Original Article

Prevalence of bacterial contamination in uncooked sticky rice samples from markets in Japan

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Abstract

We investigated the prevalence of bacterial contamination in uncooked sticky rice samples obtained from markets in Japan. Between June and August, 2019, a total of 25 sticky rice samples were purchased from shops in the Tokyo area. Twenty-two (88.0%) and 10 (40.0%) of 25 sticky rice samples harbored 3.06±0.54 log spc/g of standard plates counts and 2.17±0.23 log cfu/g of enterobacteriaceae bacteria counts, respectively. Three species of Enterobacteriaceae, *Pantoea dispersa*, *P. ananati*, and *Kosakonia cowanii*, were identified by MALDI-TOFMS-based test. Three (12.0%) of 25 sticky rice samples harbored 2.00 log cfu/g of *Bacillus* species. *Bacillus* spp. was not isolated after heat treatment (98 °C for 20 min.) of sample homogenates. Two isolates were identified *B. cereus* by both API 50 CH and MALDI-TOFMS based test. However, one isolate was *B. mycoides* by API 50 CH, but *B. cereus* by MALDI-TOF MS based test. These 3 *Bacillus* strains harbored no gene encoding the enzyme responsible for cereulide synthesis and had not produced enterotoxin. Our study suggested that some uncooked sticky rice has *Enterobacteriaceae* bacteria and *Bacillus* spp. contamination, however there is no isolate from heat treatment samples in this study. Isolated *P. dispersa*, *P. ananati*, and *K. cowanii*, are usually in the environment and these organisms have been isolated from patients with opportunistic infections. Preventing cross-contamination, from sticky rice to cooked food may be important during preparation and storage in kitchen.

Keywords: Bacterial Contamination, Sekihan, Sticky Rice, Japan

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Introduction

Bacillus cereus is a Gram-positive, rod-shaped, aerobic, motile, beta-hemolytic, spore-forming bacterium commonly found in soil, grain and food (Logan and Vos, 2009). Some strains are harmful to humans and cause foodborne illness, while others can be beneficial as probiotics for animals (Ryan, 2004). Foodborne illnesses due to *B. cereus* occur when bacterial spores survive in food that is improperly cooked and stored (Logan and Vos, 2009; Ryan, 2004). For example, *B. cereus* is often the cause of "fried rice syndrome", which can occur from consumption of fried rice dishes that have been kept at room temperature for hours (Asaeda *et al*., 2005). *B. cereus* is responsible for only a minority of foodborne illnesses (2–5%) but causes severe nausea, vomiting and diarrhea (Kotiranta *et al*., 2000). *B. cereus* isolated from patients produces two types of toxin: an emetic toxin (cereulide) and an enterotoxin (Agata *et al*., 1994; Thompson, 1984).

In Japan, national food poisoning data is collected by the Ministry of Health, Labour and Welfare. In Japan, *B. cereus* was detected in 8 of 1,330 (0.6%) outbreaks of food poisoning and 86 of 17,282 (0.5%) affected patients in 2018. Meanwhile, in Thailand, no cases of *B. cereus* food poisoning have been reported in recent years, possibly because the symptoms are not severe.

Sticky rice (*Oryza sativa* var. *glutinosa*) is a type of rice grown mainly in Southeast and East Asia and the eastern parts of South Asia and has opaque grains, very low amylose content and is especially sticky when cooked. Sticky rice and rice are normally boiled at temperatures of more than 98 °C for 20 mins (Seki and Kainuma, 1986). However, some *B. cereus* spores can survive these cooking conditions. Cooked rice is often stored at room temperature and for many hours, allowing any surviving spores to grow vegetatively. The growing bacteria can then synthesize cereulide or enterotoxin in the cooked rice.

In Japan, “Sekihan” literally means "red rice", and it is steamed sticky rice with boiled red beans. It is cooked in red water colored by red beans. Japanese enjoy “Sekihan” as a special celebratory dish for happy occasions because of red being a lucky, amulet color in Japan. Usually, Japanese like eating “Sekihan” with salt and sesame. Our previous report (Kato *et al*., 2019) suggested that some commercial Sekihan (8/34) and ingredients (2 of 10 cowpeas samples, 1 of 7 adzuki samples and 3 of 12 sesame samples) have *Bacillus* spp. contamination. In addition, sesame contains 2.00-4.84 log cfu/g of *Bacillus*, therefore, sesame is one of the high-risk factors for food poisoning in Sekihan in Japan. However, we have no results about main and raw materials in sticky rice contamination in the report.

We therefore examined bacterial populations of *Bacillus* spp., Enterobacteriaceae bacteria, and standard plate counts bacteria in cooked and uncooked sticky rice samples from markets in Tokyo, Japan. Isolated *B. cereus* samples were subjected to PCR and reverse passive latex agglutination to detect the genes encoding enzymes responsible for cereulide (CRS) and enterotoxin synthesis, respectively. Isolated Enterobacteriaceae bacteria were subsequently identified at the species level.

Materials and Methods

***Sticky rice samples and laboratory handling*:** Between June and August, 2019, a total of 25 sticky rice samples were examined in this study. The samples were purchased from shops in the Tokyo area. The sticky rice samples were held at room temperature and were kept in refrigerators at 3–5 °C, and analyzed for bacterial populations within one week of collection.

Standard plate counts, Enterobacteriaceae bacteria counts, identification of Enterobacteriaceae bacteria and statistical analysis: Uncooked sticky rice samples (10 g) were homogenized gently by hand in 90 mL of phosphate buffered saline (PBS; pH 7.0) for approximately 30 secs. Each homogenate was diluted 10^0- to 10^2-fold in PBS. A portion (100 µL) of the PBS dilution from each sample was inoculated onto SPC agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and VRBG agar (Oxoid Ltd., Basingstoke, UK). We used 2 SPC plates and 2 VRBG Plates for each diluted sample. Inoculated standard agar plates were incubated at 35 °C for 48 h and the VRBG agar plates were incubated at 35 °C for 24 h. Bacterial colonies were then counted. Growth of 1 - 3 enterobacteriaseae isolates on VRBG agar were identified using a commercial identification kit (ID Test EB-20, Nissui Pharmaceutical Co., Ltd.). When the bacteria could not be identified by the commercial kit, the bacilli were sent to the ICLAS monitoring center (https://www.iclasmonic.jp/en/microbiology/mquick.html) for identification using a Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) based test.

In addition, 1 mL of sample homogenate was transferred to microcentrifuge tubes (Safe-Lock, 1.5 mL, Eppendorf, Hamburg, Germany) and subjected to heat treatment at 98 °C for 20 mins on a heat block (THB-2, Dry block bath, AS ONE, Oosaka, Japan). A portion (50 µL) of the heat-treated PBS dilution was also inoculated onto SPC agar by the spiral plating method (EDDY JET2W, IUL instruments, Barcelona, Spain).

When one colony grew on one SPC agar or a VRVG agar and a negative one on the other SPC plate or VRBG agar, we calculated them as 100 spc or cfu/g. When a bacteria count was negative, the bacteria count for that sample was set as 1 cfu/g; then we used a logarithmic mean with standard error of the samples. Differences between means were considered significant at $p < 0.05$ by an independent two-sample *t*-test.

B. cereus counts, bacterial identification and toxin tests: Uncooked rice samples (10 g) were homogenized gently by hand in 90 mL PBS (pH 7.0) for approximately 30 secs. Two parallel 1 mL homogenate samples were collected in Safe-Lock microcentrifuge tubes; one sample was subjected to heat treatment at 98 °C for 20 mins in a heat block and the other was used as a control and not subjected to heat treatment. A portion (50 µL) of each of the heated samples and the

non-heated control samples was inoculated onto Brilliance Bacillus Cereus Agar (Oxoid Ltd) by the spiral plating method. The inoculated agar plates were incubated at 35 °C for 18 h, and then typical colonies were counted. *B. cereus* colonies were blue/green on Brilliance Bacillus Cereus Agar. The colonies that grew on Brilliance Bacillus Cereus Agar were identified using a commercial identification kit (API® 50 CH, bioMérieux, Marcy-l'Étoile, France) and the API_50_CHB database of APIweb™ (https:// apiweb.biomerieux.com) and MALDI-TOF MS based test. When the bacterial strains identified using APIweb™ included *B. cereus* species such as *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*, then, *B. cereus* (CRS gene) PCR Detection Kit (TaKaRa Bio Inc., Shiga, Japan) was used to detect the gene encoding the enzyme responsible for CRS synthesis and the gene encoding lecithinase. Assays were also performed to detect enterotoxin using a Reverse Passive Latex Agglutination kit (CRET-RPLA, Denka Seiken Co., Ltd., Tokyo, Japan). Bacteria in the *B. cereus* group containing the gene encoding lecithinase were identified as *B. cereus*.

Results

Standard plate counts and Enterobacteriaceae counts of uncooked sticky rice samples and identification of Enterobacteriaceae (Table 1) : Twenty-two of 25 (92.0%) sticky rice samples harboring 3.06±0.54 log spc/g. 2.17±0.23 log cfu/g of Enterobacteriaceae bacilli were isolated from the 10 (40.0%) samples. The Enterobacteriaceae isolates could not be identified by ID test EB-20, however, three species of Enterobacteriaceae, *Pantoea dispersa*, *P. ananati,* and *Kosakonia cowanii*, were identified by MALDI-TOF MS based test.

***Prevalence of* B. cereus *and toxin assays of isolated* B. cereus** (Table 1): In samples obtained in Japan, 3 of 25 (12.0%) sticky rice samples harbored 2.00±0.00 log cfu/g of *Bacillus* species. *Bacillus* spp. was not isolated after heat treatment of sample homogenates at 98 °C for 20 mins. The isolates from the Sample 16 and Sample 17 were identified *B. cereus* by both API 50 CH and MALDI-TOFMS based test. The isolate from the Sample 22 was *B. mycoides* by API 50 CH, but *B. cereus* by the MALDI-TOF MS based test. These 3 *Bacillus* strains harbored no gene encoding the enzyme responsible for cereulide synthesis and had not produced enterotoxin.

Table 1 Bacterial counts and species of isolates from samples

Sample ID	Standard plate counts (Log spc/g)	Enterobacteriaceae counts (Log cfu/g)	Identification method of Enterobacteriaceae isolates		Bacillus spp. counts (Log cfu/g)	Identification method of Bacillus isolates	
			ID Test EB-20	MALDI-TOF MS-based test		API 50 CH	MALDI-TOF MS based test
1	3.72	2.48	N.I. [a]	*Pantoea dispersa*	0.00		
2	0.00	0.00			0.00		
3	0.00	0.00			0.00		
4	3.20	0.00			0.00		
5	2.78	0.00			0.00		
6	3.45	2.48	N.I.	*Pantoea ananati*	0.00		
7	2.00	0.00			0.00		
8	2.95	0.00			0.00		
9	4.17	2.48	N.I.	*Pantoea ananati*	0.00		
10	3.43	2.30	N.I.	*Pantoea dispersa*	0.00		
11	3.08	2.00	N.I.	*Pantoea ananati*	0.00		
12	3.92	2.00	N.I.	*Pantoea ananati*	0.00		
13	2.30	2.00	N.I.	*Pantoea ananati*	0.00		
14	2.70	0.00			0.00		
15	3.11	0.00			0.00		
16	2.48	0.00			2.00	*B. cereus*	*B.cereus*
17	3.11	2.00	N.I.	*Kosakonia cowanii*	2.00	*B. cereus*	*B.cereus*
18	2.00	0.00			0.00		
19	2.90	0.00			0.00		
20	3.04	0.00			0.00		
21	3.11	2.00	N.I.	*Pantoea ananati*	0.00		
22	3.20	2.00	N.I.	*Pantoea ananati*	2.00	*B. mycoides*	*B. cereus*
23	3.36	0.00			0.00		
24	3.32	0.00			0.00		
25	3.00	0.00			0.00		
Mean±S.E.	3.06±0.54 [b]	2.17±0.23 [b]			2.00±0.00 [b]		

[a] No Identificaiton

[b] Geometoric mean of the bacteria counts in positive samples(Mean ±standard diviation log cfu/g).

Discussion

In Japan, some commercial "sekihan" have *Bacillus* spp. contamination and ingredients such as cowpeas, adzuki and sesame samples harbored the organisms. However, we have no results that suggest sticky rice is a main and raw material. We investigated the prevalence of bacterial contamination in uncooked sticky rice samples obtained from markets in Japan to

find the risk factors for the food poisoning in "Sekihan" in Japan.

Our previous report (Kato *et al*, 2018) in Japan showed that 28 of 29 (96.6%) rice samples from non-farmers home harbored 3.54±0.09 log cfu/g of SPCs. In these results, 23 of 25 (92.0%) sticky rice samples harbored 3.06±0.54 log cfu/g of SPCs. SPCs of sticky rice samples were lower than rice samples (p<0.05). Though in coliform group bacteria in rice samples, 6 of 29 (20.7%) samples from non-farmers home harbored 2.68±0.20 log cfu/g and the species of isolates were *Enterobacter amnigenus*, *Cronobacter sakazakii*, *Seratia ficaria*, and unidentified bacteria (Kato et al, 2018). In this study, there was no coliform group bacteria in sticky rice samples, however *Enterobacteriaceae*, such as *Pantoea dispersa*, *P. ananati*, and *Kosakonia cowanii* were isolated. *Pantoea* is a Gram-negative, non-encapsulated, non-spore-forming, ubiquitous straight rod which can be isolated from geographical and ecological sources such as plant surfaces, buckwheat seeds, human feces and the environment (Asai *et al*., 2019; Gavini *et al*., 1989; Walterson and Stavrinides, 2015). *P. dispersa* and *P. ananatis* were producing opportunistic infection especially in elderly patients, and *P. dispersa* was resistant to almost all the conventional antimicrobial agents in human clinics (Panditrao and Panditrao, 2018). *Kosakonia cowanii* (formally Enterobacter cowanii) was also frequently isolated from plants, soil and infant formula (Brady *et al*., 2009), though the type of strain was first isolated from patients (Inoue *et al*., 2000). Isolates of rice samples and sticky rice samples seem to be different in these studies but all isolates live in the environment and some bacteria are the source of opportunistic infections.

One of 29 (3.4%) rice samples from non-farmers in Japan and 4 of 7 (57.1%) rice samples from markets in Thailand harbored 2.30 log cfu/g, and 2.54±0.14 log cfu/g of *B. cereus* (Kato *et al*, 2018). In sticky rice samples in Japan, 3 of 25 (12.0%) samples had 2.00 log cfu/g of *Bacillus* species. Some samples of rice or/and sticky rice were contaminated *Bacillus* species, therefore, rice or/and sticky rice had the potential for *B. cereus* food poisoning, though *Bacillus* isolates possessed no gene encoding the enzyme responsible for cereulide synthesis, and did not produce enterotoxin in this study.

Many unidentified coliform bacilli by biochemical tests were isolated from rice and edible roots such as carrots and edible burdock samples in our previous reports (Kato *et al*., 2018; Kato *et al*., 2019). In recent years, MALDI-TOF MS based test has revolutionized routine identification of bacteria (Pavlovic *et al*., 2013). But the test still failed to distinguish *B. cereus* complex (*B. anthracis*, *B. cereus* and *B. thuringiensis*), *Escherichia coli* and *Shigella* group, and *Enterobacter cloacae* complex, and *Pseudomonas putida* complex (Rahi *et al*, 2016). In this report, none of the Enterobacteriaceae isolates from the samples could identify the species of bacteria by ID Test EB-20 basing biochemical test. However, all isolates were identified by the MALDI-TOF MS based test. The method seems to be a useful tool for identification of bacteria especially isolates from the environment and food samples. In this study, one strain was identified *B. mycoides* by API 50 CH and *B. cereus* by the MALDI-TOF MS based test. While we identified *Bacillus* spp of isolates by some identification tests, detecting pathogenic genes and/or producing toxins will be needed.

B. cereus were isolated from 41.9% (13/31) of cooked rice, brown rice and sticky rice, 33.3% (2/6) of cereal flour (rice, wheat, and corn), and 18.7% (3/16) of noodles (rice, wheat, mung beans), however the number in these samples were 1.7-3.0 log cfu /g, and the volume showed a safe level in Thailand reports (Chitov *et al*., 2008). In Japan, rice cookers with a warming function could decrease the incidence of *B. cereus* food poisoning. After cooking, cooked rice should be held at a temperature of approximately 65 °C to prevent surviving spores from growing vegetatively in rice cookers. However, many regions in Asian countries where rice is eaten do not have electricity and /or electric rice cookers.

In this study some uncooked sticky rice samples were contaminated with *B. cereus* in Japan. Since *B. cereus* and opportunistic pathogens such as *P. ananati, P. dispersa, and Kosakonia cowanii* are found in a wide range of environments, including soil, water, sewage, and food, it is very important to prevent cross-contamination of cooked rice, including sticky rice, during food preparation and storage.

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