Bangladesh J Microbiol, Volume 39, Number 1, June 2022, pp 21-29

Original Article

Identification and Differentiation of Closely Related Members of *Bacillus cereus* Group by Multiplex PCR

Sushmita Roy[1], Avirup Saha[1], Shafayet Imtiaze Khan[1], Md. Mahmud Hasan[1,2], Muhammad Manjurul Karim[1], Marufa Zerin Akhter[1], Md. Mozammel Hoq, Shakila Nargis Khan[1*]

[1]Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh, [2]National Institute of Biotechnology, Ganakbari, Savar, Dhaka-1349, Bangladesh

Highly similar genetic and phenotypic traits of at least eight bacterial species forming the '*Bacillus cereus* group' create their precise identification and differentiation quite difficult. The present study explores the applicability of a previously suggested multiplex-PCR method for the accurate identification of the candidate *Bacillus* species. Out of the 257 *Bacillus* isolates collected from soil, 44 were identified as *B. thuringiensis* and 39 as *B. cereus* by chromogenic cultural method using *Bacillus* agar, although few of them shared similar colony characteristics. Identification by the multiplex PCR in a thermo cycler using 5 different sets of primer-pairs, however produced distinct amplification corresponding to the bacterial species. Four of those pairs, named BMSH, BCJH, BTJH and BASH were designed based on *gyrB* gene that produced amplicons of four different sizes: 604, 475, 299 and 253 bp and were specific for *B. mycoides*, *B. cereus*, *B. thuringiensis*, and *B. anthracis* respectively. The remaining one of the sets was used as an internal control which was a universal primer-pair, BCGSH, designed targeting a housekeeping gene, *groEL* that could produce an amplicon of 400 bp in polymerase chain reactions for all members of the *B. cereus* group, When probing the chromosomes extracted from 257 *Bacillus* isolates by multiplex PCR; 48, 39 and 5 were identified as *B. thuringiensis*, *B. cereus*, and *B. anthracis* respectively, however the rest of the isolates did not any amplification. Interestingly, the phylogenetic tree, constructed based on partial sequences of 16S rRNA genes of selected isolates including the reference strain of *B. thuringiensis* (HD-73, sotto) could not differentiate the species, instead posited those in a single cluster. The multiplex PCR, therefore, proved to be a sensitive and reliable method for the identification of the bacterial candidates of *Bacillus cereus* group than that of cultural and rRNA gene sequence analyses.

Keywords: *Bacillus cereus* group, identification, multiplex PCR.

Introduction

The '*Bacillus cereus* group' is one of the largest and most ubiquitous microbes of high economic, medical and biodefense importance, comprised of at least eight phylogenetically very closely related *Bacillus* species. These are *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis*[1]. With the exception of *B. cytotoxicus*, which is the most divergent of the group with a chromosome of 4.085 Mb, the genomes of member species are highly conserved, with sizes of 5.2 to 5.5 Mb having very similar 16S rRNA gene sequences[2]. The most studied members of the group, *Bacillus anthracis, B. cereus*, and *B. thuringiensis* are well known and possess substantial pathogenic potential. While *B. anthracis* is the causative agent of anthrax, few members of *B. cereus* group are commonly recognized as food poisoning agents, some can also cause localized wound and eye infections as well as systemic diseases[2]. Certain *B. thuringiensis* strains are entomopathogens and are being used commercially as biopesticides, however, some strains have been reported to cause infections in the immunocompromised individuals[2].

Despite the presence of high degree phylogenetic relatedness that prompted several researchers to consider the members of the *Bacillus cereus* group as a unique species, the members demonstrated differences in plasmid content and expression of key regulatory genes. Several approaches were employed to differentiate *B. cereus* group members including whole-genome DNA hybridization[8], sequence analysis of the 16S-23S operons, the *gyrB* - *gyrA* intergenic spacer region, multilocus enzyme electrophoresis, pulsed-field gel electrophoresis analysis[4], amplified fragment length polymorphism[6], virulence factors, arbitrary PCR, multiplex PCR and PCR-restriction fragment length polymorphism[6]. However, the need to adopt a rapid and simpler method remained an issue to ponder. .

HiCrome^{TM} *Bacillus* Agar is a selective and differential media which is recommended for the isolation and differentiation amongst various species of *Bacillus.* This medium is based on the formulation of MYP (Mannitol, egg yolk, polymyxin) agar where different *Bacillus* sp. show different colored colonies[9]. However, culture-based method of identification does not always

*Corresponding author:
Dr. Shakila Nargis Khan, Professor, Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh

produce correct results and becomes confusing rather than being conclusive demanding further genetic analysis for confirmation. The current work took the advantage of a work by Park *et al*. (2007)[10] who used the *gyrB* and *groEL* genes as diagnostic markers that would simultaneously detect 4 different species of the *B. cereus* group using a multiplex PCR. While the assay yields a 400 bp amplicon for the *groEL* gene from all the *B. cereus* group of bacteria, single amplicons each for *B. anthracis*, *B. thuringiensis*, *B. cereus*, and *B. mycoides* of sizes 253, 299, 475 and 604 bp respectively were produced in addition amplified from *gyrB*. Here, a comparative analysis of the multiplex PCR method with that of the culture-based and rRNA gene sequence analytical methods were done for the locally-isolated *Bacillus* species collected from soils across Bangladesh to deduce a simple but an appropriate technique for species differentiation.

Materials and Methods

Bacterial strains

Two hundred and fifty-seven strains of *Bacillus* spp were collected from soil samples of different areas of Bangladesh[11], and stored in the Fermentation and Enzyme Biotechnology Laboratory, Department of Microbiology, University of Dhaka. Three *Bacillus thuringiensis* reference strains (HD-73, japo and sotto) from American Type Culture Collection (ATCC) were procured for this study (Table 1).

Media and growth condition

LB (Luria-Bertani) agar medium was used for the routine growth of the *Bacillus* isolates. A loopful of the bacterial isolate stored in 20% glycerol at -80°C was streaked on LB medium and incubated overnight at 37°C. A single colony grown on the medium was picked to inoculate in a 5 ml LB broth and incubated at 37°C and 150 rpm in a shaking incubator.

Traditional methods for identification of Bacillus spp.

Twenty-three strains were chosen for the traditional experiments: Dsh4, HD-73 (reference strain), ksa2, Jsd1 for *B. thuringiensis*; Spsd3, MyIa1, kbsb2, Ssb1, Jdb1 for *B. cereus*; RaSd1, Nasc3, 23s, kksd1, 45s for *B. megaterium*; rhsb2, 43s, kbsa1 for *B. subtilis*; insect-06, Rhsb3, 22s for *B. anthracis*; and jsb1, jsb2, jsc2 for *B. coagulans*[11].

Gram staining and Biochemical Tests

Gram staining, Indole production test, Methyl red test, Voges-Proskauer test, Starch hydrolysis test and Gelatinase tests were carried out following the procedures described in Bergey's Manual of Determinative Bacteriology.

Salt tolerance test

The growth and salt tolerance ability of the isolates was challenged by their ability to grow on LB broth containing 7% NaCl.

Phase contrast Microscopy

Bacillus strains were cultured on LB agar medium and incubated for 3 days at 30ºC. An aliquot of 10µl sterile distilled water was placed at the middle of the slide, a single colony was mixed with it and covered with a cover slip. The slide was placed under a phase contrast microscope (Primo star, Carl Zeiss, Germany) and the presence of spores and crystal proteins were observed.

Chromogenic colonies on HiCrome™ Bacillus Agar

HiCrome™ *Bacillus* Agar was used for the isolation and differentiation between various species of *Bacillus* from a mixed culture by chromogenic method. The medium contains peptic digest of animal tissues and meat extract as nitrogenous compounds. Mannitol serves as the fermentable carbohydrate, fermentation of which can be detected by colour change of the phenol red indicator. Mannitol-fermenting organisms like *B. megaterium* yields yellow colonies. The chromogenic mixture present in the medium is cleaved by the enzyme beta-glucosidase found in *B. cereus* resulting in the formation of blue colonies. *B. thuringiensis* also grows as blue/green colonies on this medium. *B. cereus* and *B. thuringiensis* are biochemically identical. However, on this medium they produce colonies with slightly different characteristics: *B. cereus* show light blue, large, flat colonies with blue centres, while *B. thuringiensis* produce light blue, large, flat colonies with irregular margins. *B. subtilis*, *B. megaterium* and *B. coagulans* show green, yellow and pink colonies respectively.

Preparation of total DNA

Total DNA was prepared from the *Bacillus* isolates by following a technique described by Bravo *et al*.[12]. Briefly, a single colony from LB agar was transferred into 100 µl of sterile distilled water and mixed well. The suspension was held at -20°C for 20 minute before transferring immediately into boiling temperature for 10 minutes. The resulting cell lysate consisting of chromosomal and plasmid DNA was briefly centrifuged at 12,000g for 1 min and the supernatant solution was used as the DNA template for polymerase chain reactions (PCR).

16S ribotyping

The polymerase chain reaction (PCR)-based amplification of the gene for 16S rRNA was carried out using the extracted DNA from bacterial isolates as template. These reactions were carried out in a Thermal Cycler. The amplification was carried out using primer pair mentioned in Weisburg *et al*., 1991[13] (Table 1). The PCR-amplified products were resolved by gel electrophoresis on an 1.2% agarose (Sigma) gels (w/v) and stained with ethidium bromide (40 µg/ml) before the images were captured under a UV transilluminator (Gel Doc, Bio-Rad, USA). The 100 bp ladder (Invitrogen, UK) was used as the molecular weight marker.

Extraction of DNA fragments from agarose gel

The PCR-amplified products were purified from the gels as per the instructions of the manufacturer (FavorPrep™ gel /PCR purification kit, Taiwan).

Table 1. *Primers used in the study*

Species	Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
B. cereus group	BCGSH-1F	GTG CGA ACC CAA TGG GTC TTC	*groEL*	400	Park *et al.,* 2007)[10]
	BCGSH-1R	CCT TGT TGT ACC ACT TGC TC			
B. anthracis	BASH-2F	GGT AGA TTA GCA GAT TGC TCT TCA AAA GA	*gyrB*	253	-do-
	BASH-2R	ACG AGC TTT CTC AAT ATC AAA ATC TCC GC			
B. thuringiensis	BTJH-1F	GCT TAC CAG GGA AAT TGG CAG	*gyrB*	299	-do-
	BTJH-R	ATC AAC GTC GGC GTC GG			
B. cereus	BCJH-F	TCA TGA AGA GCC TGT GTA CG	*gyrB*	475	-do-
	BCJH-1R	CGA CGT GTC AAT TCA CGC GC			
B. mycoides	BMSH-F	TTT TAA GAC TGC TCT AAC ACG TGT AAT	*gyrB*	604	-do-
	BMSH-R	TTC AAT AGC AAA ATC CCC ACC AAT			
Universal	Forward (20F)	GAG TTT GAT CCT GGC TCA G	16S rRNA		Weisburg *et. al*., 1991[13]
	Reverse (1500R)	GTT ACC TTG TTA CGA CTT			

Sequencing of the Gel Purified DNA

The gel purified DNA products were sequenced in First Base Laboratories (Malaysia). Partial sequences, obtained using forward and reverse primers, were assembled to get desired partial sequences (1400–1500 bp) via the DNA Baser Assembler (v 4.36.0) and were aligned with sequences in the Gene Bank database of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) to identify phylogenetically related microorganisms. A phylogenetic tree was constructed by using partial 16S rRNA nucleotide sequences of the isolates and reference sequences through neighbour joining analysis by using the MEGA software. The percentage of replicate trees in which the associated taxa clustered together (bootstrap test = 1000 replicates) is shown next to the branches. The model to find evolutionary distance was set into Kimura 2-parameter method[14].

Identification by Multiplex PCR

Oligonucleotide primers

The primer sequences used for the multiplex PCR was retrieved from Park *et al.*, 2007[10] (Table 1), and were synthesized by the Bionics Corporation (Seoul, Korea).

Multiplex PCR Conditions

The multiplex PCR was performed in a thermocycler (Aeris™, 96 wells, Esco Micro Pte. Ltd., Singapore). The total reaction volume of 25 ìl of the PCR mixture contained 1×PCR buffer ($MgCl_2$ plus), 0.1 mM dNTP, 10 pmol/ìl of the primers, 25 ng template DNA, and 1 U Takara Ex Taq polymerase (TaKaRa, Shiga, Japan). The reaction parameters were as follows: an initial denaturation at 94! for 5 min, 30 cycles of amplification with denaturation at 94! for 30 sec, annealing at 63! for 30 sec and an extension at 72! for 30 sec, followed by a final extension at 72! for 5 min before leaving the reaction mixture at 4!.

Results

Analysis of Cultural and Biochemical Characteristics

The strains were inoculated on LB agar medium for 24 hours at 30°C to observe the colony characteristics. The colonies were white to off white in colour, opaque, having slightly raised elevation with regular margin (Figure 3). These colony characteristics were found consistent irrespective of isolates of *Bacillus cereus* group (*B. cereus*, *B. thuringiensis*, *B. mycoides*, *and B. anthracis*), *B. megaterium*, *B. coagulans* and *B. subtilis* on LB agar plates. They also showed similar characteristics when tested by cultural and biochemical techniques. This observation was found working on twenty-three pre-identified strains, chosen based on their molecular and cultural response on chromogenic media. Furthermore, most of them showed similar biochemical characteristics (Table 2). Identification of *B. mycoides* was not evidenced irrespective of techniques applied.

Fig. 1. *Colony characteristics of Bacillus cereus group isolate on LB agar plate*

Microscopic examination for spores and crystal proteins

Observation of crystal proteins and spores with the aid of phase contrast microscope is an important insight to distinguish the members of *Bacillus cereus* group. *B. cereus*, *B. anthracis*, *B. subtilis and B. megaterium* produce spores, while *B. thuringiensis* produce crystal proteins in addition (Figure 4). The parasporal crystal proteins were observed as spherical dark

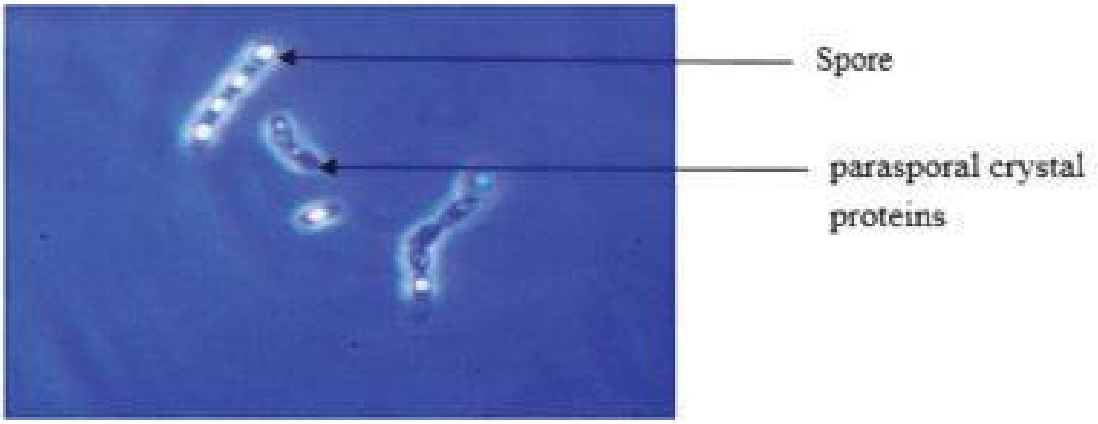


Fig. 2. *Phase contrast microscopic observation of B. thuringiensis Dsh4 upon sporulation.*

bodies beside the endospores. The spore position in the vegetative cells were sub-terminal.

Growth in chromogenic HiCrome^{TM} Bacillus Agar

Two hundred and fifty-seven *Bacillus* strains were cultured on HiCrome^{TM} *Bacillus* Agar with a view to separating them based on their chromogenic appearances. A representative figure of the cultural appearances for some of the isolates is presented in Figure 1 a, b and c. While the majority (38%, n=98) was constituted by *B. megaterium*, the selective appearance was noted for *B. coagulans* (25%), *B. cereus* (20%), *B. thuringiensis* (12%) and *B. subtilis* (3%); only 2% (n=5), however, failed to grow in the medium (Fig. 1, d).

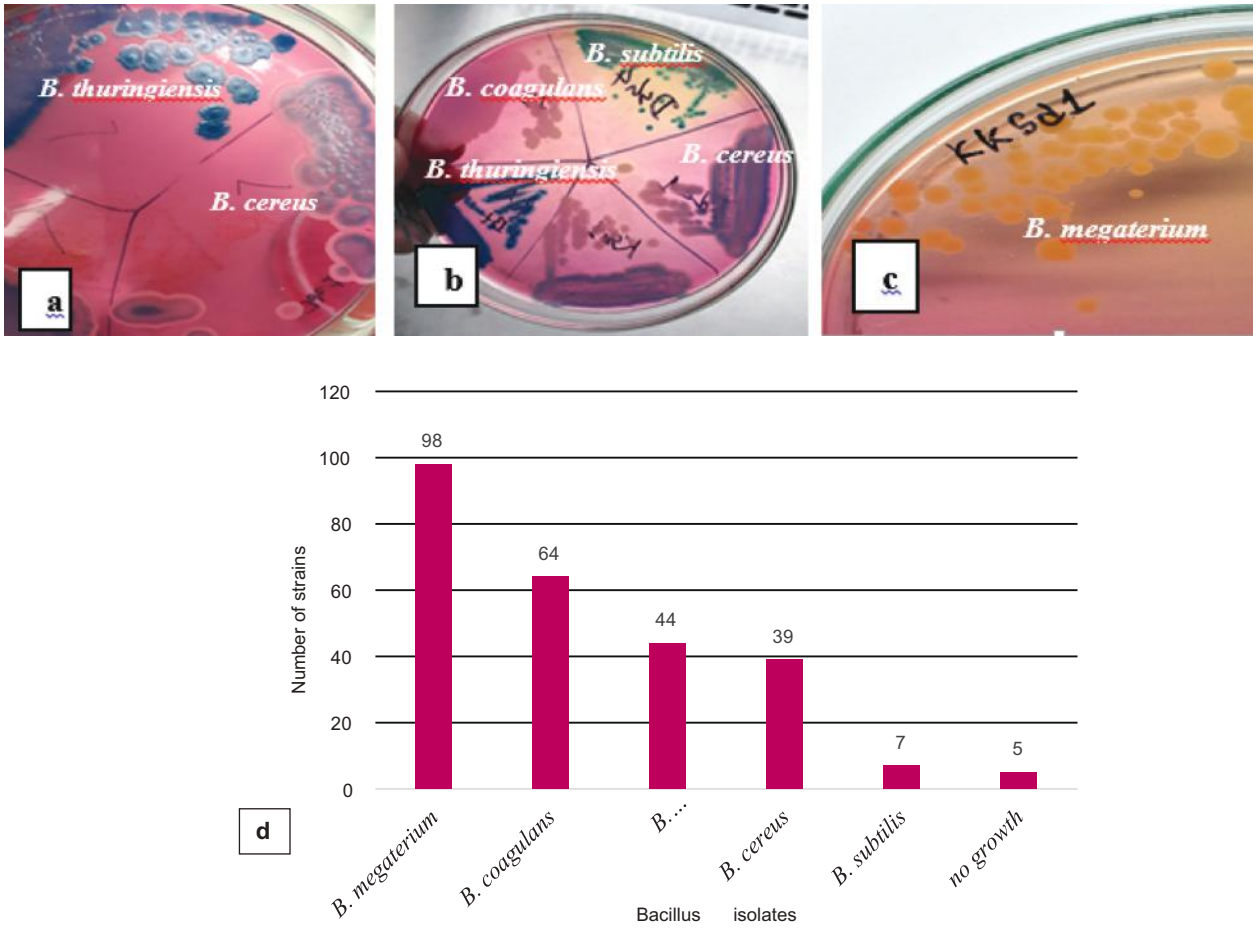


Fig. 3. *Distinguishing chromogenic colonies of different Bacillus isolates: B. thuringiensis Dsh1 and B. cereus MyIa1 (a), B. coagulans Jsc2, B. subtilis Rhsb3, B. cereus Kbsb2 and B. thuringiensis Ksa2 (b), and B. megaterium Kksd1 (c) grown on HiCrome^{TM} Bacillus agar. The distribution of bacterial isolates (n= 257) are presented (d).*

16S ribotyping

Fifteen potential isolates were chosen for 16S ribotyping in order to observe the effectivity of 16S ribotyping in differentiating species of the genus *Bacillus* with special emphasis to *B. cereus* group. Pre-identified *B. thuringiensis* (57s, 19s, 34L, Hd-73, 1i, 45L, Dsh4, leaf31, soil57, sotto), *B. cereus* (Sasb1, kksc1, kbsb2) and *B. anthracis* (Rhsb3, 22s) isolates were chosen for amplification of respective 16S rRNA gens for subsequent sequencing. Sequence alignment through NCBI database blast showed similarly sequenced microorganisms (Figure 4). In most of the cases, with different 16S partial RNA sequences of the isolates, BLAST results were similar producing no clue to be used in species differentiation.

A phylogenetic tree was constructed (Fig 5) from the partial sequences obtained based on NCBI-BLAST results by using MEGA 6 neighbour joining tree[15]. Kimura-2 parameter was taken as parameter to compute evolutionary distance where *Staphylococcus aureus* S33R (Accession no- NR-037007.2) served as theoutgroup. The tree produced no significant difference among the isolates including the reference strain, *B. thuringiensis* (HD-73, sotto), although they belonged to different species. Sequences of pre-identified isolates and other neighbouring strains of *B. cereus*, *B. thuringiensis* and *B. anthracis* obtained from NCBI-BLAST results shared similar sequences and were placed in a single position of the phylogenetic tree. Hence, no significant differences were demonstrated among the isolates of *B. cereus* group by 16S ribotyping.

Multiplex PCR analysis of the isolates

Five primer sets were selected to simultaneously detect 4 different species of the *B. cereus* group using a single multiplex PCR[10]. The annealing temperature of the multiplex PCR was optimized at 63! that enabled amplification of specific bands, such as, amplicons of 475, 299, and 253 bp for *B. cereus, B. thuringiensis* and *B. anthracis* respectively. The presence of a 400-bp amplicon was evident in all members of *B. cereus* group, which could otherwise serve as an internal control. The presence of a 604-bp product, specific for *B. mycoides* however, was not detected in any of the samples, indicating absence of the organism in the studied samples. Likewise, no band was evident from 165 strains. Absence of non-specific amplicons generated from the reaction mixtures attests the high specificity of the assay (Figure 2A). Based on the analyses, 39, 48 and 5 were detected as *B. cereus*, *B. thuringiensis* and *B. anthracis* respectively out of 257 strains tested. Overall, the PCR analysis matched mostly with that of the one produced from chromogenic culture media, except there was an increase of 9 bacteria scored in the *B. cereus* (Fig. 2B).

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_115526.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_043403.1
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_121761.1
Bacillus tropicus strain MCCC 1A01406 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157736.1
Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157735.1
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157734.1
Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157733.1
Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157732.1
Bacillus mobilis strain MCCC 1A05942 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157731.1
Bacillus luti strain MCCC 1A00359 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157730.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157729.1
Bacillus paranthracis strain MCCC 1A00395 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_157728.1
Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_152692.1
Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_114582.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_114581.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	99.35%	NR_115714.1

Fig. 4. *NCBI-BLAST result of Bacillus thuringiensis HD-73.*

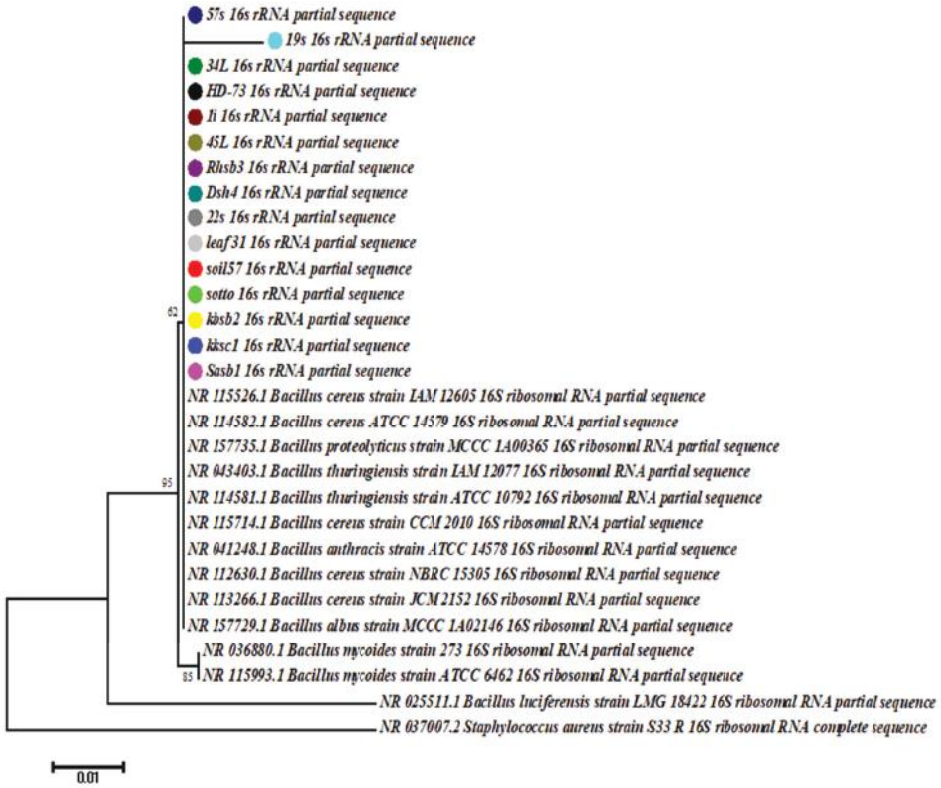
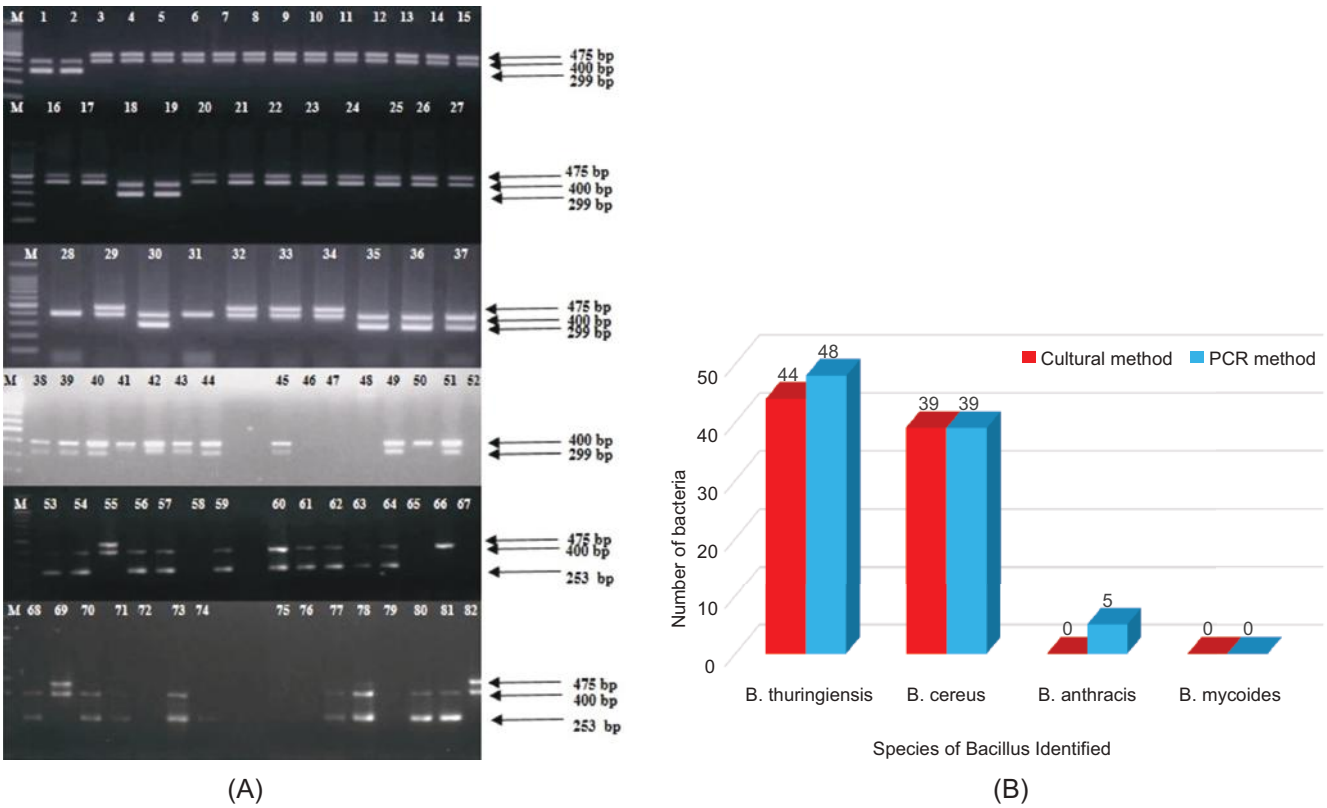


Fig. 5. *Phylogenetic tree based on the partial sequences of 16S rRNA genes of some selected isolates of Bacillus and corresponding references using MEGA6 (colour circle marks indicate the isolates).*



(A) (B)

Fig. 6. *(A) Representative illustration of 82 isolates of Bacillus cereus group in a multiplex PCR. B. cereus: lanes 3-17, 20-27, 29, 32-34, 55, 69, 82. B. thuringiensis: lanes 1, 2, 18, 19, 30, 35-37, 38-40, 42-45, 49, 51. B. anthracis: lanes 53, 54, 56, 57, 59-64, 68, 70, 71, 73, 74, 77, 78, 80, 81. B. cereus group: lanes 28, 31, 41, 50, 66.* Non *B. cereus group: lanes 46-48, 52, 58, 65, 67, 72, 75, 76, 79. (B) Comparative analysis between cultural and PCR-based methods for the identification of bacteria within B. cereus group.*

Discussion

The *Bacillus cereus* group members hold a number of pathogenic properties making them important as a research topic. Accordingly, there has been an effort to identify and differentiate these group members precisely in several reports. Among the various techniques employed so far, whole genome DNA hybridization[16], sequence analysis of 16S-23S operons[17], *gyrB-gyrA* intergenic spacer region[17], multilocus enzyme electrophoresis[18], pulsed-field gel electrophoresis (PFGE)[18], amplified fragment length polymorphism (AFLP)[19] and multiplex PCR analysis can be named. Considering the labour intensiveness, time requirement, required expertise and sensitivity, multiplex PCR has the potential to be the best choice among all the other methods. This method also has the advantage of simultaneous detection and differentiation amongst the species of this group. The present study used a previously described multiplex PCR method of Park *et al.* (2007) that used the "*gyrB"* DNA gyrase gene as the species specific sequence and "*groEL"* encoding molecular chaperonin as the group specific sequence. Using chromosomal genes like *groEL* and *gyrB* had the added advantage of not getting lost in the course of time like *cry* gene of *Bacillus thuringiensis* and toxin encoding pXO1 and capsule encoding pXO2 genes of *Bacillus cereus* that are plasmid borne. The four species specific primer pairs along with the group specific one showed excellent results in this present study. However, another study carried out by Forminska *et al.* (2012)[20] did not get the expected outcome although they used the same primers narrated by the study of Park *et al*. (2007)[10]. The successful differentiation of the species in the present study might have been possible as a result of combined observation of chromogenic agar colonies and the multiplex PCR method.

The non-responsive finding in the PCR amplification of the 165 strains could be the strains of *B. megaterium*, *B. coagulans* and *B. subtilis* which were identified by the cultural method. This identification was validated by appearance of no 'internal control' band for the *Bacillus cereus* group in the agarose gel electrophoresis, as these bacteria do not fall under this group. There were 4 strains in this study, which showed yellow colonies on HiCrome^{TM} *Bacillus* Agar medium and were identified as *B. megaterium*. However, these were identified as *B. thuringiensis* by the multiplex PCR method. Due to the similarity of growth colour on the chromogenic media, it was difficult to distinguish the thirteen strains and whether to identify them as *B. cereus* or *B. thuringiensis*. The multiplex PCR identified them as *B. thuringiensis*, hence, this method was found to be reliable. A similar multiplex PCR method has been described by Lee *et al*. (2008)[21] that used *groEL* sequence as the group marker and enterotoxin gene *nheA* and cereulide synthase gene *ces* to successfully identify and differentiate between enterotoxin producing and emetic toxin producing *Bacillus cereus* strains. In another study, Ogawa *et al*. (2015)[22] developed a multiplex PCR as a sensitive and practical method for detecting *B. anthracis* and also discriminated the virulence of this organism from other *B. cereus* group strains. These workers used three target genes namely *pag* on pXO1, *cap* on pXO2 and the Ba813 region on the chromosome as markers of *B. anthracis*. Since pXO1 and pXO2 are closely related to virulence of *B. anthracis* strains, possession of which was valuable information to know their virulence. Kim *et al*. (2005)[23] also successfully used a multiplex PCR method for rapid genotypic detection of *Bacillus anthracis* and the *Bacillus cereus* group and demonstrated that the four plasmid genotypes of *B. anthracis* and *B. cereus* group near-neighbors could be differentially and simultaneously discriminated by this assay.

Conventional usage of single PCR to detect *Bacillus* sp. individually is labour intensive, expensive and in many instances does not work well due to sequence variations in target genes[24]. A comparative analysis of the genomes of *B. cereus, B. anthracis*, and *B. thuringiensis* reveals that the chromosomes of these species are highly similar and syntenic, showing a conserved gene order. In addition, only a small subset of genes is considered unique to any species, most of which being annotated as hypothetical and located at the terminus of replication, indicating that genome plasticity occurs in this region[6]. The limitation can be overcome by establishing a multiplex-PCR assay that incorporates multiple specific primers that amplify simultaneously in a single PCR reaction. Under optimization (result not shown) of the single PCR reaction condition, the multiple primers require concordant annealing temperatures and reduction of any possible formation of primer dimers. The multiplex-PCR reaction system was carefully optimized to obtain maximal sensitivity and specificity in the presented study. When multiplex PCRs were performed with templates from *B. anthracis, B. thuringiensis, B. cereus, and B. mycoides* strains, only amplicons of the expected sizes were produced. In the multiplex PCR assays, an annealing temperature of 63°C proved to be (figure not shown) best suited for maximising amplification success as at lower and higher temperatures, some fragments were less effectively or not amplified when mixtures of DNA were tested. Surprisingly, amplification was successful also at lower/higher temperatures when templates of single strains were assayed in singleplex. The annealing time did affect the sensitivity of the present multiplex PCR system (figure not shown).

The Chromogenic medium used in the present study to differentiate *Bacillus cereus* group was assigned for identifying *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus coagulans*. On the other hand multiplex PCR based media could identify *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis* and *Bacillus mycoides* with an internal control (*groEL*) for *Bacillus cereus* group. As these two methods combined can identify a large range of *Bacillus* isolates, it is useful when there is a high number of *Bacilllus* isolates to be identified. From this chromogenic media based morphological study, 98 *Bacillus megaterium*, 64 *Bacillus coagulans* and 7 *Bacillus subtilis* were identified which could

not be determined through multiplex PCR as no primers were used for these species. On the other hand, five strains showing no growth on the chromogenic media were identified as *Bacillus anthracis* (insect 06, 22s, Rhsb3, Rdsc1, and Bysa2) by the multiplex PCR assay. As such, the combination test could identify and cover a larger variety of *Bacillus* isolates than the individual methods. As *Bacillus cereus* and *Bacillus thuringiensis* showed almost similar morphologies on the chromogenic media, a confusion was created in identifying 13 strains. However, after multiplex PCR, they were confirmed as *Bacillus thuringiensis*. Therefore, multiplex PCR worked here as the confirmatory test especially in differentiating *Bacillus cereus* and *Bacillus thuringiensis* as they are almost identical by phylogenetic, morphological and biochemical characteristics.

There was an anomaly in this combined test where four isolates (NaSb2, RaSb1, Sase1, and TaSa1) showed similar growth results like that of *Bacillus megaterium* on the chromogenic agar media but gave multiplex PCR results resembling *Bacillus anthracis*. This phenomenon can be considered as an unfortunate event of cross contamination among some isolates. As 4 isolates out of the 257 showed ambiguous results, the percentage of effectiveness of this combined test was 98.44% which indicated the significance and potentiality of this experiment.

Several conventional tests (biochemical tests, phase contrast microscopy, 16s ribotyping) were carried out to compare and evaluate the effectiveness of the combined test in differentiating *Bacillus* isolates. A series of biochemical tests were carried out on 23 pre-identified isolates. Maximum isolates showed desired test results although almost similar to each other, preventing these methods to be used as tools for their differentiation. *Bacillus cereus* and *Bacillus thuringiensis* isolates could not be differentiated by biochemical tests. There were 5 strains (Ssb1, Jdb1, Rasd1, Nasc3, 45s) of *Bacillus cereus* and *Bacillus megaterium* that showed ambiguous and overlapping biochemical results. The misinterpretation, complexity and small proximity in the difference of the results made these conventional methods less helpful in differentiating isolates of *Bacillus* sp. Phase contrast microscopy was used to differentiate only *Bacillus thuringiensis* isolates from other species because they produced visible crystal proteins with spores but sometimes it became tough to identify the crystal proteins under phase contrast microscope. The identification and differentiation of *Bacillus* sp. isolates on the basis of 16S rRNA gene sequencing has been earlier reported to be difficult[15]. In the present experiment, 15 isolates of *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus anthracis* were used to construct a phylogenetic tree with neighbouring microorganisms (obtained from NCBI-BLAST) where all the experimental isolates were placed in a single branch of the tree. This result indicated that 16s ribotyping was also ineffective in significant differentiation among the *Bacillus* isolates. The *gyrB* gene has been proven to be a useful phylogenetic discriminator for members of the *Bacillus cereus* group[25].

Conclusion

Phenotypic characteristics and 16s ribotyping analysis of the *Bacillus* isolates could not produce effective and meaningful results, while multiplex PCR of chromosomal marker *gyrB* in combination with the growth results of isolates on chromogenic media produced significant, reliable and reproducible results and this combination created a new effective way to differentiate among isolates of *Bacillus* sp.

References

1. Liu Y, Lai Q, Göker M, Meier-Kolthoff JP, Wang M, Sun Y, Wang L and Shao Z. 2015. Genomic insights into the taxonomic status of the *Bacillus cereus* group. *Sci Rep* 5: 14082 10.1038/srep14082.
2. Ehling-Schulz, M, Lereclus D and Koehler TM. 2019. The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential. In Gram-Positive Pathogens (eds V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portnoy, M. Braunstein and J.I. Rood).
3. Chang YH, Shangkuan YH, Lin HC, and Liu HW. 2003. PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. *Appl. Environ. Microbiol*. 69: 4502-4510.
4. Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carm agnola L, Brusetti L, Rizzi A, Chanishvili N, Sharp R, and Borin S. Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. *Appl. Environ. Microbiol*. 2006;72:1295-1301.
5. Henderson I, Duggleby CJ, and Turnbull PC. Differentiation of *Bacillus anthracis* from other *Bacillus cereus* group bacteria with the PCR. *Int. J. Syst. Bacteriol*. 1994;44:99-105.
6. Rasko DA, Altherr MR, Han CS and Ravel J. 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev.* 29: 303–329.
7. Helgason E, Økstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I and Kolstø AB. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl Environ Microbiol.* 2000;66:2627–2630.
8. Ash C, Farrow JA, Dorsch M, Stackebrandt E and Collins MD. Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int. J. Syst. Bacteriol*. 1991;41:343-346.
9. Mortimer PR and McCann G. 1974. Food-poisoning episodes associated with *Bacillus cereus* in fried rice. 1(7865): 1043-5.
10. Park SH, Kim HJ, Kim JH, Kim TW and Kim HY. Simultaneous detection and identification of *Bacillus cereus* group bacteria using multiplex PCR. *J Microbiol Biotechnol.* 2007;17(7):1177-82.
11. Shishir M, Roy A, Islam N, Rahman A, Khan SN and Hoq MM. Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their cry genes profile. *Front. Environ. Sci.* 2014;2:20.
12. Bravo A, Sergio S, Lorena L, Hernesto O, Carolina A, Anabel O, Miriam O *et al*. Characterization of cry genes in a Mexican *Bacillus thuringiensis* strain collection. Applied and environmental microbiology. 1998;64(12):4965-4972.
13. Weisburg W G, Barns SM, Pelletier DA and Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*. 1991;173(2):697-703.
14. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*. 1980;16(2):111–120.

15. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 2013;30(12):2725-2729.

16. Ash CJ, Farrow JAE, Dorsch M, Stackebrandt E and Collins M. Comparative Analysis of *Bacillus anthracis, Bacillus cereus*, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA. *International journal of systematic bacteriology*. 1991;41(3):343-346.

17. Harrell LJ, Andersen GL and Wilson KH. Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol*. 1995; 33(7):1847-50.

18. Carlson CR, Caugant DA, Kolstø AB. Genotypic Diversity among *Bacillus cereus* and *Bacillus thuringiensis* Strains. *Appl Environ Microbiol*. 1994;60(6):1719-25.

19. Chang YH, Shangkuan YH, Lin HC and Liu HW. PCR assay of the *groEL* gene for detection and differentiation of *Bacillus cereus* group cells. *Appl Environ Microbiol*. 2003;69(8):4502-10.

20. Formiñska K, Zasada AA and Jagielski M. Evaluation of multiplex PCR to identify the species of microorganisms from *Bacillus cereus* group. *Med Dosw Mikrobiol*. 2012;64(2):101-8.

21. Lee D, Kim K, Kwon K and Hong K. A Multiplex PCR Assay for the Detection and Differentiation of Enterotoxin-producing and Emetic Toxin-producing *Bacillus cereus* Strains. *Food Science and Biotechnology*. 2008;17(4):761-765.

22. Ogawa H, Fujikura D, Ohnuma M, Ohnishi N, Hang'ombe BM, *et al*. A Novel Multiplex PCR Discriminates *Bacillus anthracis* and its Genetically Related Strains from Other *Bacillus cereus* Group Species. *PLOS ONE.* 2015;10(3):e0122004.

23. Kim K, Seo J, Wheeler K, Park C, Kim D, Park S, Kim W, Chung SI and Leighton T. Rapid genotypic detection of *Bacillus anthracis* and the *Bacillus cereus* group by multiplex real-time PCR melting curve analysis. *FEMS Immunol Med Microbiol*. 2005;43(2):301-10.

24. Hansen BM and Hendriksen NB. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl Environ Microbiol*. 2001;67(1):185-9.

25. van Tongeren SP, Roest HI, Degener JE & Harmsen HJ. *Bacillus anthracis*-like bacteria and other *B. cereus* group members in a microbial community within the International Space Station: a challenge for rapid and easy molecular detection of virulent *B. anthracis*. *PLoS One*. 2014;9(6):e98871.