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Environment contaminant of *Bacillus cereus* isolated from ready to eat meat curry collected at various locations in Malaysia

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Abstract

The aim of the study was to isolate and identify *Bacillus cereus* from meat curry and to subtype the isolated B. cereus using RAPD-PCR and antibiotic resistance pattern. Ready to eat (RTE) meat curry samples purchased from 12 different restaurants at Kajang, Serdang and KL Sentral regions located in Selangor and Kuala Lumpur, Malaysia. Twenty-four isolates biochemically identified as *B. cereus*. Antimicrobial resistance analysis demonstrated that *B. cereus* isolates were highly resistance to ceftriaxone (100%), vancomycin (87.5%), clindamycin (91.6%) and nalidixic acid (100%). None of the *B. cereus* isolates were resistance towards ciprofloxacin (100%), streptomycin (91.6%) and chloramphenicol (83.4%). The *B. cereus* isolates were examined for randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) using primer S30 (5'-GTGATCGCAG-3') and discriminated into nine profiles. The antimicrobial analysis showed seven patterns and phenotypically less heterogeneous when compared to RAPD-PCR. A total number of nineteen types of *B. cereus* have produced by a combination of phenotype and genotype methods. These results demonstrated that both typing method provides evidence of the presence of similarity and diversity of the *B. cereus* strains from RTE meat curry.

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Introduction

The situation of foodborne ailments is a widely recognised problem that involves a broad spectrum of ailments instigated by parasitic, bacterial, viral or chemical contamination of food. While viruses are responsible for half of all the foodborne diseases, most hospitalisations and deaths related to foodborne illnesses are because of bacterial agents. Diarrheal ailments are the common signs of food poisoning, which can sometimes cause death. The sicknesses occur either due to toxins from disease-stimulating microbes or by the body's reaction to the bacteria (Teplitski *et al*., 2009). *B. cereus* acknowledged as a foodborne pathogen, is a spoilage microorganism which has linked to the evolution of quality flaws. The microorganism can trigger two kinds of foodborne infections – vomiting and diarrhoea. Symptoms are usually gentle and fleeting (up to 24 hours). *B. cereus* can often observe in different environments, such as soil, and various kinds of foods. Spores can survive even in severe conditions such as normal cooking temperatures (Schoeni and Wong, 2005). Till date, no official data is available on the prevalence of *B. cereus* poisoning in Malaysia (Nor Nadiah *et al.*, 2011). A local report has however acknowledged the existence of the bacterium in foodstuffs (Lee et al., 2009). Thus, this study was conducted to evaluate the microbiological quality and scrutinise the phenotypic, and genotypic diversity of the *B. cereus* isolates from ready-to-eat meat curry offered in Kuala Lumpur and Selangor (Malaysia). The intent is to shed light on the intrinsic risk linked with these foodstuffs.

Material and Methods

Sample collection

A total number of 72 mutton meat curry samples purchased from 12 different restaurants at Kajang, Serdang, and KL Sentral. Samples collected at two altered times in March 2015. Group1 have the code K1-4, S1-4 and kl1-4 while group2 have the code K5-8, S5-8, and KL5-8. All samples were collected after 6 hours of cooking and were placed in clean, dry sterile bags and transported in the icebox to the laboratory for bacteriological analysis.

Sample preparation

The mutton meat curry sample was aseptically cut into smaller pieces using a sterile knife. Twenty-five gram of the food samples was homogenized by a stomacher in 225 ml of peptone water for 1 minute.

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Several dilutions were achieved (1×10^1 to 1×10^5) up to fivefold for each prepared by transferring 1 ml from stock homogenate to 9 ml of sterile peptone water, mixing well with vigorous shaking, carried out to obtain separated colony (Roberts and Greenwood, 2003).

Plating of presumptive Bacillus cereus

The spread-plate procedure done in a selective media mannitol-egg yolk-polymyxin (MYP) agar (Oxoid), and incubated at 30°C for 24 hours. The total counts of *B. cereus* are based on percentage of colonies tested that were presumptively positive toward *B. cereus*, and expressed as CFU/g of sample as follows N = C / V(n) × D, where C is the sum of colonies on all plates count, V is the volume applied to each plate, n is the number of plates and D is the dilution from count obtained. Five or more colonies of presumptive *B. cereus* were randomly selected from MYP agar and subcultured onto nutrient agar slant (Oxoid) and incubate for 18-24 hours at 30°C. The samples are store at 4°C until use for identification and confirmation (Roberts and Greenwood, 2003).

Identification and confirmation of Bacillus cereus

Characterization and identification of the samples achieved by initial morphological examination and biochemical characteristics including lecithinase production, Gram stains, catalase production test, nitrate reduction test, glucose fermentation test, lactose fermentation test, Hydrogen sulfide (H2S) production test, gas production test, motility test, and indole production test. Physiochemical identification carried out as described by BAM (Tomlinson and Tomlinson, 1992).

Antibiotic sensitivity test

Antimicrobial susceptibility was determin by the disc diffusion agar method (CLSI, 2006). The antimicrobial test discs namely Ceftriaxone 30 µg, Streptomycin 25 µg, Chloramphenicol 30 µg, Ciprofloxacin 5 µg, Vancomycin 30 µg, Clindamycin 2 µg and Nalidixic acid 30 µg (Himedia) placed aseptically, and the plates incubated at 37°C for 14-19 hours. The zones were measured as follow, ≥15 sensitive and ≤14 resistance according to the standard methods (CLSI, 2006).

DNA extraction

DNA extraction was done using boiling method (Sahilah *et al.*, 2010) and Promega Wizard Genomic DNA purification kit (Promega, USA).

Primer

The random primer has the 50% G+C content gene sequence (10-mer), the S30 (5'-GTGATCGCAG-3') (First Base Malaysia) was selected for the study as it provides a reproducible and discriminatory pattern (Lee *et al*., 2011).

RAPD-PCR amplification

The RAPD-PCR fingerprinting assay was performed in a 25 µl volume each tube contained GoTaq Green Master Mix (First Base Malaysia) (2.5 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.1) and 0.1% Triton™X-100), 1.5 µl 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.4 µl of 5 units of Taq DNA polymerase) and 1.0 µl of 100 mM primer (S30), 6.5 µl water nuclease-free and 5 µl of 10 ng DNA template. A negative-DNA control was performed by adding one µl of sterile ultrapure deionized water (Sahilah *et al*., 2010). Amplification was conducted in Eppendorf Master-cycler nexus GSX1 (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 5 minutes followed by 40 cycles of denaturation at 94°C for 15 second, annealing for 15 second at 36.5°C and polymerization at 72°C for 2 minutes. Final elongation was at 72°C for 4 minutes (Lee *et al*., 2011).

Separating DNA fragments by agarose gel electrophoresis

The amplification products were analyzed by electrophoresis in a 2% agarose in 1.0 X TAE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 100 V for 45 minutes (Lee *et al.*, 2011). Gels stained with maestro-safe nucleic acid pre-stained (2 µl in 100 ml TBE). The amplified fragments were visualized digitally by using UV-trans illuminator (SYNGENE G-Box). The size of amplification products was determined using a100 bp (plus) DNA Ladder (First Base Malaysia).

Results and Discussion

The ostensible *B. cereus* colonies showed up as a dry-rough surface, red-purple in colour and encircled by white precipitated egg yolk on MYP Agar. Table 1 depicts the sum of the mean count (CFU/g) and the standard deviation of presumptive *B. cereus* for all samples. The microbe exhibited a considerably higher level of contamination at 30°C, and that means they are increasingly menacing to humans. This remarkable increase in bacterial numbers in Kuala Lumpur Sentral and Serdang in comparison to Kajang may be because of poor processing methods or sanitation practices. In a comparison between

Table 1. Isolation and biochemical characterization of *Bacillus cereus*

	Mo	Lec	Gr	Cat	Ind	Nit	Sta	Glu	Suc	Lac	H_2S	Gas	Mt
K1	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K2	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K3	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K4	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K5	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K6	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K7	Rod	+	+	+	–	+	+	+	–	–	–	–	+
K8	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S1	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S2	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S3	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S4	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S5	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S6	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S7	Rod	+	+	+	–	+	+	+	–	–	–	–	+
S8	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI1	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI2	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI3	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI4	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI5	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI6	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI7	Rod	+	+	+	–	+	+	+	–	–	–	–	+
KI8	Rod	+	+	+	–	+	+	+	–	–	–	–	+

(Mo); Morphology (Lec); Lecithinase (Gr); Gram-stains (Cat); Catalase (Ind); Indole (Nit); Nitrate reduction (Sta); Starch (Glu); Glucose (Suc); Sucrose (Lac); Lactose (H2S); Hydrogen sulfide production (Gas); Gas production (Mt); motility

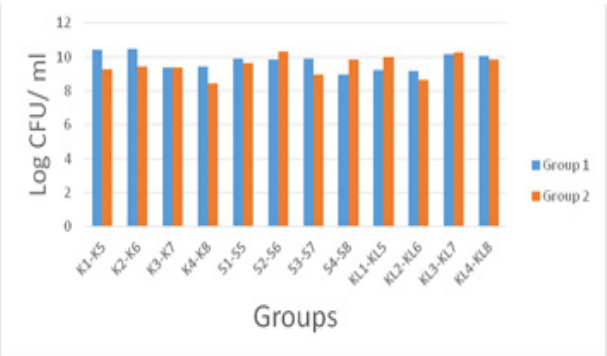

Figure 1. Comparison between two group (group1and group2) using t-test (paired) based on 95% confidence

group 1 and group 2, the T-test (paired) was utilised to construe the result by 95% confidence. As can be seen in Figure 1 there is no noteworthy (p-value = 0.277) difference between the two groups for Kajang restaurants; likewise, there is no notable (p-value = 0.623) difference between the two groups for Serdang restaurants. There is no significant (p-value = 0.258) difference between group 1 and group 2.

Corroboration of Bacillus cereus

The presumptive *B. cereus* colonies' biochemical attributes were ascertained and compared with the ones outlined in BAM (Tomlinson and Tomlinson, 1992). A total of 24 isolates demonstrated consistent phenotypic properties and substantiated as *B. cereus* (Table 1).

Antimicrobial resistance pattern

Table 2 shows the zone of inhibition of various antibiotics utilised of *B. cereus* isolates. All *B. cereus* isolates showed resistance to vancomycin (87.5%), ceftriaxone (100%), Clindamycin (91.6%) and nalidixic acid (100%). The isolates were susceptible to ciprofloxacin (100%). Furthermore, most *B. cereus* sensitive to chloramphenicol (83.4%) and streptomycin (91.6%). The observations of this study match those of other researchers (Guven *et al*., 2006; Vijaya Kumar *et al*., 2012; Agwa *et al*., 2012), excluding the samples which were injected with vancomycin as they were remarkably resistant towards the isolated strains. The percentage variations might be because of the differences in the concentration of the antimicrobial agents.

Random augmentation of polymorphic DNA through PCR

The DNA concentration range of all isolates was ≥150 ng/ul, as denoted by the Maestro Nano spectrophotometer. A UV trans-illuminator was used to visualise the isolates' RAPD-PCR fingerprints (SYNGENE G-Box) (Figure 2). In the current study, 62.5% of these isolates could produce a decent number of polymorphic bands. These bands positions differed between the isolates. Around 32.5% of the isolates were not able to distinguish the bacterial cultures with the same primer, because the RAPD-

Table 2. Zone of inhibition of different antibiotics used

	Cro	C	Cip	Da	S	Na	Va
K1	R	S	S	R	R	R	R
K2	R	S	S	S	S	R	R
K3	R	S	S	S	S	R	S
K4	R	S	S	R	S	R	R
K5	R	S	S	R	S	R	R
K6	R	S	S	R	S	R	R
K7	R	S	S	R	S	R	R
K8	R	S	S	R	S	R	R
S1	R	S	S	R	S	R	S
S2	R	S	S	R	R	R	R
S3	R	S	S	R	S	R	R
S4	R	S	S	R	S	R	R
S5	R	R	S	R	S	R	R
S6	R	R	S	R	S	R	R
S7	R	S	S	R	S	R	R
S8	R	S	S	R	S	R	R
KI.1	R	S	S	R	S	R	R
KI.2	R	S	S	R	S	R	R
KI.3	R	S	S	R	S	R	R
KI.4	R	S	S	R	S	R	R
KI.5	R	S	S	R	S	R	R
KI.6	R	S	S	R	S	R	R
KI.7	R	R	S	R	S	R	S
KI.8	R	R	S	R	S	R	R

R: resistance ≤ 14 mm
S: sensitive ≥ 15 mm
Tested for Ceftriaxone (Cro); Streptomycin (S); Chloramphenicol (C); Ciprofloxacin (Cip); Vancomycin (Va); Clindamycin (Da); and Nalidixic acid (Na).

PCR offer tiny or nil amplified DNA band. Such weak reactions elucidated by certain genetic dissimilarity in the target sequence of the primer in these genes. Bands are exhibiting similar migration distance deemed as a profile. The RAPD-PCR along with the primer S30 pattern proves the presence of likeness and variety of the *B. cereus* strains. Earlier research utilised the same primer S30 wherein all the *B. cereus* strains assessed spawned two bands – 0.91 kb and 0.5 kb. The latter is an inner part of ytcP, and an important marker for bacilli, while the 0.91 kb band appears beneficial as a *B. cereus* species-specific marker (Lee *et al*., 2011). The RAPD evaluations performed with primer S30 led to nine diverse profiles (P1, P2, P3, P4, P5, P6, P7, P8, and P9) comprising numbers of bands with same molecular weight. Antibiotic resistance delivers seven patterns (R1, R2, R3, R4, R5, R6 and R7) and the R signifies a set of antibiotics which are resistant to the strains. The outcome shows that the RAPD-PCR showed greater variety than antibiotic resistance patterns. Through an amalgamation of two different methods, 19 kinds of *B. cereus* have been identified (Table 3).

Table 3. Typing among RTE meat curry isolates of *B. cereus* using antibiotic resistance patterns and RAPD-PCR profiles

Strains no.	Antibiotic resistance patterns and their groups		RAPD-PCR profiles	*Bacillus cereus* types
K1	CroCDaNaSVa	R1	N	1
K2	CroNaVa	R2	N	2
K3	CroDaNaVa	R3	P1	3
K4	CroDaNaSVa	R4	P2	4
S1	CroCNaVa	R5	N	5
S2	CroCDaNaSVa	R1	P3	6
S3	CroDaNaVa	R3	P4	7
S4	CroDaNaSVa	R4	P5	8
KI1	CroDaNaVa	R3	N	9
KI2	CroDaNaVa	R3	N	9
KI3	CroDaNaVa	R3	P6	10
KI4	CroDaNaVa	R3	P5	11
K5	CroCDaNaVa	R6	P5	12
K6	CroDaNa	R7	P7	13
K7	CroDaNaVa	R3	P2	14
K8	CroCDaNaSVa	R1	P2	15
S5	CroCDaNaVa	R6	P2	16
S6	CroDaNaVa	R3	P8	17
S7	CroDaNaVa	R3	N	9
S8	CroDaNaVa	R3	N	9
KI5	CroCDaNaVa	R6	P3	18
KI6	CroDaNaVa	R3	P9	19
KI7	CroDaNaVa	R3	N	9
KI8	CroDaNaVa	R3	N	9
Total		7	9	19

R; Group of antibiotic that are resistance by the strains.
P; Number of profiles with same molecular weight
N; No patterns

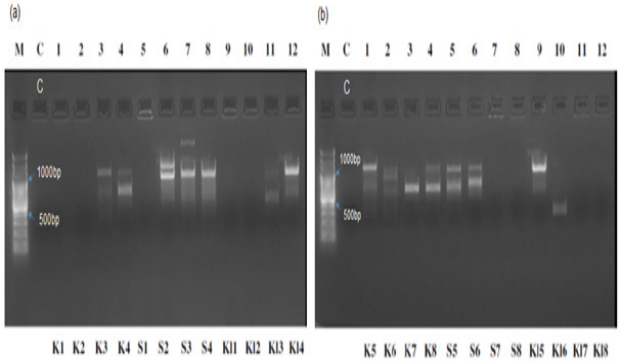

Figure 2. RAPD-PCR profiles of B. cereus (group1 and 2)
M, DNA ladder of (100-3000) bp. C, positive control (ATCC11778)
Lane (1-4), Kajang isolates. (Lane 5-8), Serdang isolates. (Lane 9-12), Kl Sentral isolates.

Conclusion

This study provides an overview of distribution and presence of *B. cereus* in meat curry. RAPD-PCR fingerprinting from these isolates can be used to obtain differentiation among strains. Comprehensive studies on this microorganism in sufficient numbers are necessary for the future. It is important to educate

food handlers about their responsibilities for food safety and train them on personal hygiene policies and essential practices for safe food handlings. Ensuring good quality raw materials, adequate lethality treatment, and effective sanitation of both the equipment and processing environment are crucial in preventing contamination of RTE meats.

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