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Genetic diversity of *Bacillus cereus* isolated from fried rice

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Abstract : Thirty five (n=35) genomic DNA of *Bacillus cereus* among fried rice isolates were determined their genetic diversity using plasmid profiling and arbitrarily primed polymerase chain reaction (AP-PCR) analysis. The *B. cereus* isolates, were isolated from 3 locations; Kajang (17), Bangi (11) and UKM's cafeteria (7) from August 2013 to July 2014. Plasmid profiling revealed 2 patterns either consist 23.kb or 23 Kb and 3.6 kb of plasmid, indicated that plasmid patterns were less heterogeneous when compared to AP-PCR analysis. Genetic fingerprinting of 35 *B. cereus* isolates was examined by AP-PCR analysis using AP1, AP2 and AP3 primers. The results of the AP-PCR were analyzed using Gel ComparIIsoftware to form dendrogram of *B. cereus* phylogeny. Dendrogram AP-PCR with primer AP1 discriminated the *B. cereus* isolates into 5 clusters and 4 single isolates, AP2 primers into 3 clusters and 3 single isolates and AP13 primers into 5 clusters and 3 single isolates at 70% similarity level examined. Results in the presence study demonstrated a wide heterogeneity among rice fried isolates of *B. cereus*.
Keywords : Genetic diversity, plasmid, *Bacillus cereus*, fried rice, AP-PCR analysis.

Introduction

Food poisoning cause by *Bacillus cereus* can cause either diarrhea or emetic. This Gram-positive and spore-forming bacteria is commonly found in soils and variety of dried foods such as grains, spices, starches and legumes[1,2]. They are also found in other food product such as raw meat, processed foods, wet wheat noddle, vegetables, seafoods, dairy, bakery products and fried rice [1, 3, 4].

In Malaysia, the first outbreak due to *B. cereus* was reported in year 1984 which affected 114 Malay students staying at a hostel in Klang secondary School[5]. Since then, most cases food poisoning outbreaks was reported from starchy foods such noodles, *nasilemak* (rice cooked with coconut milk) and *nasibriyani* (rice cooked in spices)[6]. No outbreaks of food poisoning *B. cereus* due to fried rice in Malaysia. The *B. cereus* was survived and grew in boiling rice before serving as fried rice due to the storage temperature were range from 4-55^{o}C, spores germinate and regeneration of vegetative [7].

In this study, we examined the genetic diversity among *B. cereus* fried rice isolates using plasmid profiling and arbitrarily primed polymerase chain reaction (AP-PCR) analysis. All *B. cereus* isolates were

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analyzed for antibiotic resistance and enterotoxin genes[4,8]. Strains differentiation of *B. cereus* is important in surveillance of possible public health risk for predictive value in epidemiological control. It also provides data in controlling disease outbreaks and determining their sources. Besides that, it also helpful in monitoring of the virulent strains.

Materials and methods

Bacillus cereus

A total of 35 (n=35) *Bacillus cereus* isolates were from Laboratory of Food Sciences, Universiti Kebangsaan Malaysia, Selangor. *B. cereus* isolates BC1-BC17 were from Kajang; BC18-BC28 were from Bangi; and isolates BC29-BC35 were from UKM cafeteria, Selangor. All *B. cereus* isolates were isolated in August 2013 to July 2014.

Plasmid profiling

An active colony of *B. cereus* culture was grown in into 250 ml of Luria Broth (LB) media for 20 hours at 30°C with shaking at 225 rpm. A total of 1 ml overnight culture was transferred into 1.5 ml centrifuge tube and spun down for 1 min at 13,000 rpm via a benchtop centrifuge (Minispin, Eppendorf). The DNA Plasmid extraction was done using Plasmid Maxiprep kit (Promega, MY) as described in the manufacturer's manual.

DNA extraction and AP-PCR analysis

A colony of *B. cereus* were grown in 10 ml of Nutrient broth (NB) at 30 °C for 20 hours. The cells were spun down at 13,000 rpm for 3 min.Bacteria DNA was extracted using DNA kit (Promega, USA). The primerswere AP1 (5'-CCGAGTCCA-3'), AP2 (5'-CCGGCGGCG-3')and AP13 (5'- GAGGGTGGCGGCTCT-3')[9, 10]. The AP-PCR was performed according to the method describe by [9].The assay was performed in a 25 µl volume containing GoTaq green master mix (1st Base, MY) 12.5 µl and 1.0 µl of 100 mM primer (AP1, AP2 and AP13),6.5 µl water nuclease-free and 5 µl of 10 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water.A temperature program for amplification was set; initial denaturation at 94 °C for 4 min followed by 38 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 50 °C and elongation at 72 °C for 2 min,in Eppendorf thermal-cycler (Eppendorf, Germany). Final elongation was at 72 °C for 5 min. The amplicons were analyzed on 1.5% (w/v) agarose gel in 1 X TAE buffer (40 mMTris-OH, 20 mM acetic acid and 1mM of EDTA; pH 7.6) at 100 V for 40 min and MaestroSafeTM dye was used to stain the gel. The marker was1 kb DNA ladder (Fermentas, Lithuania) and visualized using Gel Documentation System (Syngene, USA).

Data analysis

The agarose gel photos were analyzed using Gel ComparII image analysis software (Applied Math, Kortjik, Belgium). The band matching coefficient of Dice was used to form the dendrogram.

Results and Discussion

The outbreaks of food poisoning due to *B. cereus* are quite serious in Malaysia especially it implicates the school children and secondary students staying in the Hostel. Most cases of*B. cereus* food poisoning was reported from starchy foods such noodles, *nasilemak* and *nasibriyani*[6]. The prevalence of *B. cereus* in various samples of was reported by several researchers [3, 8, 11]. In this study, we further examined our *B. cereus* isolates using plasmid and AP-PCR analysis. Bacterial subtyping is important in the studying of epidemiology of infectious and foodborne diseases. It provide of great value of epidemiologic investigations where typing of bacterial isolates would allow differentiation below the species level. It also help to determine the virulent isolates previously recognized in a set of isolates.

All *B. cereus* isolates were harboredplasmids with 23 kb molecular weight in size. Two *B. cereus* isolates (BC1 and BC27) were harboring2 plasmids with 23 kb and 3.6 kb molecular in size (Fig. 1 and 2). The plasmids profiling was not showed heterogeneity of *B. cereus* isolates when compared to AP-PCR fingerprinting. The presence of plasmids hasbeen reported in many bacteria [12]. Plasmids are autonomousself-

extrachromosomal DNA element. They encoded varies of genes and functions such as fertility, resistance to antibiotic, resistance to heavy metals, production of bacteriocin and etc. Plasmids can serve as markers of various bacterial strains when plasmid profiling is used as typing system. Several studies have demonstrated that plasmid profile analysis could be reliable as epidemiologic tool for the differentiation of epidemic and non-epidemic bacteria isolates outbreaks [13]. Our finding was in contrast with other researchers who reported plasmid profiles of *B. cereus* can be a useful epidemiological marker for *B. cereus* and they found 72% (113/156) strains of *B. cereus* isolates carried plasmids with different patterns[14].We could not find any specific plasmid profiling though the *B. cereus* was isolated from three different geographical locations due to less heterogeneity. While in term of plasmid size, the large plasmid observed in this study was 23 kb in size. This finding was in contrast when compared to 15 kb molecular weight in size in *B. cereus* isolates[14]. The largest plasmid in *B. cereus* was reported of 350 kb in size[15]. The different of plasmids profiling and size may probably due to plasmids distributed across a variety of environment and different geographical location from where the *B. cereus* isolated. Thus, plasmid profiling is less useful to differentiate the *B. cereus* among fried rice isolates.

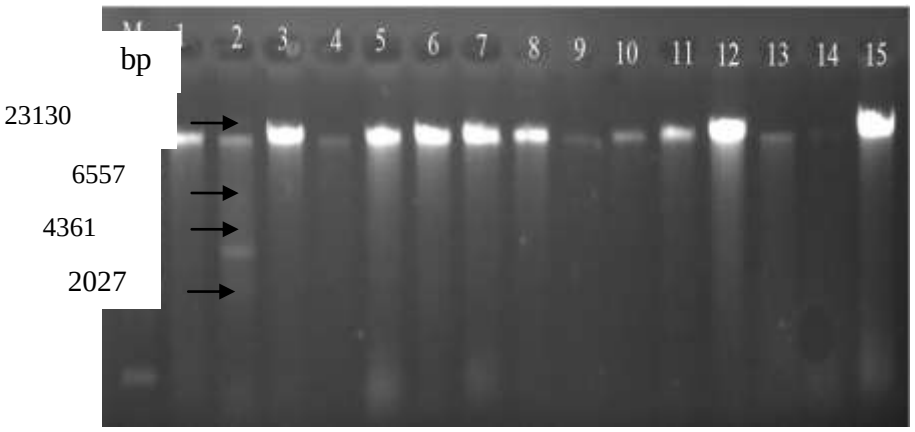

Figure 1Plasmid DNA isolated from *Bacillus cereus* strains (BC1-BC15). M: 23 kb λ Hind (*Lambda-Hindlll Digest*); Lane 1: Positive control of *B. cereus(*ATTC 11778); Lane 2-15: *B. cereus* isolates B1-B15.

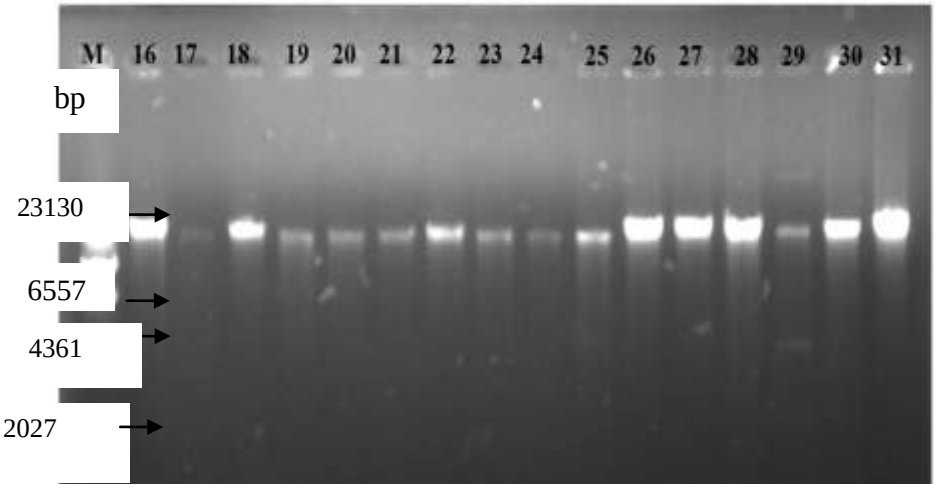

Figure 2 Plasmid DNA isolated from *Bacillus cereus* strains (BC 17-BC31). M: 23 kb λ Hind (*Lambda-Hindlll* Digest); Lane 16: Positive control of *B. cereus*(ATTC 11778). Lane 17-30: *B. cereus* isolates B16-B31.

AP-PCR fingerprinting was applied in the present study to subtype the *B. cereus* isolates isolated from fried rice samples. Subtyping would estimate their intraspecies diversity. Three primers were used in the AP-PCR analysis. AP-PCR with primer AP1 discriminated the *B. cereus* isolates into 35 AP-PCR fingerprinting. While, using dendrogram analysis it can be differentiated into 5 clusters and 4 single isolates. For AP2 primer, it could discriminate the *B. cereus* isolates into 31 AP-PCR fingerprinting and can be group 3 clusters and 3

single isolates. While, RAPD13 primers produced 32 AP-PCR fingerprinting and can be differentiated into 5 clusters and 3 single isolates at 70% similarity level examined. Results in the presence study demonstrated the AP-PCR analysis show a wide heterogeneity among rice fried isolates of *B. cereus* (Table 1, Fig. 3, 4 and 5).Our finding was consistent with other researchers who reported the AP-PCR analysis revealed large degree of strain-to-strain heterogeneity in *B. cereus*[9]. In their study, *B. cereus* and *Bacillus thuringiensis* have large degree of heterogeneity, while *Bacillus anthracis* completely homogeneous indicating a clonal lineage indicated distinct to other group of *Bacillus* and stand as a species on its own. The AP-PCR analysis has been use for subtyping many bacteria because of its reproducibility and no detailed knowledge of the genomic sequences needed. In the present study, all AP primers have been shown able to type the *B. cereus* isolate except for *B. cereus* isolate BC3 using primer AP1. This is probably due to it lost specific site on its genome, this result was consistent when repeated experiments were conducted. Combination between plasmid profiling and AP-PCR analysis produce 35 genome types.

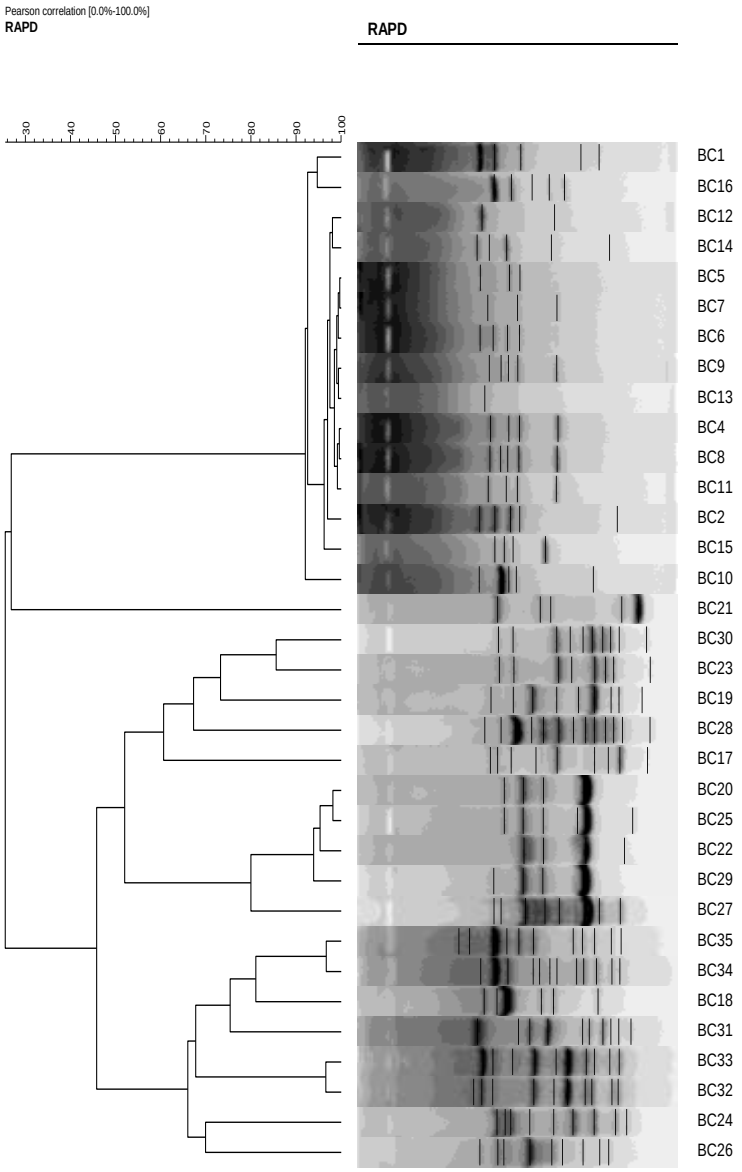

Figure3Dendrogram of typable *Bacillus cereus* isolates produced from AP-PCR analysis using AP1 primer

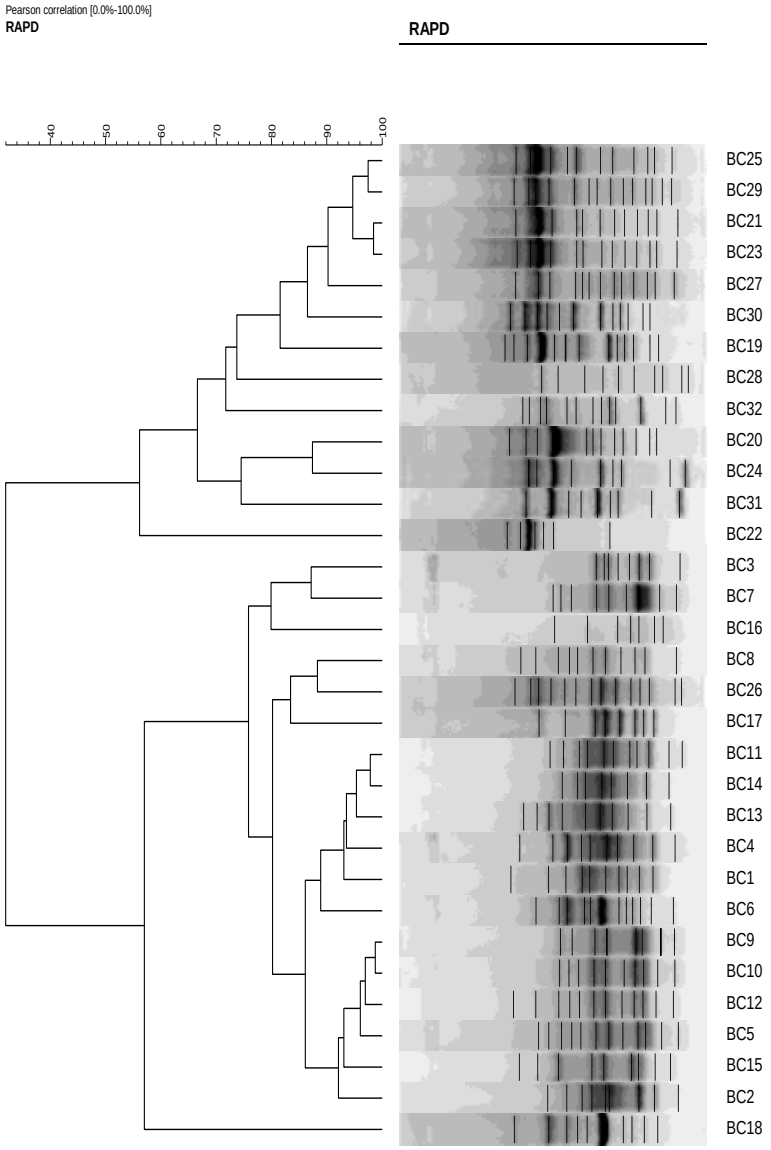

Figure 4 Dendrogram of typable Bacillus cereus isolates produced from AP-PCR analysis using AP2primer

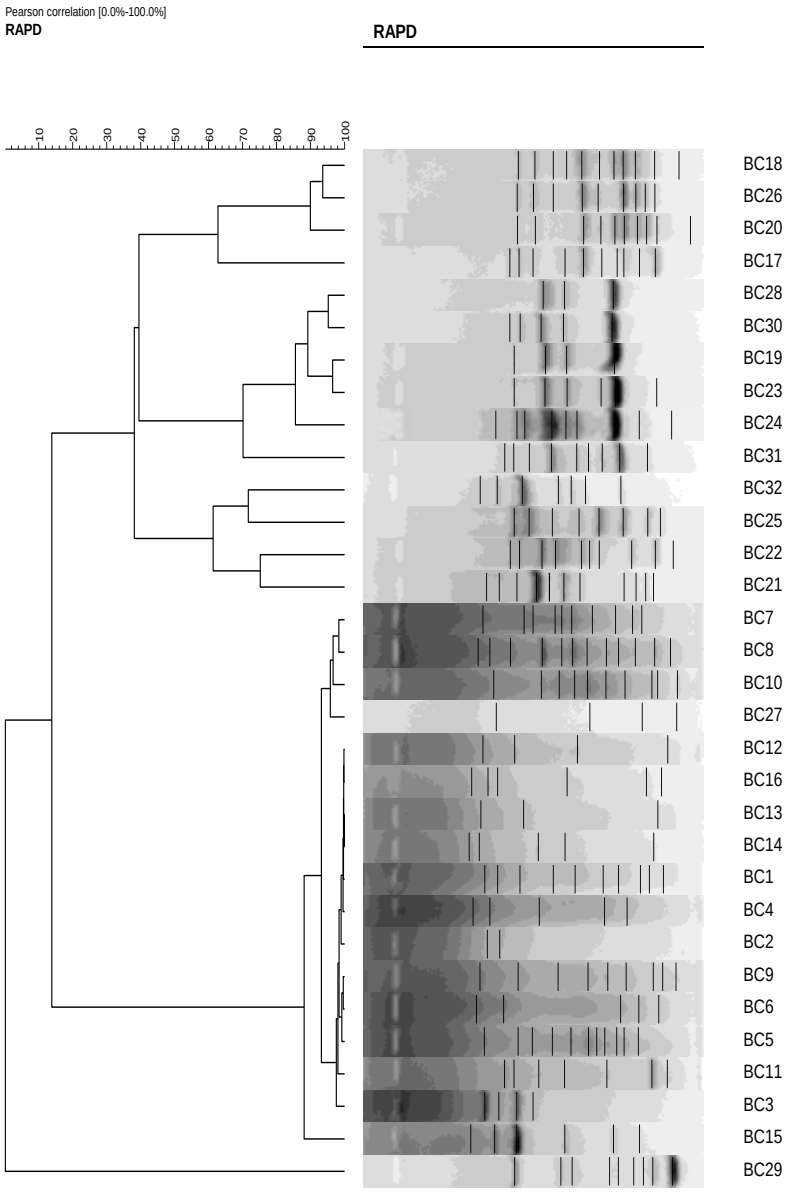

Figure 5: Dendrogram of typable *Bacillus cereus* isolates produced from AP-PCR analysis using AP13 primer

Table 1 Genotyping of plasmid profile and arbitrarily primed polymerase chain reaction (AP-PCR) analysis of *Bacillus cereus* isolated from fried rice samples.

Isolates no.[a]	Plasmid size (patterns)[b] (kb)	AP fingerprinting using different primers			Genome types[c]
		AP1	AP2	AP13	
BC1	23, 3.6 (Q1)	R1	S1	T1	1
BC2	23 (Q2)	R2	S2	T2	2
BC3	23 (Q2)	ND	S3	T3	3
BC4	23 (Q2)	R3	S4	T4	4
BC5	23 (Q2)	R4	S5	T5	5
BC6	23 (Q2)	R5	S6	T6	6
BC7	23 (Q2)	R6	S7	T7	7
BC8	23 (Q2)	R7	S8	T8	8
BC9	23 (Q2)	R8	S9	T9	9
BC10	23 (Q2)	R9	S10	T10	10
BC11	23 (Q2)	R10	S11	T11	11
BC12	23 (Q2)	R11	S12	T12	12
BC13	23 (Q2)	R12	S13	T13	13
BC14	23 (Q2)	R13	S14	T14	14
BC15	23 (Q2)	R14	S15	T15	15
BC16	23 (Q2)	R15	S16	T16	16
BC17	23 (Q2)	R16	S17	T17	17
BC18	23 (Q2)	R17	S18	T18	18
BC19	23 (Q2)	R18	S19	T19	19
BC20	23 (Q2)	R19	S20	T20	20
BC21	23 (Q2)	R20	S21	T21	21
BC22	23 (Q2)	R21	S22	T22	22
BC23	23 (Q2)	R22	S23	T23	23
BC24	23 (Q2)	R23	S24	T24	24
BC25	23 (Q2)	R24	S25	T25	25
BC26	23 (Q2)	R25	S26	T26	26
BC27	23 (Q2)	R26	S27	T27	27
BC28	23 (Q2)	R27	S28	T28	28
BC29	23, 3.6 (Q1)	R28	S29	T29	29
BC30	23 (Q2)	R29	S30	T30	30
BC31	23 (Q2)	R30	S31	T31	31
BC32	23 (Q2)	R31	N	T32	32
BC33	23 (Q2)	R32	N	N	33
BC34	23 (Q2)	R33	N	N	34
BC35	23 (Q2)	R35	N	N	35
TOTAL	2	34	31	32	35

[a]*B. cereus* isolated from Kajang (BC 1-BC17); Bangi (BC 18-28); UKM (BC29-35).
[b] Plasmid size and patterns
[c] Combination within plasmid and AP-PCR profiles
N-Not determined; ND-Not detected

In conclusion, there is an evident AP-PCR analysis is useful and reliable for subtyping intraspecies discrimination of *B. cereus* isolates. It also exhibited high level of genetic diversity among fried rice of *B. cereus* isolates studied.

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