An unidentified foodborne disease associated with the consumption of raw fresh fish was noticed from 1999 in the West of Japan. In 2010, a novel multivalvulid parasite, *Kudoa septempunctata* (Myxozoa: Myxosporea) was discovered as being the causative agent of this disease and the Ministry of Health, Labour and Welfare of Japan (MHLW) named this disease “Kudoa food poisoning”. *Kudoa septempunctata* is a myxosporean with 6–7 polar capsules and shell valves in a spore. The life-cycle of *K. septempunctata* has not been elucidated yet. However, it probably involves an alternative invertebrate host such as polychaetes without direct transmission between fish. An epidemiological study elucidated that the main symptoms of “Kudoa food poisoning” are transient vomiting, diarrhea, abdominal pain and vomiting due to gastrointestinal mucosal disruption, most of which could be recovered within 24 h. The threshold for the onset of symptoms is estimated at about $7.2 \times 10^7$ *K. septempunctata* spores per person based on an epidemiological calculation of a large-scale outbreak that occurred at Ehime prefecture. In a toxicological study, oral administration of $1 \times 10^7$ spore/g live *K. septempunctata* induced the acute accumulation of fluid in the gut of suckling mice and vomiting in house musk shrews (*Suncus murinus*). *K. septempunctata* decreased transepithelial resistance in a cultured human intestinal cell monolayer, resulting in a rapid increase of permeability. These pathogenic actions of viable *Kudoa* spores elicit symptoms in human patients. Regarding analytical methods, PCR amplification of species-specific genes and microscopic observation of characteristic spores are the best methods of choice for characterizing this parasite. To prevent the disease, heating at 95 °C for 10 min or freezing at −80 °C overnight is effective while a recent study demonstrated that liquid freezing is a more practical method. Fundamentally, the biological study of *K. septempunctata* including its life-cycle, alternative and invertebrate hosts is necessary for eradicating them. Realistically, monitoring of domestic flounder in farm and imported one in the quarantine should be useful.

From the available literature, *K. septempunctata* appears to be a unique parasitic agent that induces foodborne diseases by invading the human intestinal mucosa but does not persist long enough in the tissue for further growth, eliciting a temporal increase in mucosal permeability. Further investigations are needed to elucidate the interactions between this myxosporean parasite and human tissue.

**Key words:** *Kudoa septempunctata*, food poisoning, olive flounder, human intestine, novel disease
1. Introduction

Starting 15 years ago, a foodborne disease associated with the consumption of fresh raw fish such as *sashimi*, which is a traditional Japanese meal, became a topic of concern. The disease manifested itself as diarrhea and vomiting within 12 h after eating but symptoms would disappear within 24 h. The disease has a good prognosis without any aftereffects. Specimens from patients were examined to detect known causative agents such as bacteria, bacterial toxins and viruses. Since no causative agent was identified in these endeavors, the disease was classified as an “unidentified foodborne disease”.

Yoshioka et al.\(^1\) studied the occurrence of this foodborne disease of unknown origin in 2009 by sending a questionnaire to 136 local governments and health-care centers throughout Japan. They received responses from 99 organizations (72.8% response rate) and found that 77.7% of respondents referred to complaints about this disease, suggesting that the disease occurred widely over Japan and had been increasing every year (Figs. 1 and 2). These results encouraged the Ministry of Health, Labour and Welfare of Japan (MHLW) to begin an investigation on the causative agent of this unknown and as-yet uncharacterized disease. MHLW launched a research project on this subject in 2010 to investigate the causative agent from multifaceted possibilities, including bacteria, bacterial toxins, chemical compounds and parasites. In June 2011, MHLW released a notice that a new parasite, *Kudoa septempunctata* (Myxozoa: Myxosporea: Multivalvulidae) was the apparent causative agent of this unidentified foodborne disease, naming the disease “Kudoa food poisoning” in December 2012.

![Fig. 1. Occurrence of “the unidentified food borne disease” in 2009 in Japan (Cited and modified from Yoshioka and Sone, 2012 with permission).](image1)

![Fig. 2. Annual changes in the occurrence of “the unidentified food borne disease” until 2009.](image2)
2. Discovery of Kudoa septempunctata

As the causative food, the MHLW project focused on olive flounder (Paralichthys olivaceus) based on the report of Yoshioka et al. In a survey of 30 domestic and 30 imported flounders sold in fish markets (Tsukiji in Tokyo), this novel myxosporean parasite was discovered in one imported flounder from Korea in 2010, and was named “Kudoa septempunctata” by Matsukane et al. After this discovery, metagenomic DNA sequencings by Illumina were examined by using the same Korean olive flounder specimen. Kawai et al. found possible eukaryotic sequences classified as the gene of a myxosporean of the family Kodoidae, but no potential enterovirus or bacterial DNA sequences were detected in the same specimen. They established a PCR detection method specific to members of the genus Kudoa, including K. septempunctata, based on the 18S ribosomal RNA gene (rDNA). Subsequently, 35 specimens of olive flounder supplied from the source of the unidentified foodborne disease and 16 reference samples purchased at seafood retailers in Tokyo were analyzed by this PCR method. The PCR data and microscopic observations revealed that K. septempunctata existed in 26 specimens but not in all reference samples, suggesting that this new myxosporean species was a veritable candidate causing the unidentified foodborne disease. Simultaneously, data of toxicological experiments using rodent models supported the pathogenic potential of K. septempunctata, causing diarrhea and vomiting, as described next in detail.

3. Epidemiology

When outbreaks of the unidentified foodborne disease occurred, patients were asked about foods that they had consumed before illness to identify the causative agent. Intensive epidemiological analyses were performed on patients to whom olive flounder had been provided and in which K. septempunctata was detected. Those analyses revealed that the mean incubation time ranged from 3.4 to 16.3 h and that the main symptoms were diarrhea and vomiting. The appearance of symptoms ranged from 11.9% to 100% in individuals that had eaten the parasitized flounder. Regarding four outbreaks in Kanazawa, Akita, Osazaki and Osaka prefectures, which were evidently associated with olive flounder consumption, the odds ratio ranged from 17.11 to 43.00.

In October 2010, a large-scale outbreak occurred, involving more than 100 symptomatic people among more than 400 people who had eaten the parasitized flounder. The olive flounder residue from meals that the patients had consumed was examined and K. septempunctata was detected more than once per person, which is the ingestion threshold for the development of gastrointestinal symptoms. In this epidemic, the odds ratio was 9.50 (95% confidence interval: 1.59–∞), suggesting that olive flounder was the causative food of the unidentified foodborne disease. These authors also estimated the number of K. septempunctata spores required for the illness to develop. A Monte Carlo stimulation indicated a value of $7.2 \times 10^7$ K. septempunctata spores per person, which is the ingestion threshold for the development of gastrointestinal symptoms.

According to a MHLW report on food poisoning incidents, the number of “Kudoa food poisoning” was 34, 41 and 20 in 2011 (from June to December), 2012 and 2013, respectively. Similarly, 473, 418 and 288 patients were classified with this food poisoning for these three years. The food poisoning caused by K. septempunctata in fish shows clear seasonality (Fig. 3). Approximately 75% of incidents occurred in late summer between August and November, although the frequency of olive flounder with K. septempunctata and the number of Kudoa spores in the muscle showed no seasonal change from January to August. Considering that the temperature of seawater in summer exceeds 20 °C, Ohnishi et al., tried to raise olive flounder in culture at a higher temperature (24 °C). However, the higher temperature did not affect the frequency of olive flounder with K. septempunctata, the number of spores in individual fish or its toxicity. Therefore, the seasonality of disease occurrence remains to be elucidated. Identification of the cause of seasonality could contribute to the prevention of “Kudoa food poisoning”.

4. Characterization of K. septempunctata

As described above, K. septempunctata was newly found in 2010 as a myxosporean having 6–7 polar capsules (PCs) and shell valves (SVs) from an aquacultured olive flounder, which was one of 30 examined fishes imported from Korea and sold at Tsukiji Fish Market, Tokyo. The life-cycle of K. septempunctata must involve alternative invertebrate hosts, presumably polychaetes, because no myxosporean infection of naïve fish with the kudoid occurs when infected and naïve olive flounders are kept together (unpublished observation). Reservoir hosts for K. septempunctata are presumably polychaetes.
Sugita-Konishi Y, et al: Running title: Food poisoning by Kudoa septempunctata have not been recorded yet. It is thus necessary to survey more extensively natural fish including not only olive flounder but also other fish species, around Japan and Korea to ascertain the origin and reason why aquacultured flounders have been intensively infected with this species over the last decade. As Burger and Adlard reported, recent research using molecular genetic data such as rDNA sequences have confirmed that many kudoid species have multiple hosts, including 38 host fishes for K. thyrsites, 20 host fishes for K. nova, and 18 host fishes for K. thalassoni, while 57 of 84 kudoid species still have a single recorded host. To identify reservoir fish hosts and possible alternative invertebrate host(s) of K. septempunctata, a large-scale survey needs to be conducted. Data from such a survey would allow for the development of effective prevention measures against the human disease by cultured olive flounder.

*Kudoa septempunctata* forms a pseudocyst in the myofiber of trunk muscles of the fish. However, it is impossible to know whether the apparent infection can be detected by the naked eye, or whether consumers of raw fish are able to notice muscle slices with a heavy load of myxosporean plasmodia before it is ingested. Although several *Kudoa* spp., including K. thyrsites, K. paniformis and K. neothunni, which form pseudocysts in the myofiber, are known to cause post-harvest myoliquefaction (often termed ‘jellied meat’ or ‘soft flesh’), there are no records of this phenomenon caused by *K. septempunctata*. In addition to *K. septempunctata*, *K. thyrsites* and *K. lateolabracis*, both causing post-harvest myoliquefaction, have been recorded from the musculature of olive flounders, while *K. yasunagai* has been detected in the brain.

Currently the genus *Kudoa* Meglitsch, 1947, having four or more PCs/SVs (usually *Kudoa* spores have equal numbers of PCs and SVs), contains approximately 90 nominal species, most of which have four PCs/SVs. Phylogenetic
trees based on either 18S or 28S rDNA nucleotide sequence indicate that kudoid species with more than four PCs/SVs form two closely-related but distinct clades; one includes *K. permulticapsula* with 13 PCs/SVs, *K. scomberomori* with 6 PCs/SVs, *K. neothunni* with 6 PCs/SVs, *K. hexapunctata* with 6 PCs/SVs, *K. grammatorcyni* with 6 PCs/SVs, and *K. monodactyli* with 5 PCs/SVs; the other clade includes *K. septempunctata* with 6–7 PCs/SVs, *K. thalassomi* with 6–7 PCs/SVs, *K. igami* with 6 PCs/SVs, *K. chaetodoni* with 8–9 PCs/SVs, *K. lemniscati* with 7–8 PCs/SVs, *K. yasunagai* with 6–8 PCs/SVs, *K. lethrimi* with 7 SVs/PCs, *K. neurophila* with 5 PCs/SVs, and *K. prunusi* with 5–6 PCs/SVs. The latter clade consists mainly of kudoids with brain tropism except for three *Kudoa* spp. with muscle tropism, i.e. *K. septempunctata*, *K. thalassomi* and *K. igami*, whereas the former clade contains kudoid species with muscle tropism only. Spores of *K. septempunctata* (width 11.1–13.1 µm; thickness 8.9–10.0 µm; length 7.9–8.9 µm; PC length 3.7–5.3 µm; PC width 2.2–2.8 µm) are morphologically characterized by remarkable variation in the dimensions of SVs and PCs in the same spore. Only *K. lethrimi* from the brain of *Gymnocranius andleyi* has similarly evident variation in PC size among kudoid species with more than four PCs/SVs. Furthermore, two kudoid species, *K. thalassomi* and *K. lethrimi*, have ornamentations on SVs, namely lateral projections and wings, in contrast to the spores of *K. septempunctata* and other aforementioned congeners that lack such appendages.

Intra-species variation of rDNA nucleotide sequences have been recorded for multiple species, particularly when the focus is on 28S rDNA. In the case of *K. septempunctata*, there are no or few nucleotide substitutions in either 18S or 28S rDNA sequences deposited in the DDBJ/EMBL/GenBank databases. Kuroda et al. (unpublished) found two mitochondrial DNA haplotypes of *K. septempunctata* when they examined 115 isolates from Japan and Korea, but the epidemiological significance of this finding awaits future analysis.

5. Toxicology

The toxicity of *K. septempunctata* has been examined using rodent models and a cell culture model. Oral administration of *K. septempunctata* spores elicited remarkable accumulation of fluid in the intestine of suckling mice. Four hours post-inoculation, suckling mice produced watery diarrhea and then recovered within 24 hrs. Direct feeding of live flounder meat containing *K. septempunctata* spores (3.2–5.2 × 10⁵) as well as purified spores to house musk shrews caused them to vomit after 20–30 min post-feeding. These house musk shrews recovered within 2 h after inoculation. The application of *K. septempunctata* spores to a cultured human intestinal cell (Caco-2) monolayer rapidly decreased *trans*-epithelial resistance by 80% within 1 h, indicating an increase in permeability of the intestinal cell monolayer. The decrease in *trans*-epithelial resistance recovered fully within 18 h. These findings are consistent with the symptoms displayed by human patients, demonstrating that *K. septempunctata* spores are the causative agent of “Kudoa food poisoning”.

After ingesting raw slices of olive flounder containing *K. septempunctata* spores, sporoplasms are released from the spore (Fig. 5). It is suggested that a protease such as trypsin secreted on the intestinal mucosa induces the release of sporoplasms from the spores. After their release from spores, the sporoplasms swell and exhibit an amoeba-like shape developing pseudopodia with high motility (Fig. 6). The sporoplasms intrude into the intestinal cell monolayer and reach the basolateral side within 1 h after being released from the spores. Such an intrusion results in the formation of large holes in the intestinal cell monolayer. The prevention of sporoplasm intrusion into the intestinal cell monolayer inhibited the increase in permeability of the monolayer, indicating that the damage to the intestinal cell monolayer caused by sporoplasms is one mechanism of diarrhea found in “Kudoa food poisoning”. As demonstrated in some myxosporean species, the parasite spores release sporoplasms on the intestine lining of annelids, which are alternative invertebrate hosts, and the sporoplasms invade the intestinal cells of annelid hosts and reproduce there. Similar to this process, *K. septempunctata* might invade the human intestine mucosal lining in a normal life-cycle, but might not be able to survive there, resulting in abrupt recovery of mucosal damage and a good prognosis characteristic of “Kudoa food poisoning”.

At present, the mode of action of *K. septempunctata* is speculated as shown in Fig. 7. Viable *K. septempunctata* ingested orally can survive in the stomach, because *Kudoa* spore is resistant against pepsin. *Kudoa* sporoplasms are released from spore by trypsin treatment in the intestines. The sporoplasms adhere to the villi of intestinal cells, invade into epithelial cells, and then disturb the cell permeability. Within short time, the parasite may be self-digested. This process could be one of the mechanisms of diarrhea. Information of the mode of action on vomiting is limited and thus should be studied further.

Furthermore, oral administration of *Kudoa* spores to house musk shrews induced the production of serotonin in the intestine (Ohnishi et al., unpublished data). Since serotonin is a key stimulant of the vomiting center in the brain, it is
suggested that the production of serotonin in the intestine may act as one trigger for vomiting found associated with the disease. Whether the same mechanism occurs in the human intestine needs to be confirmed.

6. Analytical Methods of Infection

There are two notifications regarding recommended analytical methods to detect Kudoa infection in Japan. One is adopted by MHLW while the other is recommended by the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF). The method adopted by MHLW is a combination of quantitative real-time PCR (qRT-PCR) and morphological observation by microscopy. For morphological observation, Kudoa spores are purified from 1 g of olive flounder muscles and their number is counted. K. septempunctata spore have six or seven PCs/SVs, exhibiting a flower petal-like morphology\(^2\). The width of spores is approximately 10 µm, which is much larger than that of bacteria. Therefore, the
differentiation of *K. septempunctata* from other microbes in olive flounder is not difficult. When the number of spores exceeds $1.0 \times 10^5$ g muscle, the fish is incriminated as being the cause of “Kudoa food poisoning.”

In addition to *K. septempunctata*, there are two *Kudoa* spp. that form pseudocysts in the myofibers of olive flounder: *K. thrysites* and *K. lateolabracis*. Since these two *Kudoa* spp. have four PCs/SVs in quadrate spores, morphological identification of *K. septempunctata* with 6–7 PCs/SVs among myxosporean species found in olive flounder is highly reliable. In the qRT-PCR method adopted by MHLW, a standard curve can be prepared using the control plasmid containing the 18S rDNA of *K. septempunctata*, constructed by the National Institute of Health Sciences, Japan. When the copy number, which is calculated using the standard curve, exceeds $1.0 \times 10^7$ copies/g sample, the fish is *Kudoa*-positive. Since the PCR primers adopted by MHLW detect a broad range of *Kudoa* spp., and are not specific to *K. septempunctata*, morphological observations must accompany qRT-PCR. Sometimes the copy number exceeds the qRT-PCR threshold value but microscopic examination of the same muscle sample detects few or no *Kudoa* spores. This might be explained by the detection of plasmodia containing a large amount of sporoblasts and few developed spores in the muscle of olive flounder (Fig. 8). Often the ratio between developed spores and sporoblasts in the plasmodia vary in different parts of trunk muscles. The use of morphological observation can only count developed spores, whereas the qRT-PCR method can quantify all cells containing nonpathogenic sporoblasts, leading to a low correlation between the results based on microscopy and those based on qRT-PCR. Since the toxicity of *K. septempunctata* is associated with the number of viable spores, morphological observation is better than the qRT-PCR-based method for assessing the cause of this food-borne disease. In addition to these notification methods, a method to detect *K. septempunctata* from fecal samples was developed. In this method, *K. septempunctata* DNA is extracted from fecal samples of suspected patients and *Kudoa* 18S rDNA is quantified using qRT-PCR. This method is convenient for incidents in which no olive flounder remnants are available for pathogen incrimination.
The method adopted by MAFF is a combination of qualitative PCR and morphological observation by microscopy. In the morphological observation, a small cut on the skin of olive flounder is made by a scalpel and a swab is inserted into the cut. A slide glass is smeared with the swab, and observed under a microscope. Regarding the quantitation of spores, this method is inferior to that adopted by MHLW, but results in minimum damage to fish and is thus applicable to fish before shipment to the market. The PCR method adopted by MAFF is qualitative PCR using K. septempunctata-specific primers that can be applied to screening both adult flounder and a lot check of flounder fry. In contrast to the MHLW methods, which emphasize the quantitative aspect, the MAFF methods stress qualitative aspects. The difference is ascribed to different viewpoints between the two ministries; MHLW methods focus on the incrimination of the cause of foodborne diseases while MAFF methods focus on preventing Kudoa contamination in fish farms.

Both qRT-PCR and conventional PCR methods need expensive equipment, and take 2–3 h to obtain results. Thus, loop-mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA)-nucleic acid chromatography are now being established as rapid screening methods for the detection of K. septempunctata. Since these methods are simple to use and can be performed without special knowledge and expensive equipment, they are expected to be convenient test tools in fish farms, public health centers and quarantine stations.

7. Prevention

Kudoa septempunctata is easily inactivated by heating at 95 °C for 10 min or freezing at −80 °C overnight. Although heating and freezing make the olive flounder safe, the quality of its texture is considerably reduced. The heat exchange of a conventional air blast freezing method, which freezes food in cold air, causes ice crystal formation in foods, leading to damage of food cells and drip exudation after thawing. To maintain the commercial value of olive flounder for consumption as “sashimi”, heating and freezing treatments on fish cannot be used to inactivate K. septempunctata spores. Under such circumstances, the preventive measures of K. septempunctata contamination in fish farms are important. Considering that no flounder farms in Japan maintain the life-cycle of the kudoid species, uninfected flounder fry should be introduced to a farm with sterilized sea water used for culture. A final check of marketable products is also effective. These preventive measures have been conducted in some production centers of olive flounder in Japan. At present, a large amount of olive flounder is imported to Japan, and the imported fish are screened for K. septempunctata from Korea at the quarantine service in Japan for preclusion of infected flounders from the market.

When considering that preventive measures might not perfectly exclude infected fish from the market, the development of a freezing method that inactivates Kudoa spores but maintains texture quality is important. Recently, freezing methods have improved dramatically, one of them being liquid freezing. Liquid freezing uses an alcoholic liquid refrig-
erant that serves as an effective heat exchanger and enables rapid cooling without developing ice crystals. When the liquid freezing method was applied to olive flounder, fish texture was well preserved comparable to the quality of unfrozen fish, and *K. septempunctata* spores in olive flounder muscles were inactivated by only 5 min of liquid freezing. Thus, the liquid freezing method could be used to prevent “Kudoa food poisoning.”

8. Conclusion

The causative agent of a novel food poisoning associated with the consumption of raw fish is a parasite of olive flounder, *Kudoa septempunctata*, which is a new kudoid species that was discovered in investigations of a project supported by MHLW. Since kudoids are generally accepted to be harmless to humans and has been ignored for long time in the field of public health, including that of foodborne diseases, finding *K. septempunctata* is the first line of evidence demonstrating that a kudoid can cause adverse health effects.

*K. septempunctata* is a unique parasitic agent among parasites involved in foodborne diseases. Despite its inability to grow in the human body, it can invade the human intestine and finally disappear. We believe that other unknown substances may be involved in diarrhea and vomiting. The symptoms of the disease might arise from not only viable-parasite behavior but also in response to toxic compounds.

We re-emphasize that it is necessary to understand the fundamental biology of *K. septempunctata* including its life-cycle, alternative invertebrate hosts, reservoir hosts, seasonality, geographical distribution, etc., none of which is known, to establish effective measures for preventing “Kudoa food poisoning.” Recently other kudoids were discovered from a fish specimen suspected of “Kudoa food poisoning.” There is urgency in shedding light on the toxicity of other kudoids.

References