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PRESENCE OF *Bacillus cereus* FROM LOCAL UNHUSKED (ROUGH) RICE SAMPLES IN SARAWAK, MALAYSIA

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Abstract: *Bacillus cereus* is a widespread endospore forming pathogen that can cause diarrhoeal and emetic food poisoning. The purpose of this study was to determine the occurrence of *B. cereus* from local unhusked (rough) rice. A total 24 local unhusked (rough) rice samples were collected from various locations in Sarawak, Malaysia. The analysis was carried out using the most probable number–polymerase chain reaction (MPN–PCR) method. The bacterial loads of *B. cereus* in all samples were found to be more than 1100 MPN/g. PCR analysis showed that all the samples were positive for *B. cereus*. The finding of this study suggests that the local unhusked rice can be one of the potential sources for *B. cereus* foodborne outbreak. This study can be used as a baseline data for future risk assessment of *B. cereus* in local food sources.

Keywords: *Bacillus cereus,* MPN-PCR, unhusked rice

Introduction

Bacillus cereus is a gram-positive, endospore-forming and motile rod-shaped bacterium that can be found in food and soil. Its common association with food spoilage is attributed by highly adhesive endospores that can taint many kinds of foodstuff. Enterotoxins produced by *B. cereus* are strongly associated with the symptoms manifested (i.e. diarrhoeal and emetic syndromes). Diarrhoea-causing enterotoxins are haemolysin BL (HBL), non-haemolysin enterotoxins (NHE) and cytotoxin K (CytK) which are heat labile. The toxins are produced by vegetative growth of the bacteria in small intestine (Granum & Lund, 1997). While, emesis-causing enterotoxin is cereulide, that is heat- and pH-stable, and toxic at low dose (Ehling-Schulz *et al.,* 2004). The toxin is produced by the bacteria in food (Granum & Lund, 1997). Hemolysin gene is related to the production of HBL and pore forming haemolysins. Pore-forming haemolysins produced by *B. cereus* are haemolysin I or cereolysin O, haemolysin II, haemolysin III, and haemolysin IV (Ramarao & Sanchis, 2013).

Rice is an important staple food in Malaysia. Production of paddy rice in Malaysia was 3,322 tonnes in 2015 and it increased as much as 16.6% from the previous year (Department of Statistic Malaysia, 2016). Sarawak is one of the states in Malaysia that endowed with rice varieties where they are cultivated in terrains, hills and lowland areas and had approximately 122,200 hectares of paddy area in 2015 (State Planning Unit, 2015). As rice is staple food for Malaysians, it is crucial to ensure the safety level of unhusked (rough) rice before being processed and consumed. Occurrence of *B. cereus* contamination on local Sarawak rice is underreported, thus the finding from this study would provide information for the related food industries and the public for any risk control measures.

Bacillus cereus is a common soil inhabitant. Taint of foods and foodstuffs (e.g. rice, sago starch, grain) with the bacteria can occur through soil contamination on the crops (Notermans & Batt, 1998). There are a few local studies on *B. cereus* occurrence in food such as noodles, spices and legumes (Rusul & Yaacob, 1995), ready-to-eat cooked rice (Sandra *et al.,* 2012),

rice grains (Ankolekar *et al.,* 2009; Bilung *et al.,* 2016; Kim *et al.,* 2014) and cereals (Lee *et al.,* 2009; Bilung *et al.,* 2013). In some reported, there are also cases of *B. cereus* food poisoning from rice consumption (Raevuori *et al.,* 1976; Rampal *et al.,* 1984; Tay *et al.,* 1982). Hence, this study was conducted to determine the occurrence of *B. cereus* in different varieties of local unhusked (rough) rice in Sarawak using the MPN-PCR method.

Materials and Methods

Unhusked (rough) rice sampling

A total of 24 samples of different varieties of local unhusked rice were obtained from various locations in Sarawak as shown in Table 1. Types and nature of the samples are shown in Table 1. The samples were transported to Molecular Microbiology laboratory, Universiti Malaysia Sarawak for further analysis.

Table 1. Type of Samples, Nature of the Samples and Location of Sampling

No of sample	Unhusked rice variety	Nature of samples	Origin [GPS location]
1	Padi Pulut	In storage	Kampung Bokah, Bau [N01°29'23.2662" E109°55'21.3209"]
2	Padi Rejat	In storage	
3	Padi Pandan	In storage	Kampung Serasot, Bau [N01°22'42.7190" E110°02'47.2367"]
4	Padi Wangi	In storage	Kampung Tebakang, Serian [N01°06'24.2878" E110°30'52.8882"]
5	Padi Paya	In storage	
6	Padi Bario Wangi	Freshly harvested	Kampung Sindang, Samarahan [N01°28'26.1682" E110°28'46.9878"]
7	Padi Jaggoi Hitam	Freshly harvested	
8	Padi Arang	Freshly harvested	Kampung Lebor, Serian [N01°09'59.1441" E110°39'13.9475"]
9	Padi Hitam	Freshly harvested	
10	Padi Entaba	Freshly harvested	
11	Padi Mamut	In storage	Agriculture Research Centre (ARC), Semonggok [N01°23'34.6942" E110°19'35.5897"]
12	Padi MR253	In storage	
13	Padi Adan	In storage	Kampung Batu Empat, Jln Trusan, Lawas [N04°49'50.2104" E115°26'56.9400"]
14	Padi Sewangi	Freshly harvested	Melugu, Sri Aman [N01°10'58.5041" E111°28'24.0432"]
15	Padi Celum	Freshly harvested	
16	Padi Bario	Freshly harvested	
17	Padi Midek	Freshly harvested	Kampung Selantik, Sri Aman [N01°05'01.7052" E111°06'14.5980"]
18	Padi Lemak	In storage	Kampung Sibuti, Miri [N03°58'00.5736" E113°45'37.7892"]
19	Padi Selepin	Freshly harvested	Kampung Stuga, Sri Aman [N01°08'21.2820" E111°38'28.8060"]
20	Padi Nyamuk	Freshly harvested	
21	Padi Pandak	Freshly harvested	
22	Padi Mawang	Freshly harvested	
23	Padi Gudang	Freshly harvested	
24	Padi 3 Bulan	In storage	Taman Millenium, 12th Mile, Serian [N01°23'50.2116" E110°20'37.1998"]

Pre-enrichment of Bacillus cereus from unhusked rice samples

Pre-enrichment was performed according to the method described by Sandra *et al.* (2012). A total of 5 g each of the local unhusked rice from each sample was added into a stomacher bag containing 45 ml of nutrient broth (Merck, Germany) and incubated at 37˚C for 24 hours.

Bacterial enumeration

Most Probable Number analysis was performed in accordance to the method by Sandra *et al.* (2012). Briefly, the homogenised fluid from each of the enriched unhusked rice samples was serially diluted into 100-fold and 1000-fold dilutions with nutrient broth (Merck, Germany) and incubated at 37˚C for 24 hours.

DNA extraction

Extraction of genomic DNA of *B. cereus* was conducted via boil cell method in accordance to Lee *et al.* (2009). One millilitre of the turbid broth was centrifuged at 12,400 rpm for one minute. The pellet was re-suspended in 500 µl of sterile distilled water. The mixture was boiled for 20 minutes followed by immediate cooling at -20˚C for 10 minutes. The mixture was centrifuged again at 12,400 rpm for 5 minutes. The supernatant was used for PCR assay.

Polymerase Chain Reaction

Detection of *B. cereus* in the samples was performed using a primer pair of BC-1 (CTGTAGCGAATCGTACGTATC) and BC-2 (TACTGCTCCAGCCACATTAC) to amplify the 185 bp fragment of hemolysin gene (Wang *et al.,* 1997). The amplification was performed in 25 µl of reaction mixture containing 5 µl of 5X Green GoTaq® Flexi Buffer (Promega, United States), 3 µl of 25 mM $MgCl_2$ solution, 0.5 µl of 10 mM PCR nucleotide mix, 0.15 µl (5 u/µl) of GoTaq® DNA polymerase (Promega, United States), 0.5 µl of each primer, 4 µl of template DNA and 11.35 µl of sterile distilled water. The amplification was conducted with initial denaturation at 94ºC for 3 minutes, 35 cycles each of denaturation at 94ºC for 45 seconds, annealing at 49ºC for 1 minute, and elongation at 72ºC for 1 minute. Final elongation was conducted at 74ºC for 7 minutes. Analysis of the PCR product was performed using gel electrophoresis of 1.0% agarose gel at 90 V for 90 minutes. A 100 bp DNA ladder (Thermo Scientific, United States) was included as a molecular marker. The agarose gel was stained with ethidium bromide and visualised under a UV transilluminator (Maestrogen, Taiwan).

Results and Discussion

The MPN result for all the 24 unhusked rice samples (10 storage samples and 14 fresh samples) was more than 1100 MPN/g as shown in Table 2. All local unhusked rice samples (100%) were positive with *B. cereus* based on the PCR analysis. The representative image of the gel electrophoresis is displayed in Figure 1. All samples were found to be contaminated with *B. cereus*. The prevalence of *B. cereus* from the local paddy rice samples are shown in Table 2.

Table 2. Result of Most Probable Number and PCR analysis of *Bacillus cereus* in local unhusked rice

Types of Sample	MPN/g	PCR analysis
Padi Pulut	>1100	+
Padi Rejat	>1100	+
Padi Pandan	>1100	+
Padi Wangi	>1100	+
Padi Paya	>1100	+
Padi Bario Wangi	>1100	+
Padi Jaggoi Hitam	>1100	+
Padi Arang	>1100	+
Padi Hitam	>1100	+
Padi Entaba	>1100	+
Padi Mamut	>1100	+
Padi MR253	>1100	+
Padi Adan	>1100	+
Padi Sewangi	>1100	+
Padi Celum	>1100	+
Padi Bario	>1100	+
Padi Midek	>1100	+
Padi Lemak	>1100	+
Padi Selepin	>1100	+
Padi Nyamuk	>1100	+
Padi Pandak	>1100	+
Padi Mawang	>1100	+
Padi Gudang	>1100	+
Padi 3 Bulan	>1100	+

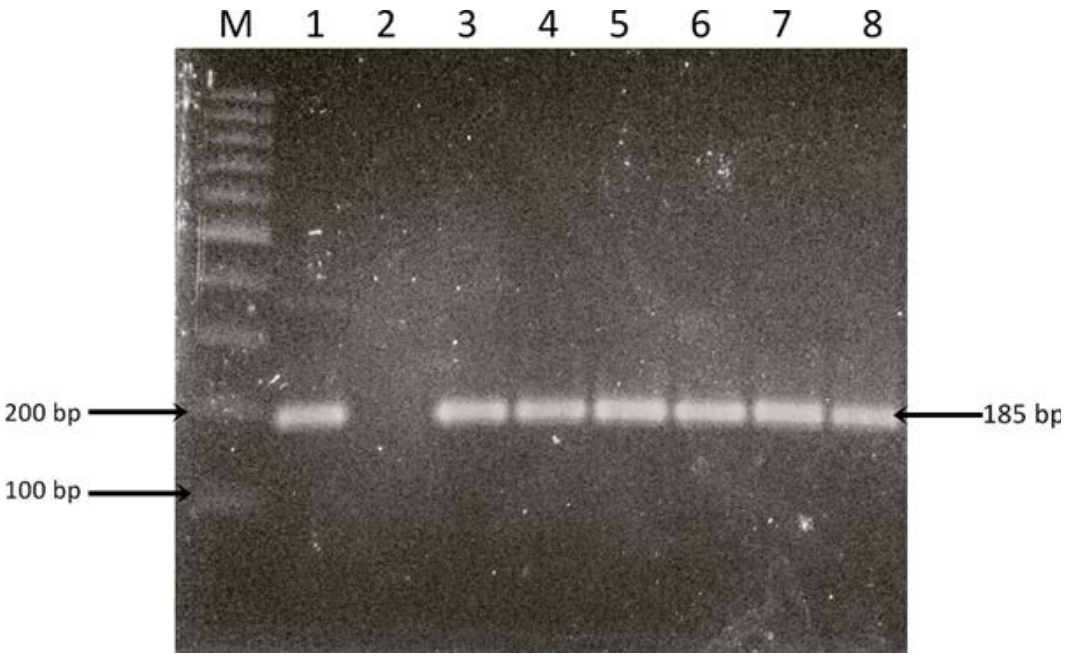

Figure 1. A representative image of agarose gel electrophoresis for MPN-PCR results. Lane M: 100 bp molecular ladder (Thermo Scientific, United States), Lane 1: *Bacillus cereus* positive control (185 bp), Lane 2: Negative control, Lane 3: Padi Rejat, Lane 4: Padi Pandan, Lane 5: Padi Wangi, Lane 6: Padi Arang, Lane 7: Padi Celum, Lane 8: Padi Mamut

The microbial load of *B. cereus* in tested samples was enumerated using MPN method and found to be more than 1100 MPN/g. This is in tandem to the finding achieved by Bilung *et al.* (2016) that obtained more than 1100 MPN/g for all *B. cereus* positive of local Sarawak and imported rice grains. At least 10^4 cells per gram of the bacteria can potentially cause emetic and diarrhoeal syndromes (Granum & Lund, 1997), thus all samples of this study can be regarded as unsafe for consumption if no killing processes are not performed. At industry level, such contamination can be reduced if proper treatments are carried out. For instance, milling removes husk and polishes rice grains and at the same time reduces the number of bacteria adhered. This reduction pattern is observable from a previous study by Kim *et al.* (2014), unhusked (rough) rice had the highest number of *B. cereus* which was 96.8% among brown rice and white rice, which were 80.6% and 57.8% respectively after milling. It was also noted by Sarrías *et al.* (2002) that husked (brown) rice has a lower prevalence of *B. cereus* than unhusked (rough) rice.

The results of PCR analysis showed that 100% (24/24) of the local unhusked (rough) rice were positive with *B. cereus.* Previous study carried out by Kim *et al.* (2014) showed that 96.8% (61/63) of rough rice samples and 57.8% (37/64) of the white rice were contaminated with B. cereus spores. Another study by Bilung *et al.* (2016) reported that 85% (17/20) of local indigenous and 100% (20/20) of imported rice were positive with *B. cereus.* In the study of Sandra et al. (2012), all raw rice samples (100%; 25/25) were positive with *B. cereus.*

However, infection is not solely subjected to consumption of the tainted food. According to Van Netten and Kramer (1992), *Bacillus cereus* can produce toxins in some desired conditions such as pH, water activity and storage temperature. It thrives on food matrix with pH 5-9, higher than 0.94 water activity

and a storage temperature of 10 – 45°C. Contamination on rice grains can be originated from various sources or occasion such as during rice production, harvesting, processing and handling where rice paddy or grains have contact with soil and irrigation water (Choi *et al.,* 2014; Haque & Russell, 2005; Laca *et al.,* 2006). Seemingly, elimination is almost impossible at cultivation sectors or marketplaces because of the widespread distribution in environment. Therefore, inactivation taken by consumers is a critical point that includes several practices such as storing rice in a dry storage (i.e. 12% - 14% of moisture content) that is unsuitable for the bacteria growth, eating rice promptly after cooking, keeping rice below 7ºC or higher than 63ºC within 2 hours upon cooking. Reheating rice can kill the vegetative cells but not cereulide, a heat-resistant enterotoxin (Haque & Russell, 2005). A study reported that the bacteria can be inactivated at 50ºC for 33.2 min, or 60ºC for 1 min, however the spores can be inactivated at 85ºC and 95ºC for 29.5 min and 2 min, respectively (Byrne *et al.,* 2006). However, these temperatures are extrapolation based on a specific *B. cereus* strain used and done on meat by the authors, which may vary in real incidences.

Conclusion

High concentration of *B. cereus* in all samples from this study prompts profound actions of controlling or reducing the bacterial contamination especially during cultivation field and paddy processing. The finding also displayed potential of *B. cereus* contaminating processed rice, thus poses risks to humans if no adequate inactivation is taken before consumption or from inappropriate handling. Further studies can be focused on survivability of *B. cereus* toxigenic endospores at different agricultural processing.

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