid larvae should be removed both for preventing human infection and maintaining marketability of fish. Deep freezing or adequate cooking are required to kill the larvae. Physical elimination of the larvae by visual

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examination is also common because deep freezing affects the texture of fish muscle, thus changes palatability of fish muscle when thawed (Podolska et al., 2019; Šimat and Trumbic, 2019).

Traditionally, many recipes with raw or undercooked fish exist worldwide and condiments such as lemon juice, vinegar, salt are added for marinating or brining them (Sánchez-Monsalvez et al., 2015; Šimat and Trumbic, 2019). In Korea, soy bean sauce, wasabi, red pepper paste are the common condiments consumed when eating raw fish. They have been customarily used for giving extra flavor and increasing storage property. In addition, customers vaguely expect germicidal effect of these condiments when eating raw or undercooked fish. But it is not clear if they have killing effect against anisakid nematodes. In this study, we selected several condiments and other media, and evaluated the *in vitro* effect on the survival of anisakid nematode L3 isolated from chub mackerel (*Scomber japonicus*).

Materials and Methods

Chub mackerel were bought from a local fisheries market located in Jumunjin, Gangwon, Korea. They were freshly caught and immediately transported to the laboratory. After measurement, the body cavity was opened and the larvae were collected with fine forceps. All the larvae were examined under the stereomicroscope, and only actively moving larvae without any physical damage were selected and washed with sterile sea water several times to remove any host tissue debris.

To evaluate the viability of the L3 in different media, the following solutions were prepared: sterilized sea water, absolute ethanol (99.9%), soju, soy bean sauce, wasabi, vinegar, red pepper paste and NaCl solution (5, 10, 20%). All the condiments and soju were commercially available products. 3 g of wasabi paste or red pepper paste was mixed with 30 ml of sterilized sea water for the experiment, and wasabi + soy bean sauce was prepared by mixing 3 g of wasabi paste with 30 ml of soy bean sauce. Soy bean sauce, vinegar, soju, absolute alcohol (99.9%, Sigma, USA) was directly used and sterilized sea water was prepared by autoclave. NaCl solution were prepared with distilled water (DW). 3 ml of each prepared solution were added into each well of 12 well plate (SPL Science, Korea), then 10 live L3 were added into each well and incubated at room temperature (20°C). The survival of the larvae was checked microscopically over incubation time. The larvae were considered alive if spontaneous or stimulated movements were observed, while those were considered dead if no movement were observed even when stimulated with a fine forceps. All the experiments were triplicated and the randomly selected larvae in each experimental group were identified by the PCR-RFLP targeting mitochondrial cox2 DNA sequences after the experiments, according to the method described elsewhere (Bak et al., 2014).

Results and Discussion

The viability of *Anisakis* species L3 in different condiments or media is represented (Fig. 1, 2). When exposed soy bean sauce, the L3 started to die after 1 hr incubation and all the L3 were died after 4 hr incubation. In case of wasabi, the L3 started to die after 1 hr incubation and all of them died after 4.5 hr incubation. When exposed to a mixture of soy bean sauce and wasabi, the L3 started to die at 30 min's incubation and all of them died after 3 hr incubation. Red pepper paste had no effect on the L3 until 6.5 hr incubation in this study and vinegar started to kill the L3 after 3.5 hr incubation (Fig. 1).

Sterilized sea water had no larvicidal effect, while absolute ethanol had immediate larvicidal effect when exposed; all the L3 were killed in seconds when exposed to absolute alcohol. Soju (ethanol concentration: 16.9%) started showing larvicidal effect after 1 hr incubation. Different concentration of NaCl sol-

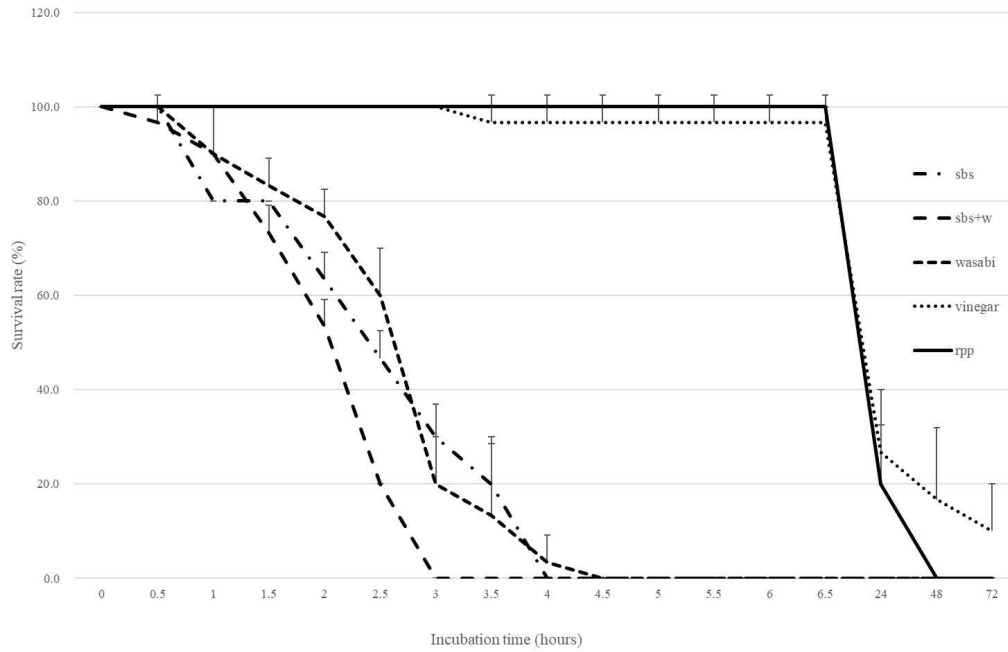

Fig. 1. Survival of *Anisakis* sp. L3 in various condiments. Abbreviations: sbs, soy bean sauce; rpp, red pepper paste; sbs+w, a mixture of soy bean sauce and wasabi. All the data were triplicated and expressed as means with standard deviation.

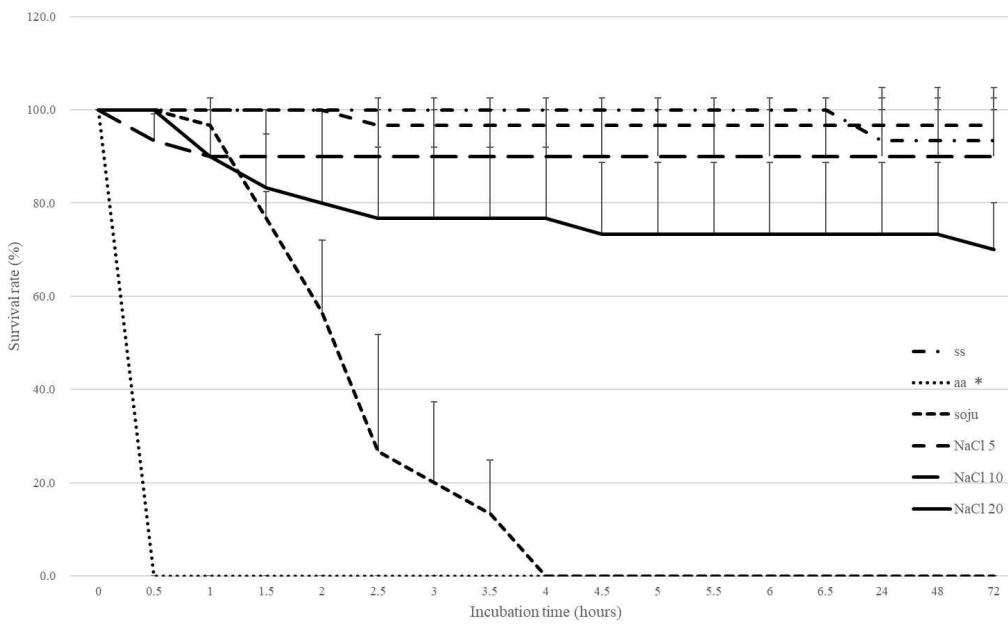

Fig. 2. Survival of *Anisakis* sp. L3 in various media. Abbreviations: ss, sterilized sea water; aa, absolute alcohol; NaCl 5, 5% NaCl solution; NaCl 10, 10% NaCl solution; NaCl 20, 20% NaCl solution. *All the L3 were immediately killed in seconds when exposed to absolute alcohol. All the data were triplicated and expressed as means with standard deviation.

ution (5, 10 and 20 %) showed different larvicidal effects and the highest larvicidal effects were observed in 20% concentration in this study (Fig. 2).

Randomly selected L3 after the viability test were transferred to identification by PCR-RFLP and the result is shown in Fig. 3. All the L3 examined (n=30) showed the enzyme cutting patterns of *A. pegreffii* (Fig. 3). We previously investigated the anisakid nematodes fauna of chub mackerel and found that most of the nematodes examined were identified as *Anisakis pegreffii* (Bak et al., 2014). Therefore, we speculated the anisakid nematode L3 population used in this experiment were *A. pegreffii*, although we did not identify all of the anisakid L3 used in this study. But, we cannot rule out the possibility of existence of hybrid genotype in our specimen, which cannot be identified only with mitochondrial DNA sequences.

We observed that the survival rate of anisakid larvae were decreased in soy bean sauce, wasabi and the mixture of them. Wasabi (*Wasabia japonica*, Japanese horseradish) is an edible plant traditionally consumed as a spice or a condiment with sushi and other foods for centuries in Japan. Several studies reported that wasabi includes different kinds of phytochemicals having various powerful herbal medicinal action, such as antibacterial property against foodborne pathogens (Lu et al., 2016; Shin et al., 2004). There is only one report on anti-parasitic activity of wasabi (Ohishi, 1974), and we also observed that the survival of L3 was affected by incubation with wasabi in this study. More studies will be necessary to clarify the effective ingredient of anti-parasitic action, because the wasabi used in this study was commercially available product made with several ingredients.

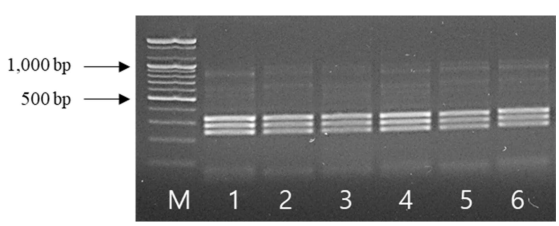

Fig. 3. Representative PCR-RFLP patterns of *Anisakis* sp. L3 in this study. PCR products were digested treated with *Hinf* I. (L: ladder, 1-6: *A. pegreffii*).

In case of soy bean sauce, the high osmotic pressure in soy bean sauce may have been stressful and consequently affected the survival of L3 in our study. Therefore, the mixture of soy bean sauce and wasabi might have shown synergic effects for killing the larvae in this study. Similarly, the red pepper paste was the commercially available product, a mixture of several condiments (e.g., red pepper powder, vinegar, sugar, garlic powder, plum extract) and it is unclear if which ingredient affected the survival of L3 in this study.

In general, *Anisakis* species L3 are known to be vulnerable to acidic condition such as the human stomach. The average survival time of *A. pegreffii* in artificial gastric juice (pH 1.8) was 2.3 days (Arizono et al., 2014). The pH of vinegar used in this study was 2.4, which is thought to be also a harsh environment and affected the survival of *Anisakis* sp. L3 in this experiment.

We reflected the actual situation of eating raw fish with the condiments for designing the incubation period in this study; we commonly put condiments to raw fish, then eat them in seconds. Therefore, the larvicidal effect of the soy bean sauce and wasabi observed in this study should be carefully interpreted. Ohishi (1974) described that soy bean sauce, vinegar, wasabi had the larvicidal effect against *Anisakis* sp. L3 in hours. Therefore, the incubation time and concentration of the condiments are thought to be the limiting factors for actual use.

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