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Original Article

Molecular detection and genotype analysis of *Kudoa septempunctata* from food poisoning outbreaks in Korea

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Abstract

Concerns about foodborne illnesses caused by *Kudoa septempunctata* are steadily growing, but reports of *K. septempunctata* in clinical and food specimens related to food poisoning in Korea are limited. This study aimed to genetically identify *K. septempunctata* in patients with acute diarrhea and in clinical and food samples related to food poisoning caused by sashimi consumption. Both real-time and nested polymerase chain reaction assays were performed to detect *K. septempunctata* 18S and 28S rDNA genes in the stools of 348 patients with acute diarrhea, 11 samples (6 stool and 5 rectal swab samples) from patients with food poisoning, and 2 raw *Paralichthys olivaceus* samples collected from a restaurant where a food poisoning incident occurred. *K. septempunctata* was identified in 5 clinical specimens (4 stools and 1 rectal swab) and 1 *P. olivaceus* sashimi sample. All detected *K. septempunctata* were of genotype ST3. This is the first study to identify *K. septempunctata* in both patients and food samples with epidemiological relevance in Korea, providing evidence that it is a pathogen that causes food poisoning. Also, this is the first study to confirm the presence of *K. septempunctata* genes in rectal swabs. Despite continuing suspected occurrences of *Kudoa* foodborne outbreaks, the rate of identification of *K. septempunctata* is very low. One reason for this is the limitation in obtaining stool and vomit samples for the diagnosis of *Kudoa* infection. We strongly suggest the inclusion of rectal swabs among the diagnostic specimens for *Kudoa* food poisoning.

Keywords: *Kudoa septempunctata, Paralichthys olivaceus*, food poisoning, olive flounder, ST3

Introduction

The genus *Kudoa* is a myxosporean parasite that infects the somatic muscles, brain, pericardium, and digestive tract of various marine fishes [1,2]. Certain species of *Kudoa* cause postmortem liquefaction known as " jelly meat", which reduces the commercial value of fish [3,4]. A study on *Kudoa septempunctata* as a causative organism of food poisoning was performed in Japan [5]. In that study, 130 of the 158 cases of unknown foodborne illness reported in 2010 were associated with the consumption of raw *Paralichthys olivaceus* (olive flounder), and *K. septempunctata* genes and spores were identified in the *P. olivaceus* remnants of patient meals. The Ministry of Health, Labor and Welfare of Japan announced *K. septempunctata* as a potential cause of food poisoning, and tests on domestic and imported *P. olivaceus* are further being strengthened [6]. Similarly, after the reports of the presence of *K. septempunctata* in cultured *P. olivaceus* in Jeju, Korea [4,7,8], the Ministry of Food and

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Conflict of interest
The authors declare no potential conflicts of interest.

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Drug Safety of Korea proposed and circulated a method for detecting *K. septempunctata* in *P. olivaceus* in 2013. Marine aquaculture has rapidly become an essential component of the global marine food supply, accounting for 44% of the global fish production in 2014. That year, Korea ranked 7th among the world's top 25 aquaculture producers [9]. In Korea, *P. olivaceus* accounted for 46.2% (37,240 metric tons) of the total fish farming yield (80,530 metric tons) in 2018 [10]. Foodborne outbreaks associated with *P. olivaceus* infected with *K. septempunctata* are crucial because they can lead to economic losses. *K. septempunctata* has 3 different genotypes: ST1, ST2, and ST3. The genotypes are determined by a combination of 2 mitochondrial genes, namely, cytochrome *c* oxidase subunit 1 (*cox1*) and large subunit rRNA (*rnl*). There are 3 alleles (*cox1*-1, *cox1*-2, and *cox1*-3) for *cox1*, which differ at 7 single nucleotide polymorphisms (SNPs). Whereas there are 2 alleles (*rnl*-1 and *rnl*-2) for *rnl*, which differ at 2 SNPs [11]. Studies have shown that *K. septempunctata* found in Japan mainly has the ST1 or ST2 genotype, whereas ST3 genotype is found in Korea [11-14].

Korean and Japanese food cultures involve frequent consumption of raw fish in the forms of sashimi and sushi. Since these cuisines are spreading around the world, identification of food poisoning caused by *K. septempunctata* is important from the public health point of view. In 2015, the Korea Disease Control and Prevention Agency (KDCA) announced foodborne illness outbreaks caused by *K. septempunctata* [15] but classified them as "other infections" that cause food poisoning [16]. This is due to the continuing controversy about whether *K. septempunctata* causes food poisoning, resulting from conflicting results on its pathogenicity in experiments using mice [5,12,13] and the possibility of confusion with toxin-type food poisoning (*Staphylococcus aureus* or *Bacillus cereus*) [17]. It is therefore important to detect *K. septempunctata* in feces or vomit samples obtained from patients with food poisoning. However, to date, only 2 studies have been performed in Korea [18,19]. In addition, although the KDCA has been monitoring 20 food poisoning pathogens for patients with acute diarrhea at cooperative hospitals nationwide through the Enteric Pathogens Active Surveillance Network (Enter-Net) every year, *K. septempunctata* has not been included in this survey. Thus, information available on the status of *K. septempunctata* infection in Korea is limited.

The present study investigated the infection status of *K. septempunctata* by detecting specific genes (18S and 28S rDNA) in stool samples from patients admitted with acute diarrhea to hospitals participating in the Enter-Net system in Busan. Patients in whom food poisoning was caused due to the consumption of seafood were included. Furthermore, raw *P. olivaceus* samples were collected from restaurants where food poisoning incidents had occurred. These samples were also used for the genetic identification and genotyping of *K. septempunctata* to investigate its potential as a food poisoning pathogen and emphasize the importance of managing and preventing such outbreaks.

Materials and Methods

Collection of samples

From November 2019 to October 2020, 348 stool samples of patients with acute diarrhea were collected from 5 hospitals in Busan. In addition, 11 samples (6 stool and 5 rectal swab samples) were collected from 6 food poisoning outbreaks that had occurred due to the

consumption of seafood. Two samples of *P. olivaceus* sashimi were collected from a restaurant where a food poisoning incident had occurred. Samples were collected at public health centers and the Environmental Sanitation Department of district offices.

Sample preprocessing and DNA extraction

The collected stool, rectal swabs (Transystem 132C, Copan, Brescia, Italy), and *P. olivaceus* sashimi samples were stored in a cool box maintained at 4-8°C and transported to our laboratory within 4 h of collection. The samples were stored at 4°C, and nucleic acid amplification assays were performed as early as possible within 48 h of the arrival of the samples.

For DNA extraction from the rectal swabs, the sample was cut with sterile scissors, placed in a 15 ml conical tube, and vortexed with sterile glass beads and 500 µl of 0.1 M phosphate buffered saline (PBS, pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) for 10 min. *P. olivaceus* sashimi sample extraction was performed as per the guidelines mentioned in the Manual for Detection of Foodborne Pathogens at Outbreaks [20]. In brief, (A) the surface of the sashimi was scratched in at least 5 places to obtain 1 g sample; (B) the collected sample was placed on a mesh of approximately 200 µm, to which 3 ml of PBS was added, (C) light mashing was performed; (D) the resultant solution collected through the mesh was centrifuged at 1,500 g for 15 min at 4°C, and (E) the solute was discarded and 1 ml of PBS was added to the precipitate.

DNA was extracted from 250 µl of the prepared homogenized solution and 0.25 g of stool. DNA extraction was performed using the QIAcube and QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Genetic identification and genotype analysis

For screening of *K. septempunctata* infection, all DNA extracts were subjected to real-time polymerase chain reaction (PCR) assay on the ABI 7500 Fast System (Applied Biosystems, Foster City, CA, USA) using the PowerChek *Kudoa* Real-time PCR kit (KogeneBiotech, Seoul, Korea). After obtaining specific fluorescence amplification curves, nested PCR was

Table 1. Primers used for PCR amplification and sequencing

Gene		Primer sequence (5′-3′)	Size (bp)	Reference
18S rDNA	1st-F	GGTGGGAGCATTTATTAGACT	333	[26]
	1st-R	AATCGAGACCACTGTCAAC		
	2nd-F	AGAAATACCGGAGTGGACCGTAAAATG		
	2nd-R	GTT CCA TGC TAT AAC ATT CAA GCG TTCG		
28S rDNA	1st-F	TGC GAG TGA AGC GGG AAA A	356	
	1st-R	GTG TTT CAA GAC GGG TCG G		
	2nd-F	GTG TGT GAT CAG ACT TGA TAT G		
	2nd-R	AAG CCA AAA CTG CTG GCC ATT T		
cox1	1st-F	TTTGTTCATCGGCACAATTC-	751	[9]
	1st-R	ATAGCCTGGAACAAGGAATC		
	2nd-F	TATGGCAAAGAAGGTCTGAT	500	
	2nd-R	TCTAGGGATTCCACAAAGAC		
rnl	1st-F	TGCCGTCAATTCTGTTGTATT	817	
	1st-R	AATACCCATGCTGTGTTCAT		
	2nd-F	GTTCCAACAAGTCCATGAA	500	
	2nd-R	GACTTTATGGACAACTCAGC		

Table 2. *Kudoa septempunctata* genotypes

Genes (Accession no.)	Genotypes		
	ST1	ST2	ST3
cox1 1 (AB915831)	+	-	-
cox1 2 (AB915830)	-	+	-
cox1 3 (LC014799)	-	-	+
rnl 1 (AB915833)	+	-	-
rnl 2 (AB915832)	-	+	+

performed to detect the 18S and 28S ribosomal DNAs. Primers of 2 *K. septempunctata* mitochondrial genes (*cox1* and *rnl*) were used for genotype analysis. The primer sets and references are presented in Table 1. PCR was performed using the ProFlex PCR System (Applied Biosystems) with AccuPower PCR Premix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions.

The amplified products were visualized on the QIAxcel Advanced System (Qiagen) and sequenced bidirectionally by Bionics Co. (Bionics Co., Seoul, Korea). The sequences were aligned by applying the BioEdit sequence alignment program (https://bioedit.software.informer.com/7.2) and analyzed using NCBI-BLAST (https://www.ncbi.nlm.nih.gov/Blast). For genotype analysis, sequences were compared with the standard strain using Clustal W in BioEdit (Table 2).

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 (IBM, Armonk, NY, USA). Mean and standard deviation were calculated for the quantitative variables, and percentages were calculated for the qualitative variables. Independent sample *t*-test and chi-square test were applied to determine the correlation between the *K. septempunctata* 18S and 28S rDNA genes and each variable. Statistical significance was set at $P < 0.05$.

Results

Prevalence and genetic identification of *K. septempunctata*

Real-time PCR for the screening of *K. septempunctata* was performed on the stool samples of 348 patients with acute diarrhea, 11 samples (6 stool and 5 rectal swab samples) from patients with food poisoning, and 2 raw *P. olivaceus* samples collected from a restaurant where a food poisoning incident had occurred. Real-time PCR amplification curves were observed in 4 patients with acute diarrhea, 5 patients with food poisoning, and 2 raw *P. olivaceus* samples, for which 18S and 28S rDNA nested PCR were performed. PCR products were sequenced and compared with the *K. septempunctata* gene for 18S rDNA (accession no. AB731754.1) and 28S rDNA (accession no. AB731755.1). Finally, 5 patients with food poisoning and 1 raw *P. olivaceus* sample were determined to be positive for *K. septempunctata*.

Epidemiological characteristics of 11 patients in whom food poisoning occurred due to the consumption of seafood in Busan during the same period are summarized in Table 3. Of the 11 patients examined, the average incubation period per patient was 4.7 h, with

Table 3. Summary of epidemiological characteristics for 11 food poisoning patients related to seafood intake in Busan

Patient No.		Out-breaks	Date of occurrence	Time interval (h)[a]	Incubation time (h)[b]	Diarrhea	Vomiting	Abdominal pain	Intake of raw *P. O.*	Sample type	PCR result of *Kudoa*[c]
1	BS20-0504-1	A	05/04/20	38	4	+	+	+	Yes	Stool	D
2	BS20-0504-2	A	05/04/20	38	5	+	+	+	Yes	Stool	D
3	BS20-0706-1	C	07/06/20	58	4	+	+	-	Yes	Rectal swab	ND
4	BS20-0707-1	D	07/07/20	20	5	+	-	+	Yes	Rectal swab	ND
5	BS20-0708-1	E	07/08/20	16	3.5	+	+	+	Yes	Rectal swab	ND
6	BS20-0708-3	E	07/08/20	16	3	-	-	+	Yes	Rectal swab	D
7	BS20-0802-1	F	08/02/20	24	4	+	+	+	Yes	Stool	D
8	BS20-0802-2	F	08/02/20	24	4	+	+	-	Yes	Stool	D
9	BS20-0504-3	B	05/04/20	25	6	+	-	+	No	Stool	ND
10	BS20-0504-4	B	05/04/20	25	8	+	-	+	No	Stool	ND
11	BS20-0708-2	E	07/08/20	16	5	-	-	+	No	Rectal swab	ND

No, Number; Date of occurrence, MM/DD/YY; *P. O., Paralichthys olivaceus*; +, positive; -, negative; D, detected; ND, not detected.
[a]Time interval (h) = Time interval between food intake and fecal sample collection.
[b]Incubation Time (h) = Time interval between food intake and symptom onset.
[c]PCR result of *Kudoa* = Nested PCR result for detecting the *Kudoa septempunctata* 18S and 28S rDNA genes.

81.8% of the patients experiencing diarrhea and abdominal pain and 54.5% experiencing vomiting. The detection rate of *K. septempunctata* was 45.5% (5 patients), with all patients reported to have consumed *P. olivaceus* sashimi. Three out of these 5 (60%) patients experienced multiple symptoms concurrently, such as diarrhea, vomiting, and abdominal pain. One out of the 5 patients experienced diarrhea and vomiting, and the other one experienced only abdominal pain. The time interval between food intake and fecal sample collection ranged from 16 to 58 h; *K. septempunctata* was not detected in the rectal swabs collected 58 h later. Of the fecal samples collected from 11 patients, 6 were stool samples (54.5%). The detection rate of *K. septempunctata* via PCR in stool samples was 66.6% (4/6), which was higher than that in rectal swabs (20.0%); however, the difference was not statistically significant. The detection rate of *K. septempunctata* via PCR in the stool samples of 8 patients who consumed *P. olivaceus* sashimi was 100.0% (4/4), which was significantly higher than that in the rectal swabs (25.0%) ($P = 0.004$). However, this study had some limitations. PCR test results obtained using each patient's stool and rectal swab should be compared, but it was not possible to do so in practice.

One *P. olivaceus* sashimi sample that was confirmed to be positive for *K. septempunctata* was collected from a restaurant where a foodborne outbreak had occurred. To the best of our knowledge, this is the first study to identify *K. septempunctata* in both patients and food with epidemiological relevance in Korea.

Genotype of *K. septempunctata*

K. septempunctata is genetically classified into 3 groups (ST1, ST2, and ST3), and its genotype is determined by the combination of 2 mitochondrial genes, *cox1* and *rnl* (Table 2) [11].

For the 5 samples collected from patients with foodborne illness (4 stool samples and 1 rectal swab sample) and 1 raw *P. olivaceus* food sample, *cox1* and *rnl* were subjected to multiple sequence alignment with sequences of standard strains. All samples were found to be of the ST3 genotype, comprising *cox1*-3 and *rnl*-2 (Fig. 1).

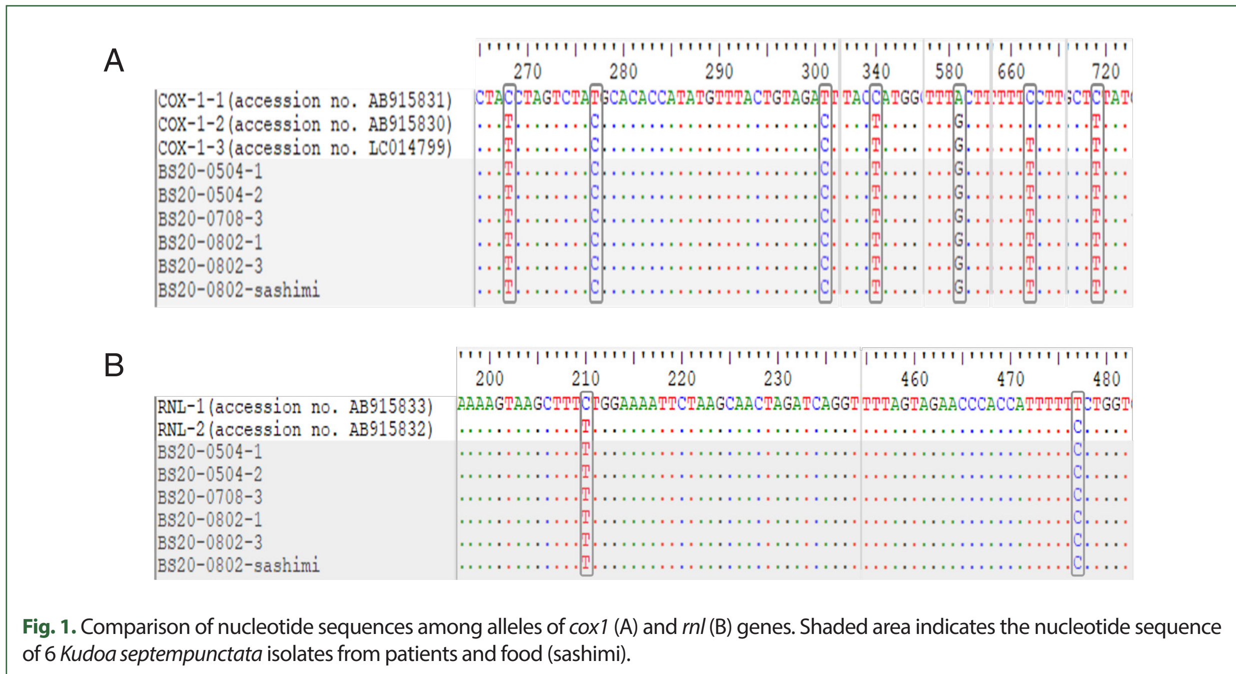

Fig. 1. Comparison of nucleotide sequences among alleles of *cox1* (A) and *rnl* (B) genes. Shaded area indicates the nucleotide sequence of 6 *Kudoa septempunctata* isolates from patients and food (sashimi).

Discussion

None of the 348 stool specimens collected from patients with acute diarrhea at the Enter-Net participating hospitals in Busan was found to contain *K. septempunctata*. This finding was in contrast to the disease surveillance statistics of Enter-Net Korea, where detection rates of acute gastroenteritis-causing bacteria and viruses were 16.3 and 17.3%, respectively, during the year 2020 [21]. The data obtained through epidemiological investigation reflect the following facts: *Kudoa* food poisoning has a short incubation period; symptoms are relieved within 24 h; and the only causative food is *P. olivaceus* sashimi infected with *K. septempunctata* [6,18]. Similar results were obtained in the present study. The 5 patients who tested positive for *K. septempunctata* presented a mean incubation time of 4 h and recovered within 3-20 h of symptom onset. The patient in outbreak C developed diarrhea and vomiting within 4 h of consuming *P. olivaceus* sashimi and recovered after 11 h. The patient's rectal swab sample was collected 58 h after ingestion of sashimi and no *K. septempunctata* was detected. This finding was consistent with that of Kim et al. [18], who suggested that epidemiological investigations should be conducted as early as possible because the detection rate of *K. septempunctata* in feces was reported to fall rapidly after a time interval of 28.5 h from food intake to epidemiological survey.

Obtaining stool samples from patients with *Kudoa* food poisoning is crucial. In this study, the detection rate of *K. septempunctata* in the stool samples of 8 patients who consumed *P. olivaceus* sashimi was 100.0%, which was significantly higher than that in rectal swab samples (25.0%). Kim et al. [18] also reported a high detection rate of *K. septempunctata* in stool samples (69.2%). Lee et al. [19] reported that 31 (52.5%) of the 59 food poisoning outbreaks related to restaurants in Chungcheongnam-do, Korea, occurred at seafood

restaurants; of these, 5 (8.5%) outbreaks were confirmed to be related to *Kudoa* food poisoning. They tested for *Kudoa* infection only in the stool among the collected fecal specimens and proposed that the detection rate of *K. septempunctata* would have been higher if more number of stool samples had been collected. During epidemiological investigation of food poisoning outbreaks, in the absence of a tool to collect stools or if the patient fails to collect them, rectal swab specimens can be used instead of stool specimens. According to the Guidelines for the Management of Waterborne and Food Poisoning Infections [22], rectal swabs are still used to detect bacterial and viral foodborne pathogens. However, only stool and vomit samples are recommended as diagnostic specimens for *Kudoa* food poisoning. Therefore, few attempts have been made to detect *K. septempunctata* in rectal swabs, even if *Kudoa* infection is suspected due to the ingestion of raw fish, such as *P. olivaceus*. This study is the first to genetically identify *K. septempunctata* in one of the 5 (20.0%) rectal swab specimens. Similarly, a previous study attempted the molecular detection of enteric pathogens, including protozoa, in rectal swabs. A total of 15 pathogens (3 viruses, 3 parasites, and 9 bacteria) were identified using a commercial multiplex PCR assay. Several protozoal pathogens (*Giardia*, *Cryptosporidium*, and *Entamoeba histolytica*) were identified in rectal swabs. The authors noted that flocked rectal swabs significantly facilitate the molecular diagnosis of diarrheal disease in children [23]. The Annual Report of the Epidemiological Investigation of Infectious Diseases in Korea, published by the KDCA, reported 377 foodborne outbreaks in public restaurants nationwide in 2018, of which 211 (55.9%) had an unknown causative pathogen [24]. However, the proportion of rectal swabs in the diagnostic specimens of these 211 foodborne outbreaks was not recorded. Nevertheless, implementing rectal swab testing for *K. septempunctata* detection could dramatically improve the identification rate of *Kudoa* infections. Therefore, although the detection rate of *K. septempunctata* in rectal swab samples was lower than that in stool specimens, we strongly recommend the use of rectal swabs as diagnostic specimens for *Kudoa* food poisoning.

K. septempunctata detected in a total of 6 specimens were all identified as the ST3 genotype. In a previous study by Takeuchi et al. [11], ST1, ST2, and ST3 genotypes of *K. septempunctata* were all reported to cause food poisoning. ST1 and ST2 genotypes are mainly detected in Japanese *P. olivaceus*, whereas the ST3 genotype is detected in Korean *P. olivaceus*. Moreover, the Korea Center for Disease Control and Prevention reported the presence of *K. septempunctata* in 84 clinical specimens from 45 foodborne outbreaks that occurred in Korea from 2015 to autumn 2016. All strains were of the ST3 genotypes [25], which is consistent with our results. To the best of our knowledge, this is the first study to detect *K. septempunctata* in both patient and food specimens (*P. olivaceus* sashimi) with epidemiological relevance in Korea. Previous animal experiments found that more than 10^6 *Kudoa* spores/mouse are needed to induce illness [5]. However, in the present study, no microscopic examination was conducted to confirm the number of *Kudoa* spores. Moreover, the *P. olivaceus* sashimi samples collected were stored in restaurants where foodborne outbreaks had occurred and were not remnants from patient meals. Despite these limitations, the epidemiological relationship between these specimens cannot be ignored because they were collected within 24 h of food poisoning, and none of the 20 known food poisoning pathogens [16] were identified (data not shown, http://heis.busan.go.kr/health/virus_007_01.aspx).

In the present study, the samples were subjected to a genotyping method for *K. septem-*

punctata based on mitochondrial (*cox1* and *rnl*) gene polymorphisms. Moreover, we performed molecular epidemiological analysis of *K. septempunctata*. Using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis on partial mitochondrial DNA (mtDNA) encoding cytochrome b and NADH dehydrogenase subunit 1, Yokoyama et al. [26] reported that *K. septempunctata* is divided into 4 types. Ohnishi et al. [27] identified 8 groups of *K. septempunctata* by applying random amplified polymorphic DNA (RAPD) analysis. This genetic diversity suggests that additional genotypes exist in *K. septempunctata*, which may impart different levels of pathogenicity in humans. Currently, there is no conclusive pathogenicity of ST3 in Korea [28], and therefore further studies are required to identify more diverse genotypes for *K. septempunctata* isolates.

In conclusion, we identified *K. septempunctata* 18S and 28S rDNA genes in 5 patients with food poisoning who ingested *P. olivaceus* sashimi and 1 sample of *P. olivaceus* sashimi. All detected *K. septempunctata* were genotype ST3, which has been commonly reported in Korea. *P. olivaceus* sashimi samples were collected from a restaurant visited by 2 patients in whom *K. septempunctata* genes were detected. This provides possible evidence that *K. septempunctata* is a causative pathogen for food poisoning. In addition, this study is the first to confirm the presence of *K. septempunctata* genes in a rectal swab sample. While suspected occurrences of *Kudoa* foodborne outbreaks continue, the identification rate of *K. septempunctata* remains low. One reason for this is the limitation in obtaining diagnostic specimens for *Kudoa* food poisoning, i.e., stool and vomit samples. Therefore, we strongly recommend obtaining rectal swabs as early as possible following symptom onset as diagnostic specimens for *Kudoa* food poisoning.

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