Original Article

Kudoa septeimpunctata-Induced Gastroenteritis in Humans after Flounder Consumption in Japan: a Case-Controlled Study

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SUMMARY: Raw fish consumption is increasing worldwide. Since around the year 2000, western regions of Japan have reported a foodborne disease of unknown cause that occurred after the consumption of flounder. In October 2010, a particularly large outbreak was reported in these regions among individuals who consumed flounder fish that had been raised in aquaculture systems. The median incubation period was 5 h (range, 4–19 h), and the most frequently reported symptom was diarrhea (80%). The risk estimate of the consumption of flounder was significantly higher than that of the development of symptoms (odds ratio = 9.50; 95% confidence interval, 1.59– ∞). According to a trace-back investigation, all of the flounder responsible for the outbreak were raised in aquaculture systems. Microscopic examination revealed that the median amount of *Kudoa septeimpunctata* present in the muscle of flounder fish from the aquaculture farm was 4.5×10^3 spores/g (range, 1.0×10^3 – 9.6×10^6 spores/g). The number of *K. septeimpunctata* spores required for the development of illness, as estimated using the Monte Carlo simulation, was 7.2×10^7 spores/g; therefore, thus this might be the minimum ingestion threshold for the development of gastrointestinal symptoms. As a public health measure, the current study results should be referred to for the prevention of the gastrointestinal symptoms related to the consumption of flounder; the national public health authority has disseminated these results. We concluded that *K. septeimpunctata*-contaminated flounder fish were associated with the gastrointestinal symptoms of this recent outbreak.

INTRODUCTION

According to a Food and Agriculture Organization (FAO) report, the consumption of raw fish such as sushi and sashimi is increasing (1,2). Many kinds of bacterial, viral, or parasitic pathogens have been detected in fishery products and are related to reported outbreaks of gastrointestinal illnesses (1,3–10). The FAO and the World Health Organization have reported that raw fish consumption is a risk factor for infectious diseases, including those caused by agents of foodborne disease outbreaks such as microbiological agents and parasites (11). Therefore, raw fish consumption is a risk to human health.

The Ministry of Health, Labour and Welfare, Japan (MHLW) reported that the number of unknown causes of foodborne disease outbreaks has increased since 2002

(12). However, it is unclear whether agents such as bacteria, viruses, bacterial toxins, or toxic chemicals have been detected in suspected foods. Following this report, the MHLW began an investigation of the unknown causes of these foodborne disease outbreaks (12). Of the 23 outbreaks documented between 2008 and 2010, several exhibited a significant association between the development of gastrointestinal symptoms and individuals who had ingested flounder (13). Fortunately, these outbreaks are self-limiting and normally have a good prognosis. Matsukane et al. reported the isolation of a new myxosporean species, *Kudoa septeimpunctata*, from the aquacultured trunk muscles of olive flounder (*Paralichthys olivaceus*) (14). Given that this parasite causes a latent infection in fish and is associated with food spoilage and the human consumption of flounder, we speculate that *K. septeimpunctata* might be involved in these food poisoning outbreaks.

However, previous outbreaks of unknown cause have either not affected a sufficient number of subjects or insufficient details regarding the onset of each symptom have been recorded to enable the clinical picture and incubation period following *K. septeimpunctata* consumption to be discussed adequately. Moreover, an estimate of the amount of *K. septeimpunctata* consumed has not been calculated.

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A large outbreak associated with flounder fish consumption was detected on October 9, 2010 following the development of gastrointestinal illnesses, which indicated that the outbreak started on October 5, 2010. The causative flounder fish was a prize promotion from a bank and was distributed to the winner of fix account depositor. Initial reports by the MHLW determined that around 400 people ingested the flounder, and that ~100 of these developed gastrointestinal symptoms. We investigated this outbreak according to the Food Sanitation Act of Japan.

The objective of this study was to investigate the clinical picture, risk estimate of onset, and incubation period of this outbreak, and estimate the threshold intake of *K. septe mpunctata*.

MATERIALS AND METHODS

Data collection: This outbreak investigation was a case-controlled study. We collected data from 73 family units (223 individuals) from the local public health center in November 2010 in a prefecture of western Japan. The subjects had ingested flounder that was given as a promotional prize by the Bank A, and the onset of any symptoms occurred on October 5–9, 2010. This case-controlled study was conducted according to the Food Sanitation Act using standardized questionnaires. The investigation of the foodborne disease outbreak was initiated by the local public health centers in a prefecture of western Japan. A standardized questionnaire gathered information regarding health status, symptoms, food intake, onset dates and times of symptoms, preserving techniques, cooking techniques, the use of pepper and seasoning, and the amount of flounder consumed. A case was defined as a person who consumed flounder that was given as a gift by Bank A on October 5–9, 2010, whereas a control was defined as the person who did not experience symptoms after the consumption of flounder on October 5–9, 2010.

The control group included family members or acquaintances that had eaten alongside the subjects but did not develop symptoms.

Flounder fish sampling: Seventy-four samples of flounder fish, which were bred in the same aquaculture pond but not consumed by the patients, were collected. The amount of *K. septe mpunctata* per 1 g of the flounder fish sample muscle tissue was determined.

Detection of *Kudoa* spores in flounder muscles (13): Flounder muscle tissue (1 g) was sieved through a 200 μ m-nylon sheet after the addition of 4 mL PBS (Takara Bio Inc., Otsu, Japan). The extract was passed through a 100- μ m cell strainer (BD, Franklin Lakes, NJ, USA) to remove debris, and centrifuged at $1,500 \times g$ for 15 min at 4°C. The pellet potentially containing *Kudoa* spores was re-suspended in 1 mL of PBS, and cells were counted using a hemocytometer. Spores were also purified from the suspension using Percoll density gradient centrifugation, as described previously (15).

Statistical analysis: The risk estimate for developed to onset of analysis was performed using logistic regression or exact logistic regression analysis. Analyses were conducted to identify contagious agents in samples of the flounder prizes. Direct microscopic observations were made, and the lead to onset of *K. septe mpunctata* intake

was estimated using a Monte Carlo simulation (11). Condition of calculation used the randomized collected both amount of intake of flounder fish and amount of *K. septe mpunctata* per 1 g of the fish. The iteration was performed 100,000 times with a threshold in the highest 25% because cases affected ~25% of the population that consumed the infected fish.

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RESULTS

The local public health centers collected 59 cases and 174 controls. There were no differences between cases and controls regarding characteristics including sex, underlying disease, and allergies (Table 1). Of the 59 cases, 36 (61%) were female. Underlying diseases were found in 39% (25/59) of cases, compared with 31% (51/167) of controls. The underlying diseases in the cases included hypertension (17%), diabetes mellitus (2%), hyperlipidemia (3%), and stomach illness (5%). Among all cases, 47 experienced diarrhea (80%), which was the

Table 1. Characteristics of Subjects

	Case (n = 59)		Control (n = 174)		P-value	Total	
	No.	%	No.	%		No.	%
Sex							
Male	23/59	39	73/172	42	0.642 ¹⁾	96/231	42
Female	36/59	61	99/172	58		135/231	61
Underlying diseases	23/59	39	51/167	31	0.235 ¹⁾	74/226	33
Hypertension	10/59	17	21/167	13	0.263 ²⁾	31/226	14
Diabetes mellitus	1/59	2	6/167	4	0.415 ²⁾	7/226	3
Hyperlipidemia	2/59	3	2/167	1	0.280 ²⁾	4/226	2
Stomach illness	3/59	5	4/167	2	0.265 ²⁾	7/226	3
Allergy	3/59	5	13/167	8	0.359 ²⁾	16/226	7
Symptom							
Fever	11/56	20					
Emesis	34/59	58					
Diarrhea	47/59	80					
Abdominal pain	25/59	42					
			Median	Range	P-value		
Age (yr)							
Case			62	13–90	0.462 ³⁾		
Control			61	1–91			
Amount of flounder fish ingested (g)							
Case			67	33–300	0.667 ³⁾		
Control			78	20–300			
Time from delivery to ingestion (h)							
Case			18	1–165	0.009 ³⁾		
Control			26	0–165			
Incubation period for symptoms (h)			5	1–22			
Emesis only (h)			5	4–19			
Emesis and diarrhea and/or abdominal pain (h)			5	4–19			
Diarrhea within 24 h (number of times)			3	1–20			
<i>K. Septe mpunctata</i> (spores/g)			4.5×10^3	1.0×10^3 -9.6×10^6			

¹⁾ Chi-square test.

²⁾ Fisher's exact test.

³⁾ Mann-Whitney U test.

Table 2. Relationship between factors symptoms

	Case		Control		OR	95%CI	P-value
	No.	%	No.	%			
Underlying disease	23/59	39.0	51/167	30.5	1.45	0.78–2.70	0.118
Hypertension	10/59	43.5	20/167	39.2	1.57	0.69–3.58	0.167
Allergy	3/59	5.1	13/167	7.8	0.64	0.17–2.31	0.359
Flounder fish eating							
Raw flounder fish eating	59/59	100.0	156/174	89.7	9.50	1.59–∞	0.008
Seasoning: soy sauce & wasabi	6/59	10.2	14/174	8.0	1.29	0.47–3.54	0.307
Preserving of flounder fish							
Room temperature	5/58	8.6	9/171	5.3	1.68	0.54–5.23	0.372
Refrigerator	28/58	48.3	109/171	63.7	0.51	0.28–0.94	0.002
Chilled room	23/58	39.7	36/171	21.1	2.42	1.21–4.82	0.012
Frozen	2/58	3.4	15/171	8.8	0.37	0.08–1.66	0.192

OR, odds ratio; 95%CI, 95% confidential interval.

Table 3. Elapse time from deliver to ingestion by each storage facility

	No.	Preservation period (h)		<i>P</i> value ¹⁾
		Median	Range	
Storage facility				
Room temperature	14	12.3	4.0–162.0	0.1306
Refrigerator	128	20.0	0.0–179.0	
Chilled	58	24.0	1.0–162.0	
Freezer	16	34.0	0.0–165.0	
				Z score <i>P</i> value
Multiple comparison of preservation period by storage facilities ²⁾				
Storage facility 1		Storage facility 2		
Room temperature	vs	Refrigerator	1.610	0.3730
Room temperature	vs	Chilled	1.425	0.4836
Room temperature	vs	Freezer	0.420	0.9750
Refrigerator	vs	Chilled	0.096	0.9997
refrigerator	vs	freezer	0.200	0.9972
Chilled	vs	freezer	0.342	0.9863

¹⁾: Mann-Whitney test.

²⁾: Steel-Dwass test.

most frequently reported symptom, followed by emesis (34 cases; 58%). The median age and amount ingested did not differ between the cases and controls. The median age was 62 years (range, 13–90 years), and the median incubation period was 5 h (range, 1–22 h). The median amount of flounder consumed by each case was 67 g (range, 33–300 g), compared with 78 g (20–300 g) in the controls. The time that elapsed from delivery to consumption (18 h) was significantly shorter in the cases than in the controls (26 h). The preservation period of the flounder fish did not differ significantly between each storage facility (Table 1). The median amount of *K. septempunctata* was 4.5×10^3 spores/g (range, 1.0×10^3 – 9.6×10^6 spores/g). All flounder were raised in aquaculture systems in the local area.

The relationship between consumption and the development of symptoms is shown in Table 2. Raw flounder consumption (odds ratio [OR] = 9.50; 95% confidence interval [CI], 1.59–∞) was significantly associated with disease onset. Preservation using a refrigerator (OR = 0.51; 95% CI, 0.28–0.94) was sig-

Table 4. Estimation of percentiles of *Kudoa septempunctata* numbers

Percentile	<i>K. Septempunctata</i> (spores/g)
0th	2.00×10^4
25th	7.50×10^4
50th	3.00×10^5
75th	7.20×10^7
100th	2.89×10^9

nificantly associated with protecting against the symptom onset, whereas a chilled room (OR = 2.42; 95% CI, 1.21–4.82) was significantly associated with the onset of symptoms. According to a Mann-Whitney U test, there were no significant differences in the preservation periods in the different facilities (Table 3). In addition, multiple comparisons of the preservation periods in each facility revealed no significant differences among the groups.

We used microscopic observations of the spores and questions related to flounder intake in the questionnaire to calculate an estimated the threshold intake amount of *K. septempunctata*. Of the 215 people who ingested flounder, 59 (27%) developed symptoms; therefore, we assumed that around three fourth had not developed symptoms. Thus, the 75th percentile was an estimation of the minimum threshold for illness; the 75th percentile for the amount of *K. septempunctata* was 7.2×10^7 spores/g (Table 4).

DISCUSSION

The outbreak described in this study was investigated using epidemiological investigations and microbiological tests. A case-controlled study conducted during the investigation showed that the consumption of raw flounder fish was significantly associated with the development of gastrointestinal illness. In this outbreak, *K. septempunctata* was identified from samples of flounder fish microscopically, which is consistent with a previous report (13). We concluded that the consumption of flounder fish infected with *K. septempunctata* caused the gastrointestinal illness in this outbreak.

The illnesses described included at least one gastrointestinal illness such as emesis, diarrhea, and/or abdomi-

nal pain. Diarrhea was the most common symptom; of the 59 cases, 58 developed emesis and/or diarrhea, and only one case developed abdominal pain. Kawai et al. also reported that the most common symptom was diarrhea; therefore, we conclude that the main symptom associated with infection by *K. septe mpunctata* is gastrointestinal symptoms such as vomiting and diarrhea (13).

The current investigation showed that the median incubation period in these cases was 5 h (range, 4–22 h). Kawai et al. reported that the incubation periods of 24 flounder-related outbreaks had a median incubation period of 3–16 h (range, 0–25 h) (13). The MHLW distributed a notification that they were performing a foodborne disease investigation. The document described the time to the onset of symptom development, and also reported that some symptoms had widely variable incubation periods; thus, the case definitions might differ among outbreaks. We performed accurate case definitions, and assumed that the incubation period could indicate another disease. We concluded that the median incubation period of *K. septe mpunctata* was 5 h, and that the main symptoms were emesis, diarrhea, and/or abdominal pain.

K. amamensis and other species affect muscle myoliquefaction and cause what is known as “jelly meat” (16–18). Wills (19) and Patashnik et al. (20) reported that parasite-produced enzymes cause post-mortem muscle degeneration in fish. Moran et al. reported that myoliquefaction (21), and St-Hilarie et al. (22) and Moran et al. (17) reported that jelly meat decreased the value of fish to the extent that they could not be sold. Matsukane et al. (14) reported that *K. septe mpunctata*-positive muscles showed no myoliquefaction. However, *K. septe mpunctata* form spores that cannot be seen in flounder muscle by the human eye. Therefore, there is currently inadequate gross evidence of *K. septe mpunctata* in flounder.

The amount of flounder ingested did not differ significantly between cases (median, 67 g; range, 33–3,000 g) and controls (median, 78 g; range, 20–300 g). Moreover, the median amount of *K. septe mpunctata* was 4.5×10^3 spores/g (range, 0.1×10^3 – 9.6×10^6 spores/g). The results of microscopic observation suggested that the amount of *K. septe mpunctata* in each flounder varied. Thus, the amount of flounder ingested might not predict the onset of gastrointestinal symptoms. We also estimated the threshold intake of *K. septe mpunctata*, which might be useful as a public health measure. Controlling of flounder contaminated with *K. septe mpunctata* is an important factor for preventing foodborne disease in Japan.

One risk factor for the development of symptoms was preservation in a chilled room, whereas refrigeration afforded protection. Comparisons of the elapsed time of delivery to consumption were significantly longer than previously thought. However, the length of preservation time was not significantly different among each storage facilities (Table 3) and was not related to the development of gastrointestinal illness. According to the current study, the standard of the storage facilities and chilled rooms was significantly associated with development of gastrointestinal illness, and there was a significant association between the use of refrigerators and

preventing gastrointestinal illness. The Division of Food Safety of the Council on Public Health of the MHLW reported that refrigeration decreases the pathogenicity of *K. septe mpunctata* in laboratory animals (<http://www.mhlw.go.jp/stf/shingi/2r9852000001ahy8-att/2r9852000001aib5.pdf>). This investigation showed that the length of time and preservation temperature between delivery and consumption were significantly shorter in subjects compared with controls. However, the current investigation did not collect data regarding preservation temperature. As such, further investigations into both the length and temperature of refrigeration are needed to evaluate the prevention of this illness.

Martínez et al. (23) reported that the consumption of *Kudoa spp.*-infected fish was a possible cause of human allergy-induced gastrointestinal symptoms, as indicated using skin prick tests. In the current study, we collected data regarding the history of allergies in each subject, and compared this information with their symptoms after flounder consumption. The resulting epidemiological data were not associated significantly with allergies. However, we did not test for IgE antigen; thus, we did not have the biological data to assess potential association. Therefore, a future study could investigate the relationship between *K. septe mpunctata* intake and allergic reactions in cases of gastrointestinal symptoms.

This investigation has advantages over previous studies because it involved a large outbreak in a large number of subjects (13,24); thus, the estimates were improved by an increased sample size (25). According to logistic regression analysis the consumption of flounder fish was associated with the development of gastrointestinal symptoms. These results were consistent with another flounder-related outbreak (13). All flounder given as prizes were raised in aquaculture systems. The current tests detected *K. septe mpunctata* in fish samples, and estimated that the required amount of consumed *K. septe mpunctata* for the development of symptoms was 7.2×10^7 spores/g; therefore, this study suggests that the estimated ingestion threshold is at least this amount.

In conclusion, flounder consumption was associated with gastrointestinal symptoms in the current outbreak. Microscopic observation detected *K. septe mpunctata* in flounder samples. The main symptoms were emesis and diarrhea, and the median incubation period was 5.0 h. An estimate of the ingestion threshold of *K. septe mpunctata* for developing symptoms was 7.2×10^7 spores/g. These results could therefore be used as a public health measure to prevent gastrointestinal symptoms related to the consumption of flounder.

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Conflicts of interest None to declare.

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