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Detection of *Bacillus cereus* group and emetic *Bacillus cereus* group strains in milk using multiplex polymerase chain reaction

Jintana Pheepakpraw[1], Chanita Sinchao and Thararat Chitov[1*]

[1] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

***Corresponding author**: thararat.chitov@cmu.ac.th*

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ABSTRACT

Bacillus cereus group is one of the most frequently contaminated foodborne bacterial pathogen groups in milk and dairy products. Members of this group can cause emetic foodborne illness because of their ability to produce the emetic toxin. In this study, we aimed to investigate the occurrence of *B. cereus* group in raw milk and to develop a multiplex PCR method for detecting emetic *B. cereus* group strains. A total of 286 raw milk samples were collected from various dairy farming areas, including Mae Jo, Mae Wang, Sankampaeng, and Mae On, in Chiang Mai province, Thailand. The presence of *B. cereus* group species was examined using Bacara chromogenic agar, through which *B. cereus* group was found in 34.27% of the milk samples from all farming areas. A duplex PCR that detected *its* (universal gene) and *motB* (gene specific for the *B. cereus* group) was also performed, through which 47.90% of the milk samples were detected positive for the *B. cereus* group. Furthermore, a multiplex PCR targeting the *motB, cesA*, and *its* genes was developed. The developed method was successfully used to identify emetic *B. cereus* isolates from raw milk.

Keywords: foodborne pathogen, emetic toxin, rapid method, milk quality, dairy industry

INTRODUCTION

Bacillus cereus group is a specific group of Gram-positive, endospore-forming, rod-shaped bacteria that are closely related at the *16S rRNA* gene level (Ehling-Schulz et al., 2019). *B. cereus* group is comprised of many closely related species, including *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. anthracis*, *B. weihenstephanensis*, *B. cytotoxicus*, *B. toyonensis*, and *B. wiedmannii* (Miller et al., 2018). *B. cereus* and some closely related species are known to cause foodborne illnesses due to their ability to produce toxins. The illnesses can be divided into two distinct types: diarrheal and emetic, which are associated with enterotoxins and emetic toxin, respectively. There are many types of diarrheal enterotoxins, including hemolysin BL (HBL), non-hemolytic enterotoxin (NHE), and cytotoxin K (Sehoeni and Wong, 2005), all of which are heat-labile proteins (Wang et al., 2014). It is believed that enterotoxins are released in the small intestine after cells disintegrate (Jeßberger et al., 2017). Emetic toxin is a single-entity toxin, a cyclic peptide with a molecular weight of 1.2 KDa. It is also known as "Cereulide" and has a similar structure to the antibiotic Valinomycin (Granum and Lund, 1997). Cereulide is made up of alternating amino and hydroxy acids, which are synthesized by nonribosomal peptide synthetases (NRPS), encoded by the genes in the cereulide synthetase (*ces*) cluster (Marxen et al. 2015). The toxin is highly resistant to heat, extreme pH, and proteolytic enzymes and is released in food by living cells (Rajkovic et al., 2008).

Because *B. cereus* group bacteria are widely distributed in the environment, they can easily contaminate many types of food (Stenfors Arnesen et al., 2008). Dairy products are among the types of food that are most frequently contaminated by the *B. cereus* group, which mainly comes from raw milk (Vidal et al., 2015; Coorevits et al., 2008). Maintaining the quality of raw milk is crucial as it may harbor *B. cereus* and serve as a source of contamination at milk processing plants. However, the data on the prevalence of *B. cereus* in raw milk in Thailand was very limited. Furthermore, the spores of *B. cereus* can survive pasteurization and can germinate and multiply in dairy products, producing toxins under favorable conditions. The heat-stable emetic toxin, once produced in dairy products, is difficult to eliminate and can pose health risks to consumers. Therefore, rapid methods for detection of *B. cereus* group species and its emetic toxins are needed for the dairy industry.

In this study, we aimed to investigate the occurrence of *B. cereus* in raw milk from local dairy farms in four dairy cooperatives located in different parts of Chiang Mai province, one of the most important dairy production areas in the northern part of Thailand. Also, because there is no simple, industrially applicable assay for detection of emetic

B. cereus group species, we also aimed to develop a multiplex PCR assay for the rapid detection of emetic *B. cereus*.

MATERIALS AND METHODS

Collection of raw milk samples

Samples of raw milk from dairy farms affiliated with dairy cooperatives in different areas of Chiang Mai, Thailand, which included the areas of Mae Jo, Mae Wang, Sankampaeng, and Mae On, were collected from January to July 2020. In total, 286 samples were collected from individual farms from the receiving tank at the dairy cooperatives with which the dairy farms were affiliated. Duplicate milk samples (50 ml) were taken from the receiving tank immediately after all the milk from each individual farm was combined. The samples were stored on ice after collection and during transportation, kept at 4 °C in the laboratory, and analyzed within 24 h.

Isolation on a chromogenic agar plate

B. cereus group was isolated from raw milk samples according to the method described in the Bacteriological Analytical Manual (BAM, US FDA). In brief, a 1-ml portion of each sample was transferred into 9 ml of Butterfield's phosphate buffer dilution water and mixed. The homogenate was serially diluted (10-fold) in the same buffer until the desired dilutions were obtained. A 100-µl portion of each dilution was plated on Bacara agar (BioMérieux, Marcy-l'Étoile, France) using the spread plate method. Plating was performed in duplicate. The plates were incubated aerobically at 30 °C for 24 h. Bacterial colonies that were pink or orange surrounded by an opaque zone of egg yolk hydrolysis, which were typical *B. cereus* group colonies, were selected from each plate and subcultured on Tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) to obtain pure cultures. The pure cultures were stored at 4 °C and as frozen glycerol stock cultures.

Duplex PCR for detection of B. cereus group in raw milk

Extraction of Bacterial DNA from Raw Milk

Bacterial DNA was extracted from raw milk using the cetyltrimethylammonium bromide (CTAB) extraction method, modified from Worden (2009). Raw milk samples were centrifuged at 7000×g for 10 min. The pellet from each sample was resuspended in 600 µl of CTAB buffer [1 M Tris (pH 8.5), 5 M NaCl, 0.5 M EDTA, and 0.2% (w/v) CTAB]. Proteinase K (15 µl) was added to each sample, vortexed, and incubated at 60 °C for 1 h. Then, an equal volume of chloroform was added to the mixture. The content was mixed and centrifuged at 12,000×g for 15 min at 4 °C. The supernatant was transferred to a new tube, an equal volume of isopropanol was added, and the microtube was incubated at -20 °C for 1 h or overnight. The DNA pellet was collected after centrifugation at 12,000×g for 15 min, then washed twice with 70% (v/v) ethanol, and centrifuged at 12,000×g for 15 min. The supernatant was discarded, and the DNA pellet was air-dried before being resuspended in 50 µl of TE buffer. The DNA samples were stored at -20 °C. Throughout the preparation, filtered pipette tips were used.

Detection of the B. cereus group in raw milk samples using duplex PCR

A duplex PCR was used for the detection of *B. cereus* group species, targeting the *motB* gene (encoding the flagellar motor protein specific to *B. cereus* group species) and the *its* gene (internal transcribed spacer, used as an internal control for the presence of bacterial DNA). The primers used for the amplification of these genes are shown in Table 1. The 25 µl PCR reaction mixture contained 1 µl of DNA template (0.5–5 µg/µl), 0.2 µM of each primer, 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 0.4 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 2.5 µl of 10×Taq polymerase buffer. PCR was performed in the Eppendorf Mastercycler (Eppendorf, Framingham, MA, USA). The amplification was operated with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. The amplified PCR products were analyzed using gel electrophoresis on a 1.5% agarose gel alongside a 1 Kb DNA ladder (Meridian Bioscience, Cincinnati, OH, USA). Agarose gel electrophoresis was performed in 1×TAE buffer for 80 min at 80 V. Following electrophoresis, the agarose gels, which were prestained with Safe Green dye (Vivantis, Selangor Darul Ehsan, Malaysia), were visualized under a blue light transilluminator.

Development of multiplex PCR for detection of emetic *B. cereus*

Bacterial strains and isolates

The reference bacterial strains used in this study included *B. cereus* F4810/72 (also known as B0358, an emetic strain) and *B. cereus* DSM 4384 (also known as B–4ac, a diarrheagenic strain). *B. cereus* group isolates used in this study were obtained from raw milk samples. They were isolated in this

study (see above) or in a previous study (Sinchao, 2021; received from Chanita Sinchao, an associate member of the Microbial Resource and Technology Laboratory, SCB2806, Chiang Mai University).

DNA extraction from bacterial isolates

Chromosomal DNA was prepared from *B. cereus* strains and isolates grown in Tryptone soy broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract for 24 h at 37 °C. The cell pellet from a 3-ml culture was washed twice with 500 µl of phosphate buffer saline (pH 7.2), resuspended in 600 µl of extraction buffer [200 mM Tris (pH 8.5), 250 mM NaCl, 25 mM EDTA, and 0.5% (w/v) SDS], followed by extraction with 300 µl of phenol: chloroform: isoamyl alcohol (25:24:1). After that, the mixture was centrifuged at 14,000 g for 10 minutes at 4 °C, and the upper layer was transferred to a new tube. DNA was precipitated by adding the same volume of isopropanol and incubated at -20 °C for 20 min. The DNA pellet was collected after centrifugation at 14,000×g for 10 min. The DNA pellet was washed with 70% (v/v) ethanol and centrifuged at 14,000×g for 10 min. The supernatant was removed, the DNA pellet was air dried, then resuspended in 50 µl of TE buffer, and DNA samples were stored at -20 °C. Throughout the preparation, filtered pipette tips were used.

Development of multiplex PCR

A multiplex PCR targeting *motB* (gene encoding the flagellar motor protein that is specific to the *B. cereus* group), *cesA* (the *cereulide synthetase* gene specific to emetic *B. cereus*), and *its* (universal for bacteria, used as an internal control) was developed with primers listed in Table 1. To optimize the multiplex PCR conditions, the annealing temperatures were tested from 55 to 70 °C. The multiplex PCR was performed in a 25 µl reaction volume, containing 2.5 µl of 10× buffer, 0.2 µM of each primer, 0.2 mM of each dNTP, 3 mM of $MgCl_2$, 1 unit of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µl of DNA template (0.5–5 µg/µl). The amplification was operated with an initial denaturation step (5 min at 95 °C), 35 amplification cycles (30 s at 95 °C, 45 s at 55 °C to 70 °C, 1 min at 72 °C), followed by a final extension (10 min at 72 °C). The developed multiplex PCR was tested with *B. cereus* group isolates from raw milk, *B. cereus* F4810/72, *B. cereus* DSM 4384, *B. subtilis*, and *Escherichia coli*.

Table 1. PCR primers for detection of the *B. cereus* group and emetic *B. cereus*

Target gene	Primer	Expected PCR product size (bp)	Primer sequence	T_m (°C)	Reference
its	*its* F	185	5' AATTTGTATGGGCCTATAGCTCAGCT 3'	64.7	Yang et al., 2005
	its R		5' TTTAAAATAGCTTTTTGGTGGAGCCT 3'	61.6	
mot B	*motB* F	577	5' ATCGCCTCGTTGGATGACGA 3'	60.5	Oliwa-Stasiak et al., 2010
	motB R		5' CTGCATATCCTACCGCAGCTA 3'	61.3	
ces A	*cesA* F	996	5' CCGCCAGCTAGATGAAAAAGA 3'	55.0	Sinchao, 2021
	cesA R		5' ATCACTTTCGGCGTGATACC 3'	55.0	

RESULTS AND DISCUSSION

Occurrence of B. cereus group in milk by chromogenic agar plate

From the analysis of 286 raw milk samples from four dairy farm areas, the *B. cereus* group was found in 34.27% of the milk samples tested. A parallel duplex PCR assay for detecting the occurrence of *B. cereus* group species through the presence of the *motB* gene, was also performed. It appeared that *B. cereus* group species were found in 47.90% of the samples tested. The results from this study suggest that the duplex PCR assay was more sensitive than the chromogenic agar method for detecting *B. cereus* group in milk. Due to the low levels of *B. cereus* contamination that can occur in milk, the PCR method, which is faster and more sensitive than traditional, culture-based methods, is often preferred. Even at low concentrations, it is difficult to culture them in the laboratory. The PCR assay has been suggested as a convenient method for detecting enterotoxigenic *B. cereus* isolates. (Wehrle et al. 2009). With the target of PCR detection being a species-specific gene and an emetic-toxin-related gene, performing a duplex or a multiplex PCR offers

a more rapid detection than performing multiple single reactions (Kalyan Kumar et al., 2010). Chelliah et al. (2017) found that *motB* was very specific to the *B. cereus* group, and detection of this gene could be an efficient way to identify the presence of this bacterial group. Previous studies found contamination of the *B. cereus* group in 9%–43% of raw milk samples by selective plating methods (Ahmed et al., 1983, Mosso et al., 1989, and Yobouet et al. (2014), and in 62% by a PCR method (Aksoy et al., 2021). In our study, *B. cereus* group was found in raw milk from farms in all areas examined (26.09%–36.59% by chromogenic Bacara isolation and 46.26%–53.85% by the PCR method) (Table 2), which, although within the range previously reported, indicated that the proportion of milk samples contaminated with *B. cereus* was high. Milk from some areas, such as Sankampaeng, was found to be less frequently contaminated by the *B. cereus* group species than milk from the other areas, using the Bacara isolation method. This could be because the bacteria were absent or present at a lower level than the detection limit. The contamination of the *B. cereus* group in milk from the individual farms in all the areas examined indicates that *B. cereus* group species would also be present in the bulk tank milk, where milk from individual farms in each location was combined. Since the raw milk was immediately sent through the pipeline to the bulk storage tank, where it is cooled down, the presence of *B. cereus* in the milk in the bulk tank was unavoidable. *B. cereus* in the bulk tank milk, which is used as raw material for the dairy industry, would survive pasteurization and contaminate the pasteurized dairy products. Therefore, it is important to keep the level of *B. cereus* group low through the proper storage of raw milk before processing and of pasteurized milk products to prevent the production of toxins, especially the heat-stable emetic toxin. In addition, proper cleaning and sanitizing of milking equipment, regular testing and monitoring, and maintaining milk storage temperature are necessary for ensuring milk quality and safety (Burke et al., 2018).

Table 2. Occurrence of the *B. cereus* group in milk from farms in four dairy farming areas in Chiang Mai

Area	No. of samples positive on Bacara agar (%)	No. of *motB*-positive samples (%)
Mae Jo	15/41 (36.59%)	19/41 (46.34%)
Mae Wang	18/52 (34.62%)	28/52 (53.85%)
Sankampaeng	12/46 (26.09%)	22/46 (47.83%)
Mae On	53/147 (36.05%)	68/147 (46.26%)
Total	98/286 (34.27%)	137/286 (47.90%)

Optimization of multiplex PCR for detecting B. cereus group

Annealing temperatures for a multiplex PCR designed to detect the *B. cereus* group and emetic *Bacillus* strains were tested from 55 °C to 70 °C. This temperature range was chosen from the melting temperatures of each primer (Table 1). The target genes in the multiplex PCR included *motB* and *cesA*, which are specific for *B. cereus* group and emetic *B. cereus* strains, respectively. The *its* gene was an additional target gene included in the multiplex PCR, which served as an internal control. Each primer set was first tested individually and later in the multiplex PCR, using the DNA templates from the reference strains: *B. cereus* F4810/72 (an emetic strain, *cesA*-positive) and *B. cereus* DSM 4384 (a non-emetic strain, *cesA*-negative). The PCR products from *B. cereus* F4810/72 showed clear bands for *its*, *motB*, and *cesA* at 185 bp, 577 bp, and 996 bp, respectively. The annealing temperatures at which three bands were clearly seen ranged from 56.1 °C to 63.4 °C (Figure 1 (a)). Therefore, the annealing temperature of 59 °C was chosen for the multiplex PCR analysis for the detection of emetic *B. cereus*.

The developed multiplex PCR was then used to detect emetic *B. cereus* isolates obtained from raw milk samples. The results showed that the multiplex PCR could successfully detect emetic *B. cereus* group isolates (Figure 1 (b), lanes 5–10). Many researchers had previously confirmed the specificity of *motB* for the *B. cereus* group and other species that are closely related to *B. cereus* (Oliwa-Stasiak *et al*. (2010); Chelliah *et al*. (2017)). Detection of *cesA*, the *cereulide synthetase* gene that is specific to emetic *B. cereus*, gives more information on the potential of the *B. cereus* group present in a raw milk sample to produce the emetic toxin. The internal control (*its* gene), a gene found in all bacteria, was included as a universal target gene to ensure that the PCR reaction was free of any PCR inhibitors and was correctly prepared (Yang *et al.* 2005). These primers and the optimized conditions

for the multiplex PCR could also be further applied for the detection of *B. cereus* group and emetic *B. cereus* in dairy products.

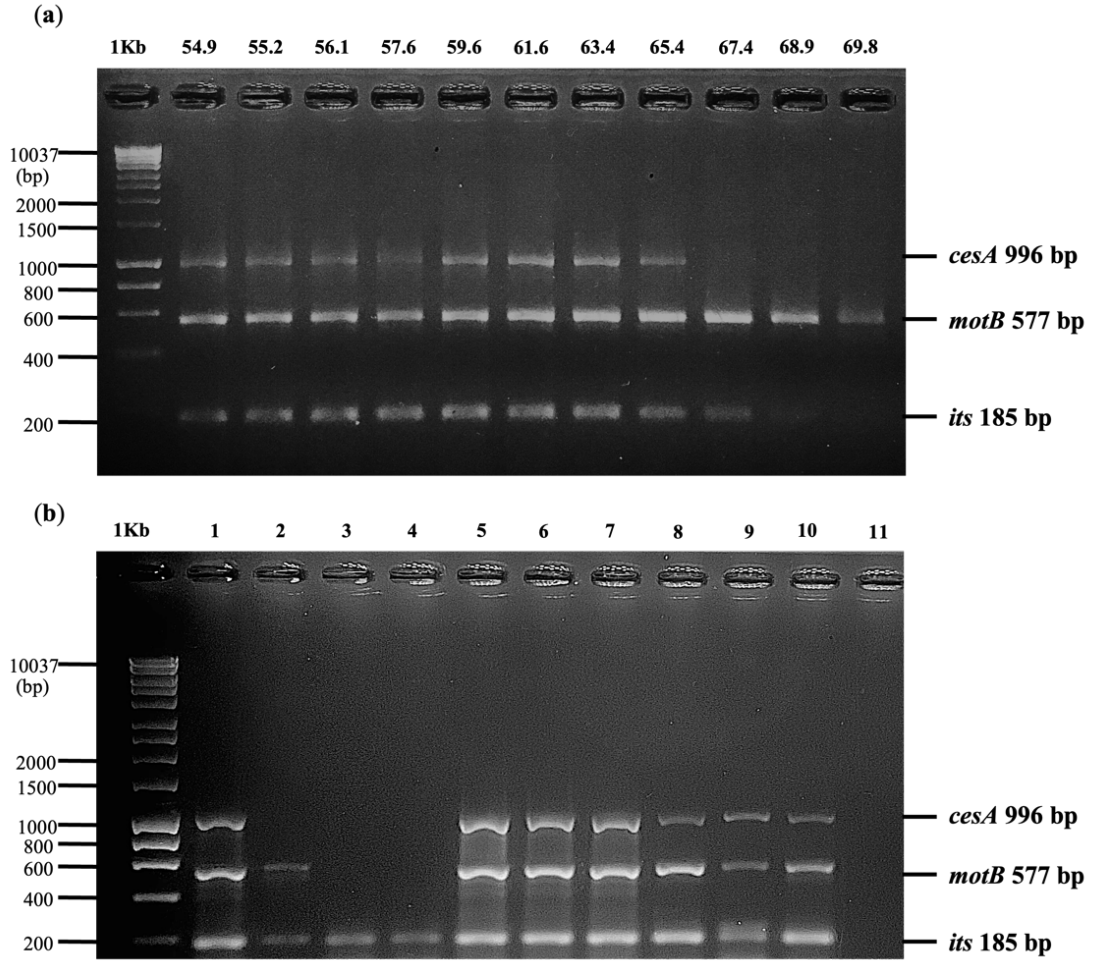

Figure 1. The amplified fragments obtained from the multiplex PCR designed for detection of the *its*, *motB*, and *cesA* genes. **(a)** Different annealing temperatures were tested to find an optimum annealing temperature, using the DNA from *B. cereus* B358 (F4810/72) (an emetic strain, *cesA*-positive) as a template. **(b)** Examples of multiplex PCR performed at an annealing temperature of 59 °C, using DNA from emetic *B. cereus* reference strains isolates from raw milk samples. Lane 1 = DNA from *B. cereus* F4810/72 (reference emetic strain), Lane 2 = DNA from *B. cereus* DSM 4384, non-emetic strain), Lane 3 = DNA from *B. subtilis (Bacillus species outside the B. cereus group)*, Lane 4 = DNA from *E. coli,* Lanes 5–10 = DNA from emetic *B. cereus* isolates from raw milk, and Lane 11 = negative reaction control (distilled water, no DNA). 1Kb = 1 kb DNA ladder (Meridian Bioscience, Cincinnati, OH, USA).

CONCLUSIONS

B. cereus group species were detected in raw milk samples from four dairy farming areas in Chiang Mai with a frequency of 34.27% and 47.90% by the Bacara chromogenic agar method and the PCR method, respectively. The duplex PCR targeting the *its* and *motB* genes was more sensitive than the chromogenic agar method and could be adopted as a method for rapid screening of the *B. cereus* group in milk. A multiplex PCR for detecting emetic *B. cereus,* which amplifies the *its, motB,* and *cesA* genes, was also developed. The PCR reaction was optimized using the DNA of *B. cereus* F4810/72, a reference emetic strain. As a result, an optimum condition was found with an annealing temperature of 59 °C. This multiplex PCR was tested with raw milk isolates of *B. cereus* and could successfully identify emetic isolates.

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