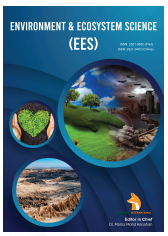
ZIBELINE INTERNATIONAL PUBLISHING

ISSN: 2521-0882 (Print)
ISSN: 2521-0483 (Online)
CODEN: EESND2

Environment & Ecosystem Science (EES)

DOI: http://doi.org/10.26480/ees.02.2020.68.72



RESEARCH ARTICLE

# DETECTION OF *HBLD* TOXIN GENE BY *BACILLUS CEREUS* ISOLATED FROM MEAT CURRY FOOD SAMPLES IN MALAYSIAN RESTAURANTS

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**ARTICLE DETAILS**

***Article History:***

Received 28 March 2020
Accepted 30 April 2020
Available online 27 May 2020

**ABSTRACT**

*Bacillus cereus* is a ubiquitous foodborne pathogen, can cause food poisoning, leading to infections, have two major types of food poisoning emetic and diarrheal. Foods rich in protein such as meat are associated with foodborne outbreaks of diarrhea caused by *B. cereus*. The aim of this study is to isolate and identify *B. cereus* from ready to eat (RTE) meat curry from restaurants in Malaysia and to detect *hblD* pathogenic gene of *B. cereus* isolates. Mannitol egg yolk polymyxin agar was used as a selective isolation medium. Commercially available kits and boiling methods were used for DNA extraction, samples acquired from restaurants were examined for the presence of Hemolysin BL gene by polymerase chain reaction (PCR). Among all isolates, twenty-four of *B. cereus* isolates detected for HBL enterotoxin production by the discontinuous pattern on HBL sheep blood agar then confirmed by biochemical tests. More than 58.33 % of the isolate showed discontinuous hemolysis pattern on HBl blood agar and 29.16% of the samples were shown positive for *hblD* gene that can cause diarrhea with the size of 807bp on gel. This study demonstrated that RTE meat curry was a potential source for entero-toxigenic *B. cereus* and the presence of the *hblD* toxin genes for the HBL complex in the isolates tested were highly associated. Therefore, these meat curry isolates should be regarded as potential toxin producers.

**KEYWORDS**

*Bacillus cereus,* Beta hemolysis, HBL gene, Meat curry, PCR.

## 1. INTRODUCTION

*Bacillus cereus* is an aerobic spore forming bacterium that is commonly found in soil, dust, air, water and vegetables, and in many raw and processed foods as a meat product, milk, and rice. *B. cereus* food poisoning may occur when foods are prepared and held without adequate refrigeration for several hours before serving with *B. cereus* reaching higher than $10^6$ cells/g (Granum et al., 1996). Various reports implicate both *B. subtilis* and *B. licheniformis* as potential food poisoning agents (Kramer et al., 1989; Choma et al., 2002; Zeighami et al., 2020; Hauge, 1955; Rampal et al., 2984; Garofalo et al., 2020; Ncube et al., 2020; Beecher et al., 1995; Ali et al., 2017). The pattern of their repeated occurrence in association with episodes of food poisoning suggests a significant involvement; there have been an increasing number of well-documented reports substantiating the role of *B. cereus* as a food poisoning organism (Kramer et al., 1989). Bacteria are a common cause of foodborne illness. Foodborne disease can also be caused by a large variety of toxins that affect the environment. The foodborne illness usually arises from improper handling, preparation or food storage and some foodborne illnesses are caused by enterotoxins (Choma et al., 2002). Enterotoxins can produce illness even when the microbes that produced them have been destroyed (Zeighami et al., 2020). According to the public health community that regular hand washing is one of the most effective defenses against the spread of foodborne illness and the action of monitoring food to ensure that it will not cause a foodborne illness is known as food safety (Granum et al., 1996).

Two types of illness have been attributed to the consumption of foods contaminated with *B. cereus*. The first and characterized by abdominal pain and non-bloody diarrhea and has an incubation period of 4-16 h, following ingestion with symptoms that last for 12-24 h. The second, which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of the contaminated food and diarrhea is not a common feature in this type of illness. *B. cereus* has three enterotoxins that have been shown to be involved in food poisoning including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE) and cytotoxin K (CytK). There are insufficient reports about the symptom for the enterotoxin T (BceT) and enterotoxin FM (EntFM) (Choma et al., 2002; Zeighami et al., 2020).

For Dose–response relationship Kramer and Gilbert (1989) summarized a large number of foodborne disease outbreaks caused by *B. cereus*. The data showed that the levels of *B. cereus* present in food consumed that had caused a diarrhoeal syndrome varied from $2\times10^3$ to $10^8$ organisms. An analysis of $10^7$ incidents of emetic syndrome food poisoning in the United Kingdom showed that the numbers of *B. cereus* present in the food (mostly rice) ranged from $1\times10^3$ to $5\times10^{10}$ organisms, with a median value of $1\times10^7$ organisms (Kramer et al., 1989). The extremely high levels of

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Cite the Article: Marwan Msarah; Ahmed Alsier and Sahilah, A.M. (2020). Detection Of *HBLD* Toxin Gene By *Bacillus Cereus* Isolated From Meat Curry Food Samples In Malaysian Restaurants. *Environment & Ecosystem Science, 4(2): 68-72*.

contamination may result from the storage conditions of the food remnants and based on this data Granum et al. (1996) proposed that the real infectious dose may vary from about $1\times10^5$ –$1\times10^8$ viable cells or spores and hence food containing $1\times10^6$ *B. cereus* may not be safe for consumption. Human volunteer studies carried out by Hauge (1955) supported the notion that *B. cereus* can indeed induce diarrhea in humans. In Malaysia, the first outbreak of food poisoning due to *B. cereus* was reported by Rampal et al. 1984, Malaysian students staying in a religious secondary school hostel in KLANG area. The students developed an illness mainly characterized by abdominal pain, nausea, vomiting and giddiness. The median incubation period in this outbreak was 2.5 hours. *B. cereus* was determined as the probable cause of this outbreak and the fried noodles the most likely vehicle for the organism. *B. cereus* appeared to be the most likely cause of this outbreak for the following reasons; the clinical manifestations and the incubation period were consistent with that expected of the vomiting toxin type of *B. cercus*. Furthermore, the *B. cereus* count in the implicated food was 2.3 x $10^6$/g which is generally considered as high enough to initiate an outbreak (Rampal et al., 2984).

Hemolysin BL (HBL), a three-component enterotoxin consists of a binding component B and two lytic components L1 and L2 encoded by *hblA*, *hblD* and *hblC* genes respectively and it's considered as a primary virulence factor in diarrheal type for its ability to cause fluid accumulation in rabbit ileal loops test. All three components are required for enterotoxic and hemolytic activity. The function of B protein encoded by the *hblB* gene, which shows high sequence homology to the *hblA* gene, is still unclear. HBL toxin exhibits hemolytic and dermonecrotic activities, increases vascular permeability (Garofalo et al., 2020; Ncube et al., 2020; Wiwat and Thiramanas; 2014).

Beta hemolysis surrounding colonies on blood agar is a diagnostic character of *B. cereus* and the hemolytic pattern surrounding HBL producing strains is discontinuous which is diagnostic for hemolysin BL (Ncube et al., 2020). PCR-based and hybridizaton-based techniques applied in the detection of HBL producing *B. cereus* isolates. The PCR-based method has been used extensively for all of the HBL-encoding genes, nevertheless, the presence of these toxin genes does not necessarily indicate that this bacterium is able to produce HBL enterotoxin and cause disease (Ncube et al., 2020; Beecher et al., 1995; Wiwat and Thiramanas; 2014). The study is to isolate, identify and detect *hblD* pathogenic gene of *B. cereus* from ready to eat (RTE) meat curry from restaurants in Malaysia.

## 2. Material and Methods

### 2.1 Samples collection

A total number of seventy-two meat curry samples acquired from twelve restaurants which located in KAJANG, SERDANG and Kl SENTRAL areas over two weeks. The samples collected six hours after cooking. Retained in clean dry and sterile bags then transported to the laboratory for bacteriological analysis. *Bacillus cereus* were isolated and purified using the plate spreading methods and streaking method on Mannitol Egg Yolk Polymyxin Agar (MYP) (Merck) as selective medium for *B. cereus* and incubated at 30°C for 24 h (Beecher and Wong, 1994).

The total counts of *B. cereus* are based on percentage of colonies tested that were presumptively positive toward *B. cereus*, expressed as CFU/g of sample as follow N = C / V(n) × D, whereas C is the sum of colonies on all plates count, V is the volume applied to each plate, (n) is the number of plate and D is the dilution from count obtained and for the standard error It is calculated by SEM = S/ n. Where SEM is standard error of the mean, s is sample standard deviation, n = size (number of observations) of the sample (Ali et al., 2017). The identity of an organism may be confirmed by demonstrating its ability to perform some biochemical reactions, each species conforming to a recognizable result pattern (Burgess and Horwood, 2006).

The production of hemolysin BL enterotoxin of *B. cereus* isolates was demonstrated by discontinuous double hemolysis pattern on blood agar plates (Beecher et al., 1995). For preparation of HBL blood agar plates, de-fibrinated sheep blood was centrifuged at 5,000 rpm 4°C for 10 min to isolate red blood cells (RBCs). The RBCs were washed with an equal volume of 0.15 M NaCl by centrifugation until the supernatant was colorless. Nutrient agar (NA) supplemented with 0.15 M NaCl was sterilized and allowed to cool to 50°C, washed de-fibrinated sheep blood were then added to the cooled molten agar with final concentrations of 5% and poured into plates in 15 mL volumes. *B. cereus* isolates were inoculated in 5 mL of brain heart infusion broth (BHI) supplemented with 0.1% glucose (BHIG) and incubated at 37°C for 18-20 h with 150 rpm shaking. The overnight culture was suspended by vortexing and inoculated on HBL blood agar plates by spot inoculation. After that, the plates were incubated at 24°C and were frequently observed between 12 and 72 h.

A simple DNA extraction was done involving boiling, chilling and centrifugation (Sahilah et al., 2010). The cells were grown in 1.5 mL of Lauria-Bertani (LB) (Tryptone, 4.0 g/L, Yeast Extract, 5.0 g/L, Sodium chloride, 10.0 g/L) at 35°C for 20 hour before harvested and centrifuged at 12,000 rpm for 1 min then the supernatant was discarded. The pellet then washed with 1.0 mL sterile distilled water and vortex. Then, it was boiled at 97°C for 10 min and immediately was frozen at -20°C for 10 minutes. The tube centrifuged at 10,000 rpm for 3 min then the supernatant used as a template.

The assay performed in a 25 µL volume, tube contained GoTaq green master mix (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, l of 100 mM forward and reverse primer (HD-1), 6.5µL nuclease free water and 5 µL of 10 ng DNA template. A negative DNA control was performed by adding 1 µL of sterile ultra-pure deionized water (Roberts and Greenwood, 2003). Amplification performed in Eppendorf Master-cycler nexus GSX1 (Germany). The samples consist of the strains cultured in BHI with 0.1% glucose and incubated overnight at 37°C. For the PCR a so-called "touch down" program was used consisting of a starting cycle of 3 min at 94°C, followed by five runs in two cycles each of 1 min at 94°C, 1 min at 60°C and 3 min at 72°C, 30 cycles of 1 min at 94°C, 1 min at 54°C and 3 min at 72°C and finally one cycle of 10 min at 72°C (Veld at al., 2001).

The PCR products were separated by gel electrophoresis using a 1.5% agarose gel in a TBE buffer supplemented with DNA gel stain (1uL in 50 mL TBE). The marker thermos scientific GeneRuler 1kb DNA Ladder was used, the ladder composed of fourteen chromatography-purified individual DNA (in base pairs). It contains three reference bands of (6000, 3000 and 1000 bp) for easy orientation. For each PCR test a positive strain (*B. cereus* ATCC 11778), a blank (no DNA added) were used as negative controls. The amplified fragments visualized with UV trans-illuminator (SYNGENE G Box). Add to make a 100 µM primer stock. HD-1 primer ordered from 1st BASE company (Malaysia) with foreword sequence (ATA TTC ACC TTA ATC AAG AGC TGT CAC G) and reverse sequence (CCA GTA AAT CTG TAT AAT TTG CGC CC) is 25.1 nano-moles forward and reverse is 27.1 nano-moles then by adding 251 µL and 271 µL of $H_2O$; a 100 µM primer stock will be created (the original primer tubes used for this 100 µM stock). The master stock primers newly suspended in $H_2O$ allowed sitting at room temperature for 10 minutes before used for working stock dilutions.

## 3. Results

### 3.1 Selective Agar

The bacteria after inoculated onto MYP agar and incubated at 37°C for 24 h. *B. cereus* colonies appeared pink in color and surrounded by precipitate zone surrounding growth. *B. cereus* colonies are pink on MYP and may become more intense after additional incubation. At the first week SERDANG restaurants samples scored the highest mean of the presumptive *B. cereus* and the second week KL SENTRAL restaurants samples is higher while KAJANG restaurants samples still lowest, perhaps this due to both SERDANG and KL SENTRAL areas is more crowded with people and the restaurants samples taken from is near the train station which means more contact with people that resulted in higher contamination than the samples collected from KAJANG restaurants which are less congested. In statistic for compare two group (week 1 and week 2) T-test (paired) was used.

The interpretation of the result based on 95% confidence showed that there is no significant difference (p-value = 0.277) between week 1 and week 2 of KAJANG restaurants, also there is no significant (p-value = 0.623) difference between week1 and week2 of SERDANG restaurants, also there is no significant (p-value = 0.258) difference of Kl SENTRAL restaurants between week 1 and week 2, no significant difference (p- value = 0.626) between week 1 and week 2 for all the restaurants **(Figure 1)**.

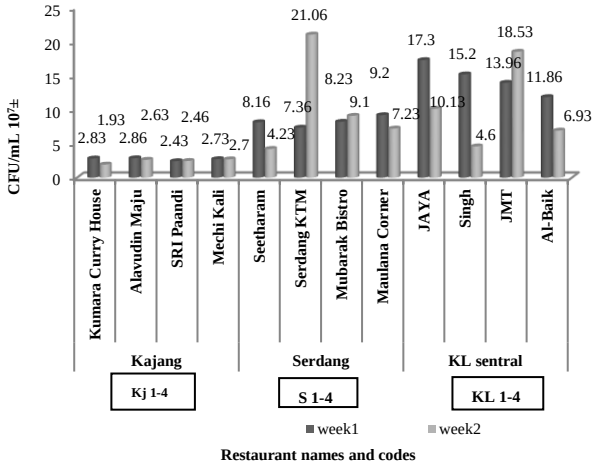


**Figure 1:** Presumptive *Bacillus cereus* collected from different areas

### 3.2 Biochemical Reactions

*B. cereus* confirmed by demonstrating its ability to perform some biochemical reactions, the biochemical characteristics of the isolated bacteria were determined and compared with those described in Bacteriological Analytical Manual (Anonymous, 1992). The results were similar in all cases, except for a small number of tests (Table 1).

**Table 1:** Results of Biochemical Tests

| | Mo | L | Gr | C | Ind | NR | Sta | Glu | Suc | La | $H_2S$ | Gas | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kj1 | rod | + | + | + | — | + | + | + | — | — | + | — | + |
| Kj2 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kj3 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kj4 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kj 1′ | rod | + | + | + | — | + | + | + | — | — | + | — | + |
| Kj 2′ | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kj 3′ | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kj 4′ | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| S1 | rod | + | + | + | — | + | + | + | — | — | + | — | + |
| S2 | rod | + | + | + | — | + | + | + | — | — | + | + | + |
| S3 | rod | + | + | + | — | + | + | + | — | — | — | + | + |
| S4 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| S 1′ | rod | + | + | + | — | + | + | + | — | — | + | — | + |
| S 2′ | rod | + | + | + | — | + | + | + | — | — | + | + | + |
| S 3′ | rod | + | + | + | — | + | + | + | — | — | — | + | + |
| S 4′ | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kl1 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kl2 | rod | + | + | + | — | + | + | + | — | — | — | + | + |
| Kl3 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| Kl4 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| KL 1 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| KL 2 | rod | + | + | + | — | + | + | + | — | — | — | + | + |
| KL 3 | rod | + | + | + | — | + | + | + | — | — | — | — | + |
| KL 4′ | rod | + | + | + | — | + | + | + | — | — | — | — | + |

Whereas:

Kj = KAJANG Area, restaurants 1-4 on 1st week and 1′- 4′ 2nd week collected samples

S = SERDANG Area, restaurants 1-4 on 1st week and 1′- 4′ 2nd week collected samples

KL = KL SENTRAL Area, restaurants 1-4 on 1st week and 1′- 4′ 2nd week collected samples

\+ = 90-100% of strains are positive

— = 90-100% of strains are negative

Mo = Morphology

L = Lecithinase

Gr = Gram-stains

C = Catalase

Ind = Indol

NR = Nitrate reduction

SH = Starch Hydrolysis

Glu = Glucose

Suc = Sucrose

La = LACTOSE

H2S = Hydrogen sulphide gas

M = Catalase

### 3.3 Test for Hemolytic Activity

*B. cereus* cultures usually are strongly hemolytic and produce a 2-4 mm zone of complete beta ß hemolysis surrounding growth. All of *B. cereus* isolates showed ß-hemolysis that is characteristic of *B. cereus*. The results showed that 14 of 24 *B. cereus* isolates gave positive discontinuous hemolysis. The results between PCR and HBL sheep blood agar correlated with the most *B. cereus* isolates. There were two isolates, however, which gave continuous hemolysis pattern was observed, but discontinuous hemolysis pattern was not clearly visible (8.33%). Therefore, the results were reported as doubtful.

### 3.4 PCR

In this study, PCR show positive result for *hblD* gene for (29.16%) (7/24) of *B. cereus* isolates which is similar to Wiwat and Thiramanas 2014 who reported that *B. cereus* isolate gave positive results with *hblA*, *hblC* and *hblD* genes, but gave negative result with *hblB* gene (Table 2). Moreover, 58.33% of *B. cereus* isolates were also detected for HBL enterotoxin production by discontinuous pattern on HBL sheep blood agar. The double hemolysis is only visible for a limited period but even when the plates judged every few hours the double hemolysis was not detected for 41.67% of the samples. Figure 2 shows the PCR of B. cereus isolated by using HD1 F-R primers resulted in amplified fragment size of 807bp. The samples that have been collected at the first week show positive outcome for KAJANG restaurants number one and four. The first and second restaurants for SERDANG restaurants and only the second restaurant for KL SENTRAL areas show positive outcome for hblD gene. The samples collected the second week show positive outcome for KAJANG restaurants number four and weak positive for restaurant three for KL SENTRAL area.

**Table 2:** Detection of *hblD* gene and HBL enterotoxin production of *Bacillus cereus* isolate

| *B. cereus* isolate code | Hbl blood agar plates[b] | PCR[a] *hblD* |
|---|---|---|
| ATCC 11778 | + | _ |
| Kj1 | - | - |
| Kj2 | - | - |
| Kj3 | + | - |
| Kj4 | + | + |
| S1 | - | - |
| S2 | - | - |
| S3 | + | - |
| S4 | +/- | - |
| KL1 | + | - |
| KL2 | - | - |
| KL3 | - | + |
| KL4 | +/- | - |
| Kj 1′ | + | + |
| Kj 2′ | + | - |
| Kj 3′ | + | - |
| Kj 4′ | + | + |
| S 1′ | + | + |

| | | |
|---|---|---|
| S 2′ | + | + |
| S 3′ | _ | - |
| S 4′ | _ | - |
| KL 1′ | + | - |
| KL 2′ | + | + |
| KL 3′ | _ | - |
| KL 4′ | + | - |
| Total (%)[c] | 58.33 | 29.16 |

Whereas:

Kj = KAJANG Area, restaurants 1-4 first week and 1′- 4′ second week

S = SERDANG Area, restaurants 1-4 first week and 1′- 4′ second week

KL = KL SENTRAL Area, restaurants 1-4 first week and 1′- 4′ second week

a = PCR reported as: +, PCR product of the expected size was observed; no PCR product was observed.

B = HBl blood agar plate results reported as +, discontinuous hemolysis pattern was observed; continuous hemolysis pattern was observed; +/-, continuous hemolysis pattern was observed, but discontinuous hemolysis pattern was not clearly visible (doubtful).

c = The total of positive results in all tests reported as the percentage and calculated by excluding the results from *B. cereus* Strain ATCC 11778.

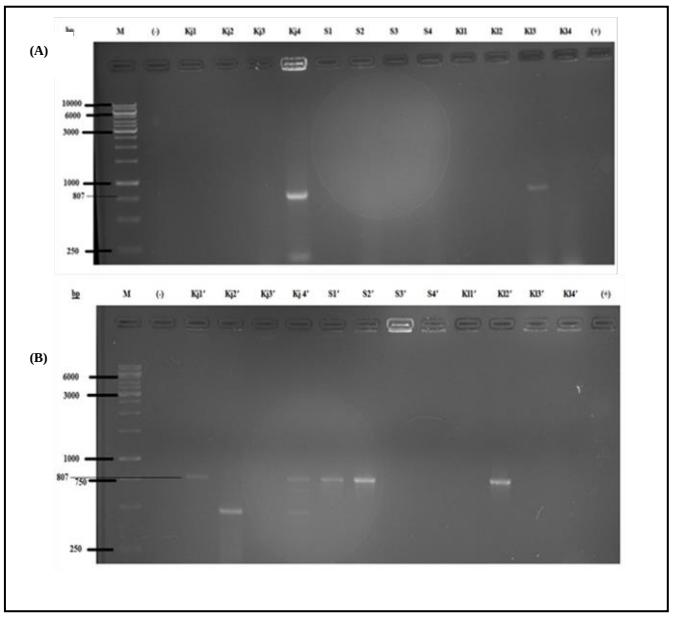


**Figure 2:** Detection of *hblD* gene of *Bacillus cereus* isolated (A) first week (B) second week by polymerase chain reaction (PCR) electrophoresed on 1.5% (w/v) Agarose gel. Lane 1: Thermo Scientific GeneRuler™ 1kb DNA Ladder, lane 2: Negative control (nuclease free water) ,lane 3-6: *B. cereus* isolated from KAJANG prepared by boiling methods, lane 7-8: *B. cereus* isolated from SERDANG prepared by boiling methods, lane 9-10: *B. cereus* from SERDANG restaurants prepared by DNA extraction kit, lane 11-12: *B. cereus* isolated from Kl Sentaral prepared by boiling methods, lane 13: *B. cereus* isolated from Kl SENTRAL prepared by DNA extraction kit, lane 14: *B. cereus* isolated from Kl SENTARAL prepared by boiling methods, lane 15 : Positive control *B. cereus* ATCC 11778 prepared by DNA extraction kit.

The results correlated for 29.16% *B. cereus* isolates that were detected by PCR with the *hblD* gene (Table 2). The *B. cereus* that was weakly positive for hemolysis on the blood agar failed to show PCR amplification for *hblD genes*. Maybe the reason is that the sequence of the primers varies in the binding site. The results showed that boiling methods were sufficient to use for DNA extraction for *B. cereus.* Veld et al. (2001) reported that the discontinuous hemolysis pattern on blood agar was only visible at a limited of time and that this method does not justify as a reliable tool for HBL detection. Therefore, the discontinuous pattern not observed in these nine isolates may result from one or all of those reasons. The results showed that 29.16% of the isolates were positive for the *hblD* gene that can cause diarrhea. Explanations can be given for the negative results, that the PCR method did not detect the gene because of some genetic variation in the target sequence for the HD-1 primer for this gene. Further research will be needed to clarify the weak PCR reactions. To explain the low incidence of food poisoning due the consumption of meat curry containing high numbers of *B. cereus* is that *B. cereus* to be able to cause food poisoning, all three components of the HBL complex must be present (Rampal et al., 2984; Garofalo et al., 2020; Ncube et al., 2020; Beecher et al., 1995; Ali et al., 2017). Therefore, further research is needed.

## 5. Conclusions

The extensive distribution of *Bacillus cereus* with the thermal resistance of spores helps to explain the wide variety of foods that have been implicating in *B. cereus* foodborne illness outbreaks. Two types of disease emetic and diarrheal have been attributing to the consumption of foods contaminated with *B. cereus*. Both types of food-borne disease are relatively mild and self-limiting. In this study 24 isolates were identified as *B. cereus,* SERDANG and KL SENTRAL restaurants samples has scored the highest in the meantime KAJANG restaurants samples is the lowest could be as a result of poor processing method, poor hygiene practice, improper and unhygienic handling of the meat curry, bad sanitation operations and use of unclean utensils. However, an important factor that significantly contributes to the increase in the count is the location of the restaurants. SERDANG and KL SENTRAL restaurants locations is near the KTM train railway and more crowded with population than KAJANG area. Foods left for a long time will encourage the growth of the spores, even under heat treated. Therefore, one should assume that *B. cereus* is present and should take preventative measures to prevent growth during food handling. The crude estimate of risk would be useful and provide important information that can attract some degree of attentions by public health authorities. Reducing the potential hazards by understanding the potential risks from meat curry and subsequently refine the analysis, implement control measure and improve the surveillance system.

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