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ISOLATION OF *B. cereus* s.l. FROM DRIED HERBS AND SPICES PRODUCTS AND MILK PRODUCTS PURCHASED FROM LOCAL MARKETS IN NEGERI SEMBILAN

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Abstract

Bacillus cereus is a spore-forming bacterium that is widely distributed in the environment and can be detected in a variety of food including dried herbs and spices and milk products. When the vegetative form of the bacteria produces toxins, these can result in food borne illness and pose a significant public health hazard. However, no or less study in food health safety related to *B. cereus* has been done in Malaysia. This study aims to isolate *B. cereus* in dried herbs and spices and milk products using selective and differential Mannitol Egg-Yolk Polymyxin (MYP) agar and Gram staining. The presumptive colonies of *B. cereus* were selected based on mannitol and lecithinase utilization on MYP agar and showed Gram-positive rod-shaped bacteria by Gram staining. These presumptive *B. cereus* were then confirmed using 16S rRNA sequencing. For the dried herbs and spices, four out of ten samples were presumptive *B. cereus*, however only three were confirmed and identified as *B. cereus* s.l. From these three samples with positive *B. cereus* s.l., two samples were contained more than 10^4 CFU/g indicating the samples contain a high number of colonies that may result in food poisoning. Meanwhile, for the milk products, out of 12 samples, three samples showed presumptive *B. cereus* on MYP plates, however identified as non *B.cereus* using 16S rRNA sequencing. Thus, further isolation and identification of the isolated bacteria in a larger sample need to be carried out to provide the data prevalence of *B. cereus* in Malaysian dried products and milk products and to serve as extra information about the safety of consuming dried items that may cause harm to people.

Keywords: 16S rRNA sequencing, *Bacillus cereus*, food poisoning, isolation, Mannitol Egg-Yolk Polymyxin agar

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Introduction

Bacillus cereus is one of the major agents that causes food borne illness outbreak. *B. cereus* is a group of spores forming Gram-positive rods commonly found in soil and environments. The spores frequently contaminate a wide range of food products such as rice, cereals, meat products, vegetables, milk and its derivative products, spices and dried herbs (Berthold-Pluta et al., 2019; Gdoura-Ben Amor et al., 2018). In fact, spores can survive even in severe environments such as normal cooking temperature (Schoeni and Wong, 2005). Food poisoning by *B. cereus* can manifest itself as an emetic or diarrheal syndrome caused by the release of the toxin cereulide and proteinaceous enterotoxins respectively.

The *B. cereus* group (also known as *B. cereus* sensu lato (s.l.) is a subdivision of the *Bacillus* genus that consists of eight genetically closely related species: *B. cereus* (sensu stricto (s.s.)), *B. anthracis*, *B. mycoides*, *B. thuringiensis*, *B. pseudomycoides*, *B. cytotoxicus*, *B. toyonensis* and *B. weihenstephanensis*.

Since these *Bacillus* species are difficult to differentiate from each other, they are reflected in the term 'presumptive *B. cereus'* commonly used in standard procedures for the detection of *B. cereus* in food based on ISO 7932:2004 and ISO 21871:2006. The confirmatory test is insufficient to distinguish between the species, however some are less commonly found in environment namely *B. weihenstephanensis*, *B. anthracis*, *B. thuringiensis*, *B. mycoides* and *B. pseudomycoides*. Typical protocol steps include the dilution of the sample, plating on the *B. cereus* selective plate mannitol egg yolk polymyxin agar (MYP) or polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA), and further confirmation on beta-hemolytic property on sheep blood agar (Peterrz et al., 1985). MYP and PEMBA agar are selective on Gram-positive bacteria as the antibiotic polymyxin inhibits most Gram-negative bacteria. Besides, the utilization of mannitol in MYP and PEMBA agar makes this agar as differential agar. *B. cereus* does not form acid from mannitol as it is unable to utilize mannitol. Both MYP and PEMBA agar contain egg yolk to test for lecithinase activity, and *B. cereus* will cause a white precipitation zone around the colonies. Although MYP and PEMBA agar are supposed to be selective for *B. cereus*, the presence of background flora such as *Bacillus* species other than *B. cereus* and *Staphylococcus aureus* may mask the growth of *B. cereus* on the plate (Tallent et al., 2011). In order to confirm the bacterial species, 16S rRNA sequencing was done and the phylogenetic tree was constructed.

In Malaysia, the outbreak of *B. cereus* was first reported by Rampal et al. (1984) where 114 students were infected with *B. cereus* after eating fried noodle. There was an outbreak in the primary school in Sabah in 2012 which the contamination was found in 'nasi kuning' or turmeric rice (Mihat, 2017). Another outbreak due to contamination of *B. cereus* in milk was reported in a news that involved 191 students in 2011 due to a distribution of UHT milk in an event named "Program Susu 1Malaysia" (Ubong et al., 2011). As far, there were few studies conducted to investigate the prevalence of *B. cereus* in local food products. This study was intended to provide the current data on the presence of *B. cereus* group in dried herbs and spices and milk products purchased from local markets in Negeri Sembilan, Malaysia. In fact, no study has been conducted as yet to check the *B. cereus* contamination in dried herbs and spices in Malaysia.

Methods

Sample collection

A total of ten samples of dried herbs and spices products and 12 samples of milk products were randomly purchased from different local supermarkets in Negeri Sembilan, Malaysia. The dried products included turmeric powder, kurma powder, soup powder, curry powder, cumin powder, black pepper, parsley flakes and cinnamon powders purchased from three different supermarkets. For milk products, there were two samples of raw milk obtained from two different cattle farms, there different commercial local pasteurized milk, UHT milk, local made cheddar cheese, home-made yoghurt, home-made ice-cream and three milk powder of different brands. The expire dates were checked before buying them and quickly stored in a refrigerator until being processed.

Isolation of *Bacillus cereus* on MYP agar and Gram staining

For each food sample, 10g or 10 ml was weighed under sterile condition, homogenized by a Stomacher in 90ml of sterile peptone saline solution (Oxoid, Basingkon, England) at 230rpm for two minutes. Serial dilutions were prepared, and 0.1ml of each diluted sample was streaked onto Mannitol Yolk Polymyxin (MYP) agar medium (Oxoid, Basingstoke, England) in duplicates. Plates were incubated for 24 hours at 37°C. MYP agar medium was prepared based on egg yolk and mannitol supplementation, and made it selective due to the additional of Polymyxin B. MYP agar is a selective medium used to isolate vegetative cells of *B. cereus*. Typical colonies of *B. cereus* are bright-pink and uniform and surrounded by a zone of precipitation indicating lecithinase production. These colonies presumptively identified to be *B. cereus* until further observed by Gram staining and confirmed 16S rRNA sequencing.

The presumptive colonies for *B. cereus* were selected for Gram staining according to method described previously in Beveridge (1990). *B. cereus* appeared as a Gram positive and has a rod-shaped cell with short to long chains.

Extraction of bacterial DNA and 16S rRNA amplification by PCR

The presumptive *B. cereus* isolates were grown overnight at 37°C in 10ml of tryptic soy broth (Oxoid, Basingkon, England). Bacterial genomic DNA was then extracted using the PrimeWay Genomic DNA Extraction Kit (Apical Scientific, Malaysia) according to the manufacturer's instructions. Next, a Polymerase Chain Reaction (PCR) targeting bacterial 16S rRNA was carried out using exTEN 2X PCR Master Mix (Apical Scientific, Malaysia). The sequence for the forward and reverse primers used were 5' AGA GTT TGA TCC TGG CTC AG 3' and 5' AGG CCC GGG AAC GTA TTC AC 3' respectively at concentration of 600nM for both. Total PCR reaction was at 50 µl. The reaction temperature was 95℃ for 2 minutes, followed by 35 cycles of three-step reaction (95℃ for 30 seconds, 54℃ for 30 seconds, and 72℃ for 90 seconds) and a final extension of 10 minutes at 72℃. Agarose gel electrophoresis was used to visualise PCR results. 2.5% TAE agarose gels (Thermo Fisher Scientific) were used for gel electrophoresis, and DNA was visualised by adding 1X Invitrogen SYBR Safe DNA Gel Stain. The electrophoresis was carried out for 45-60 minutes at 80-90 V. Amplification of 16S rRNA from bacterial DNA results in a band product of around 1300 bp and samples were sent for sequencing (Apical Scientific Sdn Bhd, Malaysia).

BLAST analysis and phylogenetic tree

The sequences received were trimmed and verified for quality before exported to NCBI BLAST (Basic Local Alignment Search Tool) database to search for the similar sequences to confirm the bacterial species. The 16S rRNA sequences of the related bacteria (Table 1) were downloaded from the GenBank. Nucleotide sequence alignments were made using ClustalW and trimmed the alignments. Then, Bioedit 7 was used for refining the entire alignment. Maximum likelihood (ML)-based phylogenetic analyses were performed with MEGA 4 using default parameters (bootstrap=100). *B. weihenstephanensis* was not included in the phylogenetic tree as the 16S rRNA sequence is not yet available in the database upon this study was carried out.

Table 1. *B. cereus* group species and strains of each species from which 16S rRNA gene sequences were analysed and compared in this study

Bacillus cereus	***Bacillus thuringiensis***	***Bacillus anthracis***	***Bacillus mycoides***	***Bacillus cytotoxicus***	***Bacillus toyonensis***	***Bacillus pseudomycoides***
NBRC 15305	NBRC 101235	ATCC 14578	ATCC 6462	NVH 391-98	BCT-7112	NBRC 101232
CCM 2010	ATCC 10792	SB1	DSM 11821		P18	DSM 12442
ATCC 14579	IAM 12077					

Result and Discussion

Table 2 showed that out of 10 samples of dried products, five were presumed to contain *B. cereus* which were sample CA2 (cinnamon powder), CA3 (kurma powder), CA4 (soup powder), CA5 (curry powder) and CA6 (cumin powder). These five samples were mannitol negative, produce lecithinase and has Gram positive bacilli shaped which fulfill the characteristics of *B. cereus* s.l. (Table 2). It is notably important to take note, among *B. cereus* s.l., only *B. cereus*, *B. thuringiensis* and *B. weihenstephanesis* would have similar morphology on MYP plates and therefore were not able to discriminate at species level of these three species in *B. cereus* s.l. (Tallent et al., 2012). Meanwhile for the milk products, there were 3 samples that were recognised as presumptive *B. cereus* which were sample EL7 (unpasteurized raw milk from farm A), EL8 (pasteurized milk) and EL9 (unpasteurized raw milk from farm B) as shown in Table 2. These presumptive colonies were restreaked on new fresh MYP agar plates to obtain purer colonies.

Table 2. Description on colony morphology of isolated bacteria on MYP agar and Gram staining

Sample code	Sample description	Morphology on MYP plates	Mannitol utilisation	Lecithinase production	Gram staining	Presumptive *Bacillus spp*
DRIED HERBS AND SPICES PRODUCTS						
CA1	Tumeric powder	Circular, pink colonies	No	No	Gram positive bacilli	No
CA2	Cinnamon powder	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
CA3	Kurma Powder	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
CA4	Soup powder	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
CA5	Curry powder	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
CA6	Cumin powder	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
CA7	Black pepper	Raised large irregular yellow colonies.	Yes	Yes	Gram positive bacilli	No
CA8	Parsley flakes	Circular, yellow colonies	Yes	Yes	Gram positive bacilli	No
CA9	Cinnamon powder	Circular, yellow colonies	Yes	Yes	Gram positive, bacilli	No
CA10	Cinnamon powder in a sterilised bottle packaging	No colony growth	N/A	N/A	N/A	N/A
MILK PRODUCTS						
EL1	Milk powder	Circular, small, yellow colonies	Yes	No	Gram positive, cocci	No
EL2	Homeade cheddar	Circular, small, yellow colonies	Yes	No	Gram positive cocci	No
EL3	Pasteurised milk	Circular, small, yellow colonies	Yes	No	Gram positive, Cocci	No
EL4	UHT milk	No colony growth	N/A	N/A	N/A	N/A
EL5	Chocolate milk	Circular, small, yellow colonies	Yes	No	Gram positive, bacilli	No
EL6	Homemade natural yoghurt	Circular, small, yellow colonies	Yes	No	Gram positive, bacilli	N/A
EL7	Unpasteurised raw milk farm A	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
EL8	Pasteurised milk	Circular, pink colonies	No	Yes	Gram positive bacilli	Yes
EL9	Unpasteurised raw milk farm B	Circular, pink colonies	No	Yes	Gram positive, bacilli	Yes
EL10	Pasteurised goat milk	Circular, yellow colonies	Yes	No	Gram positive, bacilli	No
EL11	Milk powder	Circular, yellow colonies	Yes	No	Gram positive, bacilli	No
EL12	Goat milk powder	Circular, yellow colonies	Yes	No	Gram positive, cocci	No

Sequencing based on 16S ribosomal DNA gene (16S rRNA) gene offers several advantages over conventional biochemical methods in such a way it enables to discriminate between bacterial species or strains between phenotypically identical bacteria. 16S rRNA gene was found to be highly conserved in all bacteria. Due to its essential function in ribosomal assembly yet contains variable region that can be used as fingerprints for particular species making it a gold standard technique to be used in identification, comparison and phylogenetic classification of bacteria (Martinez-Porchas et al., 2017). This study utilized 16S rRNA gene sequencing to confirm the bacterial species for the presumptive *B. cereus* identified earlier in Table 2 which were sample CA2, CA3, CA4, CA5, CA6, EL7, EL8 and EL9.

PCR amplification of this gene region produced positive band at approximately 1300 base pairs as confirmed by electrophoresis analysis (Figure 1). For the dried herbs and spices samples, four out of five samples were successfully amplified. One of the samples (sample CA5) however was not amplified, which could be due to poor quality of extracted DNA or human error in pipetting. Besides, it could be suggested that the plant chemicals from the dried spices and herbs are likely to interfere with the PCR reaction mixture and the insoluble powdery matrices cause challenges with DNA extraction (Sajali et al., 2018). Therefore, sample CA5 was not proceeded for gene sequencing.

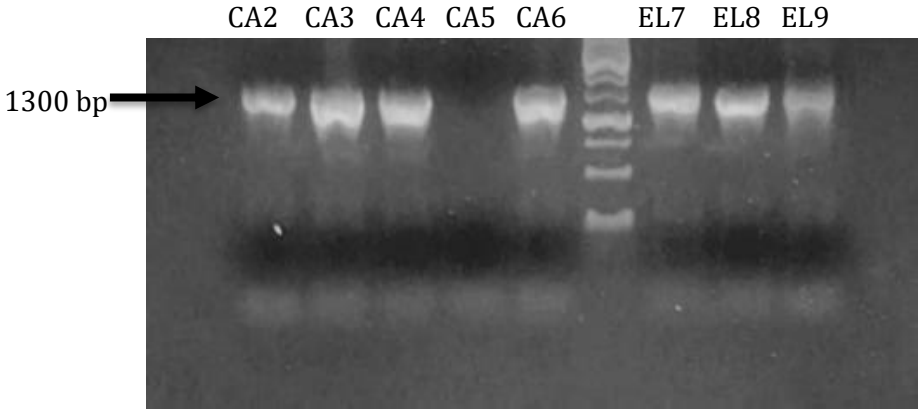

Figure 1. Gel electrophoresis of 16S rRNA amplification of the presumptive *B. cereus* in samples dried herbs and spices and milk products

The amplified PCR products were then sent for sequencing and the sequence data obtained were trimmed and verified for quality. The sequences were then imported into the BLAST database to search for the bacterial sequence similarities. According to BLAST analysis, only sample CA3, CA4 and CA6 were belonged to *B. cereus* s.l. (with 100% similarity), while sample CA2, EL7, EL8 and EL9 were identified as non-*B. cereus*. From this study, the prevalence of *B. cereus* in dried herbs and spices purchased from local market in Negeri Sembilan reported to be 30%. Two out of three samples (CA4 and CA6) containing *B. cereus* s.l. have more than 10^4 colony forming unit (CFU/g) (data not shown). Food Standards Australia New Zealand considers *B. cereus* concentrations of more than 10^4 CFU/g or CFU/ml to be unsafe for human consumption (Food Standards Australia New Zealand, 2001). This is the first reported study of isolation of *B. cereus* s.l. in dried herbs and spices in Malaysia. Fogele et al (2017) reported that *B. cereus* was found in 76% of spices and 24% in dried herbs from the local market in Latvia. In a study of nine food products in Poland, Berthold-Pluta et al. (2019a) discovered the prevalence of *B. cereus* in herbs and spices was at 63.3%. In Tunisia food products, *B. cereus*-group-like bacteria were identified in 28.9% of the spices (Gdoura-Ben Amor et al., 2018). These suggest that the use of *B. cereus*-containing spices as food additives or flavors may represent a risk in the case of inadequate heat treatment.

Despite the selectivity that have been imposed using MYP agar and Gram staining, the presumptive *B. cereus* (sample CA2, EL7, E8 and EL9) that based on the plating on MYP agar previously were confirmed as non-*B. cereus* by 16S rRNA sequencing. These non-*B. cereus* were ubiquitous bacteria that are commonly present in milk products and soil. It seems the microflora present outgrowth on the plate and less chance for the bacteria such as *B. cereus* to be recovered (Talahmeh et al., 2019). Study by Chon et al. (2012) suggested that supplementing MYP agar with antibiotic trimethoprim helps to inhibit competing flora and improve the selectivity for *B. cereus*. Streaking the presumptive colonies on blood agar could improve the selectivity as *B. cereus* appears as beta-hemolysis. Several additional biochemical tests such as catalase test, motility test and tyrosine decomposition test could be included for the bacterial isolation and characterization.

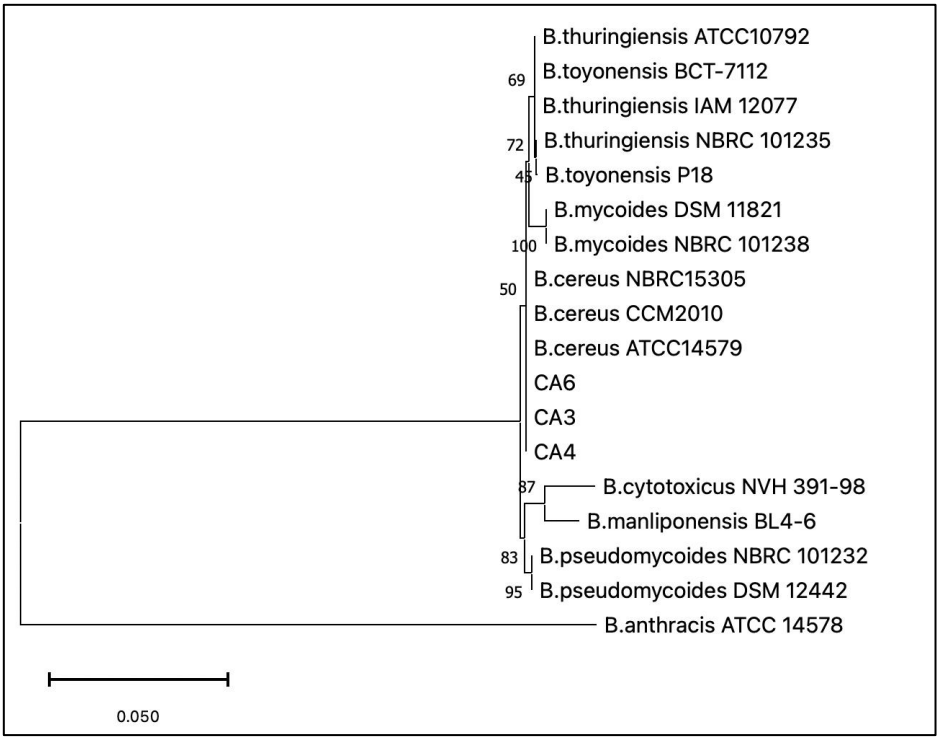

Figure 2. Phylogenetic tree of confirmed isolated *B. cereus* group (CA3, CA4 and CA6) in comparison with different species in *B. cereus* group from databank

The 16S rRNA sequencing and the phylogenetic tree (Figure 2) revealed that samples CA3, CA4 and CA6 were very closely related to *B. cereus* sensu stricto, however, is neither obviously distinguishable from other *B. cereus* group species. As mentioned previously, only *B. cereus* sensu stricto, *B. thurienginesis* and *B. weihenstephanensis* that would give similar colony morphology on MYP agar plates (Tallent et al., 2012). However, *B. thuringiensis* and *B. weihenstephanensi*s are less commonly encountered as compared to *B. cereus* s.s. Indeed, recent bioinformatic evaluation using data from whole genomic sequences (WGS) or multi-locus sequence typing (MLST) suggest that both *B. cereus* and *B. thuringiensis* should be treated as one species Cardazzo et al. (2008) and Liu et al (2015).

Some suggested amplification of *cry/cyt*-genes to identify *B. thuringiensis* (Noguera & Ibara, 2010) which eventually absent in *B. cereus* s.s. However, these gene regions have a huge diversity and make it hard to detect all variants where negative amplification does not completely indicate the absent of *B. thuringiensis*. Therefore, to discriminate *B. cereus* from *B. thuringiensis* remains a challenge. Indeed, the guidelines for identification and enumeration of *B. cereus* in feed and food (ISO 7932:2004) do not differentiate the species of the *B. cereus* but subsumed as presumptive *B. cereus*. In summary, this study able to isolate *B. cereus* s.l. from dried herbs and spices with a prevalence of 30%. However, *B. cereus* was not able to be isolated from the milk products.

Conclusion

This study provides an overview of distribution and presence of *B. cereus* s.l. in dried herbs and spices as well as in milk products. Isolation of *B. cereus* was done using MYP agar and the bacterial species were confirmed using the 16S rRNA sequencing. The prevalence of *B. cereus* s.l. was found to be 30% for dried herbs and spices purchased from local market in Negeri Sembilan. It is indeed essential to educate food handlers about food safety and hygienic handling as the possibility of *B. cereus* exist in dried herbs and spices with inadequate food heating may cause food-borne poisoning to the consumers. Additional research is required to assess the sanitary risk potential linked to *B. cereus* in which the results will determine the requirement of routine food quality control as a part of food safety programs.

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Author Contribution
The authors confirm contribution to the paper as follows: N. Zainal-Abidin: study conception and design, supervision, writing manuscript, review and editing; C.A. Calextus and H.I. Hairul-Hisham: data collection and analysis.

Conflict of Interest
Authors declare no conflict of interest.

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