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PHENOTYPIC CHARACTERIZATION, VIRULENCE POTENTIAL, AND GENETIC DIVERSITY OF *BACILLUS CEREUS* ISOLATED FROM RAW COW'S MILK OF SOME EGYPTIAN DAIRY FARMS

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ABSTRACT

This study aimed to achieve the incidence, phenotypic characterization, virulence potential, and genetic diversity of *B. cereus sensu lato* group in raw bulk tank milk (BTM) and mastitic milk, with special concern to the *B. cereus sensu stricto (s.s.).* Milk samples including122 bulk tank milk (BTM) and 67 clinically mastitic cow's milk were screened for the existence of *B. cereus s.l.* group. *B. cereus s.s.* were isolated and PCR-confirmed with a prevalence of 31.7%. Thirty-two isolates displayed typical morphology, while 28 isolates displayed atypical morphology. Among isolates, 93.4, 46.7 and 85% were hemolytic, biofilm, and slime producers, respectively. The most prevalent toxin genes were *cyt-k*, *bceT* and *nhe* genes in percentages of 86.7, 85, and 46.7%, respectively. One isolate harbored *ces*-gene (1.7%). Also, 53.3% and 50 % of *B. cereus s.s.* isolates harbored *tasA* and *sipW* genes, respectively. Comparing typical and atypical isolates revealed that 100% of typical isolates were hemolytic versus 85.7% of atypical isolates, 53.1% and 78.1% of typical isolates were biofilm and slime producers versus 39.3% and 92.9% of atypical ones. Nine toxin gene profiles were found. Prevalences of toxin and biofilm-related genes in typical isolates were 53.1, 84.4, 93.8, 0, 46.9 and 46.9 % for *nhe*, *cyt K*, *bceT*, *ces*, *tasA*, and *sipW* genes versus 39.3, 89.3, 75, 3.6, 60.7 and 53.57% in atypical isolates. The obtained finding demonstrated the presence of potentially pathogenic *B. cereus s.s*. in milk. The isolation of atypical forms of *B. cereus s.s*. which weren't less virulent than the typical form was confirmed. Therefore, efforts should be made to prevent their misdiagnosis in dairy plants.

Keywords: *B. cereus s.s.*, BTM, mastitis, PCR, typical, atypical colony morphology, hemolysis, biofilm, slime production, toxin genes, biofilm-related genes.

INTRODUCTION

Milk is a vital component of the human diet due to its nutritional content. However, it is a good medium for the enhancement of many bacterial contaminants (Adamski *et al.,* 2023). Several factors can affect the level of contamination, such as animal health status, farm hygiene, milking hygiene, as well as the storage temperature of milk (Yuan *et al*., 2022). Raw milk containing these pathogens can be considered a threat to consumers (Rolbiecki and Harnisz, 2022). Among the predominant microbial pathogens present in raw milk is the psychrotrophic *Bacillus spp.* particularly *Bacillus cereus (B. cereus)*. *B.*

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cereus is a complex species group characterized by increased phenotypic and genotypic similarities. This bacterial group can cause dairy product spoilage, as they can produce proteolytic and lipolytic enzymes, causing undesirable adverse changes in milk and causing food-borne outbreaks (Owusu-Kwarteng *et al*., 2017; Nassib *et al*., 2018; Liu *et al*., 2020).

B. cereus is one of the major mastitis-causing pathogens in dairy cows. Contaminated udders can finally result in the existence of *B. cereus* in raw milk. As these bacteria exist everywhere in dairy farm environments, they can infect the mammary gland either during lactation or dry cow therapy using unsterilized intra-mammary injections. Moreover, some *Bacillus spp*. can induce gangrenous mastitis (Alnakip *et al*., 2014; Meng *et al*., 2022; Eid *et al*., 2023). Spore-formers, especially the *B. cereus sensu lato (s.l.)* group, include seven species including *B. cereus sensu stricto (s.s.)*, considered significant contaminants of the dairy environment. It also correlates to milk's value and safety (Kumari and Sarkar, 2016; Huang *et al*., 2020).

B. cereus causes many problems in dairy industries due to many facts. First is its ability to form heat-resistant spores in milk. Second, its ability to grow in a wide variety of dairy products. Moreover, the ability to produce toxins (Markland *et al*., 2013). Also, *B. cereus* can stick to surfaces to form biofilms, which has a special focus and is of great importance for manufacturers, since it causes a risk to public health (Galié *et al*., 2018). Some strains of *B. cereus* are foodborne pathogens and their distinct toxins can induce diarrhea and/or emesis. Thus, the persistence of biofilms is of alarm to the food industry (Lin *et al*., 2022).

The pathogenic potential and virulence characteristics of *B. cereus* are complex. They are based primarily on the secretion of toxins and tissue-destructing exoenzymes (Senesi *et al*., 2010; Ehling *et al*., 2019). These virulence characteristics include biofilm formation, secretion of hemolysins, cytotoxins, trimeric complexes, proteases, enterotoxins including enterotoxin FM, diarrheal toxin Bcet, and enterotoxin, and phospholipases. The presence of lipases and proteases producing *B. cereus* strains could initiate food degradation and spoilage. This can eventually result in great economic losses for the producers (Gopal *et al*., 2015).

This study aimed to achieve the incidence of *B. cereus s.l.* group in raw bulk tank milk (BTM) and mastitic milk with special concern to the *B. cereus s.s.*. Also, to investigate the morphological diversity of *B. cereus s.s.* on the chromogenic medium. Moreover, spot the light on their pathogenic potential including hemolysin production, biofilm-forming ability, and slime production. Molecular detection of different toxins and biofilm-related genes was performed.

MATERIALS AND METHODS

1. Collection of raw milk samples:

A total of 189 milk samples were obtained from different Egyptian dairy farms among different localities including Alexandria, El-Beheira, Monufia, Gharbia, Ismailia, Giza, El Faiyum, Beni Suef, and Assuit governorates. Samples included 122 BTM and 67 individuals with clinically mastitic milk. All specimens were collected aseptically and transported to the laboratory immediately on ice bags. The animal manipulation and sample collection methods were approved by the Committee of Institutional Animal Care and Use of the Agricultural Research Center (ARC-ARRI- 74-24).

2. Enumeration, isolation and identification of *B. cereus*:

2.1. *B. cereus* culture and their morphological characteristics on selective HiCrome Bacillus agar.

One milliliter of each BTM sample was cultivated immediately on HiCrome Bacillus agar (HiMedia, India) after serial dilution for *B. cereus* counting according to Fuchs *et al*. (2022) while mastitic milk samples were enriched at 30°C for provocation, then ten

microliters were cultivated to guarantee detection of *B. cereus* group. Milk samples were grown on HiCrome Bacillus agar, and incubated at 30°C for 24-48 h. The plates were screened for bacterial growth and colony morphology. Colonies with typical morphology as depicted in the medium instructions were selected and recorded. Also, atypically grown colonies were recorded and selected for further examination. Cell shape and Gram's reaction were declared using the microscopical examination of Gram-stained smears. Moreover, motility and other biochemical examinations were applied according to Aryal (2022). The typical and atypical colonies primarily suspected to be *B. cereus* were stored in glycerol 10% at -20 ^{o}C for further examinations.

2.2. Molecular confirmation:

2.2.1.DNA extraction.

Crude DNA was extracted from bacterial isolates using the Chelex-100 method as reported by Gdoura-Ben Amor *et al.* (2018). Many loopfuls of bacteria grown on the brain heart infusion agar plate were harvested and dispersed in 200 µl of 5% Chelex-100. The suspension was boiled for 10 min followed by 10 min centrifugation to precipitate bacterial debris. The supernatant was collected and stored at -20˚C to be used as crude template DNA for PCR amplification**.**

2.2.2. Primers.

Different primer pairs were used in this study. The information concerning the used primers is depicted in Table (A).

To confirm the isolates to be *B. cereus* group *s.l.* species, amplification of the gene encoding the flagellar motor protein (*motB* gene) was performed at first using BCFomp1 and BCRomp1 primers according to Oliwa-Stasiak *et al*. (2010). To confirm the isolate to be *B. cereus s.s.,* the genus conserved gyrB gene was amplified using the BC1 and BC2r primers. Also, to exclude any *B. thuringiensis*, the cry gene was amplified using K3 and K5 primers. The three single PCR reactions were established in 20 µl reaction volume containing 5µl of DNA as template, 20 pmol of each primer, and 1X of PCR master mix (Dream Taq Green PCR Master Mix, ThermoFisher). The amplification cycles were carried out in SimpliAmp Thermal Cycler (ThermoFisher Scientific). Reaction conditions were optimized to be 94°C for 4 min. as initial denaturation, followed by 35 cycles of 94°C for 30 seconds, specific annealing temperature of each primer pair for 30 seconds and 72 °C for 30 seconds. A final extension step at 72°C for 10 min. was followed. Each run included positive and negative controls. Amplification products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. A 100bp DNA marker was run simultaneously to ensure that the PCR products were of the correct size.

3. Detection of phenotypic virulence characteristics of *B. cereus*:

Only *B. cereus s.s.* PCR-confirmed isolates were screened for different phenotypic virulence characteristics including hemolytic activity, biofilm and slime-forming abilities.

3.1. Assessment of hemolytic activity.

The hemolytic activity was evaluated by culturing isolates of *B. cereus s.s.* on blood agar containing 5% sheep blood (Oxoid, UK). After the plates were incubated at 30° C for 18-24 h, the production of hemolysin was visually checked for the existence of an incomplete or complete halo zone of hemolysis surrounding the colonies. The strains were recorded as α, β or non-hemolytic strains according to Senesi and Ghelardi (2010).

3.2. Assessment of biofilm-forming ability.

Pure bacterial isolates were grown overnight at 37^{o}C on blood agar. Three colonies were suspended in 3mL Tryptone Soya broth (TSB) and incubated at 37^{o}C for 24 h. This broth was diluted to (1: 40) using a fresh TSB ($2–7 \times 10^{7}$ cfu/mL) to be used as the inoculum

in the microtiter plate test. The test was accomplished according to both Dubravka *et al.* (2010) and Darwish and Asfour, (2013). Also, strains were interpreted according to their optical density (OD) as directed by the same authors.

3.3. Assessment of slime production.

Congo Red Agar (CRA) plate method was used to assess the capability of *B. cereus s.s.* to form slime (Mathur *et al*., 2006; Darwish and Asfour, 2013). Tryptic Soy agar containing 0.08% Congo red (Sigma) was used to prepare CRA plates. Isolates were inoculated on the plates and incubated at 37ºC for 24 h in aerobic conditions. Incubation at room temperature for 48 h was followed. Interpretation of results was performed according to colony morphology and consistency. Isolates were considered positive slime producers when colonies appeared black, dry with rough surfaces and edges. When colonies appeared as either black colonies with smooth, rounded, shiny surfaces or red colonies of dry consistency with rough edges and surfaces, the isolates were evaluated as intermediate slime producers. Red colonies with smooth rounded and shiny surfaces were considered negative.

4. Molecular detection of toxins and biofilm-related-genes:

Only *B. cereus s.s.* confirmed isolates were screened for different toxins and biofilm-related genes using specific primer pairs in single and multiplex PCR assays (Table A). Single PCR reactions were established in 20 µl reaction volume, as mentioned above. Multiplex PCR reactions were performed in 40 µl reaction volume containing 30 pmol of each primer and 1X of PCR master mix (Dream Taq Green PCR Master Mix). Reaction conditions were performed as mentioned above. To validate the results, all PCR amplifications were performed twice. To exclude false negative cases, only isolates that give good amplification signals using the genus-specific primers were used for screening toxins and biofilm-related genes.

Table A: Primer sequences, PCR product size, species and/or target gene, and annealing temperatures of different PCR assays.

Name	Sequence 5'-3'	Species&/or gene-specific	T_a ·c	PCR product size (bp)	Reference
BCFomp1 BCRomp1	ATCGCCTCGTTGGATGACGA CTGCATATCCTACCGCAGCTA	*B. cereus* group specific (*motB* gene)	54.5	575	Oliwa-Stasiak *et al.* (2010)
BC1 BC2r	ATTGGTGACACCGATCAAACA TCATACGTATGGATGTTATTC	*B. cereus s.s.* specific (*gyrB* gene)	58	365	Yamada *et al*. (1999)
K3 K5	GCTGTGACACGAAGGATATAGCCAC AGGACCAGGATTTACAGGAGG	*B. thuringiensis* (*cry* gene specific)	56	1600-1700	Kuo and Chak (1996)
mPCR: HD2 F HA4 R	GTAAATTGATGAICAATTTC AGAATAGGCATTCATAGATT	*hbl* gene	49	1091	Ehling-Schulz *et al.* (2006)
NA2 F NB 1 R	AAGCIGCTCTTCGIATTC ITIGTTGAAATAAGCTGTGG	*nhe*-gene		766	
CK F2 CK R5	ACAGATATCGGICAAAATGC CAAGTIACTTGACCIGTTGC	*cyt*K gene		421	
CesF 1 CesR 2	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	*ces gene*		1271	Ehling-Schulz *et al.* (2005)
bceT F bceT R	TTACATTACCAGGACGTGCTT TGTTTGTGATTGTAATTCAGG	*bce*T gene	58	428	Agata *et al.* 1995
tasAF tasAR	AGCAGCTTTAGTTGGTGGAG GTAACTTATCGCCTTGGAATTG	*tas*A gene	59	488	Caro-Astorga *et al*., 2015
SipW F sipW R	AGATAATTAGCAACGCGATCTC AGAAATAGCGGAATAACCAAGC	*sip*W gene	54	488	

RESULTS

Prevalence of *B. cereus* in milk samples

A total of 189 milk samples were screened for the presence of *B. cereus* group bacteria. Eighty-eight samples produced *B. cereus*-like colonies on HiCrome medium, giving a total of 88 presumptive *B. cereus* isolates. The percentage of probable *B. cereus* group bacteria in both individual and BTM samples is shown in Table (1). *B. cereus* count on HiCrome Bacillus agar media in BTM varied in positive samples from 20 to 200 cfu/ml of milk.

For molecular identification of *B. cereus s.s.*, each isolate was subjected to 3 PCR tests. One was specific for *B. cereus s.l.* group, the second was specific for *B. cereus s.s.* and the third one was specific for *B. thrungensis.*

Based on the PCR results, 78 out of 88 *B. cereus-like* isolates amplified the 575 bp amplicons, and therefore were confirmed to be *B. cereus s.l.* with an overall prevalence of 41.3% (Table 1). In comparison, the other 10 isolates (5.3%) did not amplify the specific amplicon and were therefore excluded from the *B. cereus s.l.* group. Fig.1A shows the specific 575 bp PCR products of *motB* gene of *B. cereus s.l.* group of positive representative *B. cereus s.l.* isolates. Moreover, 60 isolates from the 78 amplified the specific 365 bp fragment and were confirmed to be *B. cereus s.s.* with an overall prevalence of 31.7%. They were also negative using the *cry* gene primers specific to *B. thrungenesis*. The remaining 18 isolates (9.5%) did not amplify the specific PCR product of *B. cereus s.s.* and so were assumed to belong to the other *B. cereus s.l.* group but not *sensu stricto*. All PCR reactions were performed twice to ensure the reproducibility of the results. Fig. (1B) shows the 365bp PCR product of *gyrB* gene of *B. cereus s.s*. specific isolates.

All *B. cereus s.s.* PCR-confirmed isolates were screened for their colony's morphological characteristics. As shown in Table (2), 32 isolates displayed typical morphology, while 28 isolates displayed atypical morphological characteristics. Isolates that displayed atypical morphology were divided according to their colony morphology into 3 forms a, b, and c. The characteristics of colony morphology of all forms are shown in Fig. (2). Fig. (2A) shows the typical colony morphology of *B. cereus s.s.* on HiCrome Bacillus agar medium, which appeared as large flat (red arrow) and small atypical pinpoint colonies (black arrow), both of them with blue centers. The atypical form (a) appeared as small bluish-green colonies with a yellow background (Fig. 2B). The atypical form (b) appeared as small blue to bluish-green colonies with a pink background (Fig. 2C). Lastly, the shape of the colony of the atypical form (c) was recorded to be large light greenish with smaller bluish-green colonies in between on HiCrome ™Bacillus agar (Fig. 2D).

Phenotypic virulence characteristics

B. cereus s.s. PCR-confirmed isolates were examined for their virulence characteristics, including their hemolytic activities, biofilm-forming ability, and slime production. Based on hemolytic activities, 31 (51.7%) isolates showed α-hemolysis, while 25 (41.7%) showed β-hemolysis. Only 4 (6.6%) isolates were non-hemolytic. Considering biofilm-forming abilities, 28 isolates produced biofilm with different grades (46.7%).

As shown in Table (2); 15, 7 and 6 isolates out of the 60 *B. cereus s.s.* isolates were interpreted as strong, moderate, and weak biofilm producers, while 32 (53.3%) isolates were non biofilm producers. In general, 28 isolates were biofilm-positive in a percentage of 46.7%. Concerning slime production, 26 and 25 isolates were strong and moderate slime producers, with a total percentage of 85 (51/60). Whereas 9 isolates were non-slime producers, as depicted in the same table. Morphology of strong, moderate and negative slime producers are shown in Fig.(3).

Prevalence of Toxin genes

All PCR-confirmed *B. cereus s.s.* isolates were screened for the presence of five toxin

genes, including hemolytic enterotoxin gene (*hbl*), non-hemolytic enterotoxin gene (*nhe*), cytotoxin k-gene (*cytK*), enterotoxin T gene (*bceT*), and cerulide toxin gene (*ces*). The numbers of isolates positive for the investigated genes are depicted in Table (3). None of the isolates harbored the *hbl*-gene while the *cytk, bceT* and *nhe* genes are the most prevalent; 86.7, 85 and 46.7%, respectively (Table 3). Only one isolate from bulk tank milk samples harbored the *ces*-gene (1.7%). Fig. (4B) showed the amplified 1271bp, 766bp and 421 bp PCR products of *B. cereus s.s*. toxins *hbl, nhe, and cytK* genes, respectively, while Fig. (4B) showed the amplified 428bp PCR products of *bceT* gene.

Prevalence of biofilm related genes

B. cereus s.s. confirmed isolates were screened for the presence of two biofilm-related genes, namely *tasA* and *sipW* genes. As depicted in Table (3), 53.3% and 50% of isolates harbored the *tasA* and *sipW* genes, respectively. Fig. (5) showed the amplified PCR products of both *tasA* and *sipW* genes in *B. cereus s.s.* isolates.

Distribution of virulence characteristics among *B. cereus s.s.* isolates with diverse colony morphology

The distribution of virulence characteristics among *B. cereus s.s.* PCR-confirmed isolates with diverse morphological characteristics are shown in Table (4). When isolates with typical colony morphology were compared with those of atypical colony morphology, 100% of typical isolates were α and β hemolytic, versus 85.7% of the total atypical isolates. Also, 53.1% and 78.1% of the typical isolates were biofilm and slime producers, versus 39.3% and 92.9% for the atypical isolates, respectively. The detailed number and percentages of each virulence characteristic among different isolates with different colony morphology are depicted in Table (4).

Distribution of toxin and biofilm-related genes among *B. cereus s.s.* isolates with diverse colony morphology

The distributions of PCR-positive toxin and biofilm-related genes among *B. cereus s.s.* PCR-confirmed isolates with diverse typical and atypical colony morphology were declared in Table (5). The percentages of toxin and biofilm-related genes in typical isolates were 53.1, 84.4, 93.8, 0, 46.9 and 46.9 % for *nhe, cyt K, bceT, ces, tasA, and sipW* genes versus 39.3, 89.3, 75, 3.6, 60.7 and 53.57% in total atypical isolates, respectively. The detailed numbers and percentages of each gene in both typical and atypical isolates were cleared in Table 5.

Toxin gene profiling of *B. cereus s.s.* isolates

The results revealed that 58/60 of *B. cereus s.s.* isolates carried a minimum of one of the toxin-associated genes, with a percentage of 96.67%, while only 2 (3.33%) didn't harbor any toxin gene. According to the positive toxin genes, nine toxin-gene profiles were found and named A, B, C, D, E, F, G, H, and I. The positive toxin genes of each profile are mentioned in Table (6). Moreover, Table (6) displayed the specific toxin gene profiles of PCR-confirmed *B. cereus s.s*. isolates of both typical and atypical colony morphology. Both profiles B and C were the most common profiles, with percentages of 35 and 40%, respectively, while profiles A and G were the lowest two profiles, each with 1.67%. Toxin profile C is the dominant profile among typical (43.8) and atypical (35.7) isolates.

Phenotype-genotype correlations

The correlation between all screened phenotypes and genotypes was statistically analyzed against one another using Pearson's correlation analysis of the presence/absence data of all assays.

The association between the presence of both *tasA* and *sipW* genes was the most observed. Its correlation coefficient was 0.87 due to the presence of a strong congruence with a statistically significant dependence at p-value ≤ 0.001.

Other moderate and significant positive correlations were observed between both the

tasA and the *sipW* genes and *cytK* gene, with Pearson's correlation coefficients of 0.45 and 0.42, respectively, at p-value ≤ 0.001.

A slight and significant negative correlation was also noticed between slime formation and *cytK* gene, with a Pearson's correlation coefficient equal to 0.37 at p-value ≤ 0.005. The correlation between *tasA* and/or *sipW* genes and biofilm formation was slight and negative, but not significant.

Negative correlation between hemolysis and the presence of both *ces* and *nhe* genes, with Pearson's correlation coefficient equal – 0.29 and – 0.3 at p-value ≤ 0.05.

In general, the correlation between all the studied phenotypes and virulence genes detected by PCR was displayed as a correlation plot highlighting the significant association and other subtle correlations (Fig. 6).

Table 1: Incidence of *B. cereus sensu stricto* in the collected milk samples.

Type of samples	No. of samples	Primary isolates	*B. cereus group sensu lato* PCR-confirmed isolates		*B. cereus sensu stricto* PCR-confirmed isolates	
			No.	%	No.	%
Individual mastitic milk	67	40	36	53.7	31	46.3
BTM	122	48	42	34.4	29	23.8
Total	189	88	78	41.3	60	31.7

Table 2: Prevalence of potential pathogenicity of *B. cereus sensu stricto* isolates.

B. cereus sensu stricto PCR-confirmed isolates		Morphology				Hemolysis			Biofilm				Slime		
Source of samples	No.	Typical	atypical			α	β	N	S	M	W	N	S	M	N
			a	b	c										
Individual mastitic milk	31	23	2	2	4	20	11	-	10	4	2	15	14	14	3
BTM	29	9	8	4	8	11	14	4	5	3	4	17	12	11	6
Total (%)	60	32 (53.3)	28 (46.7)			56 (93.3)		4 (6.7)	28 (46.7)			32 (53.3)	51 (85)		9 (15)

S; strong, M; moderate, W; weak, N; negative

Table 3: Distribution of positive toxins and biofilm-related genes in *B. cereus sensu stricto* PCR-confirmed isolates from individual mastitic and bulk tank milk samples

Type of samples	*B. cereus s.s.* PCR-confirmed isolates	No. of positive Toxins-related genes (%)					No. of positive biofilm-related genes (%)	
		hbe gene	*nhe gene*	*cytk gene*	*bceT gene*	*ces gene*	*tasA* gene	*sipW* gene
Individual mastitic Milk	31	-	21 (67.7)	29 (93.5)	30 (96.7)	-	15 (48.3)	15 (48.3)
BTM	29	-	7 (24.1)	23 (79.3)	21 (72.4)	1 (3.44)	17 (58.6)	15 (51.7)
Total	60	-	28(46.7)	52 (86.7)	51 (85)	1(1.7)	32 (53.3)	30 (50)

Table 4: Distribution of virulence characteristics amongst *B. cereus sensu stricto* PCR-confirmed isolates with different colonial morphology.

Colonial morphology	*B. cereus s.s.* PCR-confirmed isolates	Phenotypic virulence characteristics												
		Hemolysis No. (%)				Biofilm No. (%)				Slime No. (%)				
		α	β	Total hemolysis	non	S	M	W	Total positive	non	S	M	Total positive	non
Typical	32	16 (50)	16 (50)	32 (100)	0 (0)	10 (31.2)	4 (12.5)	3 (9.4)	17 (53.1)	15 (46.9)	11 (34.4)	14 (43.7)	25 (78.1)	7 (21.9)
Total atypical	28	15 (53.6)	9 (32.1)	24 (85.7)	4 (14.3)	5 (17.9)	3 (10.7)	3 (10.7)	11 (39.3)	17 (60.7)	15 (53.6)	11 (39.3)	26 (92.9)	2 (7.10)
Atypical a	10	4 (40)	3 (30)	7 (70)	3 (30)	1 (10)	2 (20)	0 (0)	3 (30)	7 (70)	5 (50)	3 (30)	8 (80)	2 (20)
Atypical b	6	3 (50)	2 (33.3)	5 (83.3)	1 (16.7)	2 (33.3)	0 (0)	1 (16.7)	3 (50)	3 (50)	4 (66.7)	2 (33.3)	6 (100)	0 (0)
Atypical c	12	8 (6.7)	4 (33.3)	12 (100)	0 (0)	2 (16.7)	1 (8.3)	2 (16.7)	5 (41.7)	7 (58.3)	6 (50)	6 (50)	12 (100)	0 (0)

Table 5: Distribution of PCR-positive toxins and biofilm-related genes amongst *B. cereus sensu stricto* PCR-confirmed isolates with different colonial morphology

Colonial morphology	*B. cereus* s.s. PCR-confirmed isolates	No. (%) of PCR-positive toxin and biofilm-related genes					
		nhe gene	*cytk* gene	*bceT* gene	*ces* gene	*tasA* gene	*sipW* gene
Typical	32	17 (53.1)	27 (84.4)	30 (93.8)	0 (0)	15 (46.9)	15 (46.9)
Total atypical	28	11 (39.3)	25 (89.3)	21 (75)	1 (3.6)	17 (60.7)	15 (53.57)
Atypical a	10	4 (40)	8 (80)	8 (80)	0 (0)	3 (30%)	3 (30)
Atypical b	6	4 (66.7)	6 (100)	6 (100)	1 (16.7)	5 (83.3)	5 (83.3)
Atypical c	12	3 (25)	11 (91.7)	7 (58.3)	0 (0)	9 (95)	7 (58.3)

Table 6: Distribution of toxin gene profiles among *B. cereus sensu stricto* PCR-confirmed isolates with different colonial morphology

Morphology	Total no of isolates	A	B	C	D	E	F	G	H	I
		bceT+, ces+, cytK+ nhe+ No. (%)	*bceT+, cytK+ nhe+* No. (%)	*bceT+, cytK+* No. (%)	*bceT+, nhe+* No. (%)	*nhe+, cytK+* No. (%)	*bceT+* No. (%)	*nhe+* No. (%)	*cytK+* No. (%)	All genes are negative
Typical	32	0(0)	13(40.6)	14(43.8)	3(9.4)	0(0)	0(0)	1(3.1)	0(0)	1(3.1)
Atypical Total	28	1(3.6)	8(28.6)	10(35.7)	0(0)	2(7.1)	2(7.1)	0(0)	4(14.3)	1(3.6)
Atypical A	10	0(0)	3(30)	3(30)	0(0)	1(10)	2(20)	0(0)	1(10)	0(0)
Atypical B	6	1(16.7)	3(50)	2(33.3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Atypical C	12	0(0)	2(16.7)	5(41.7)	0(0)	1(8.3)	0(0)	0(0)	3(25)	1(8.3)
Total isolates	60	1(1.67)	21(35)	24(40)	3(5)	2(3.33)	2(3.33)	1(1.67)	4(6.67)	2(3.33)

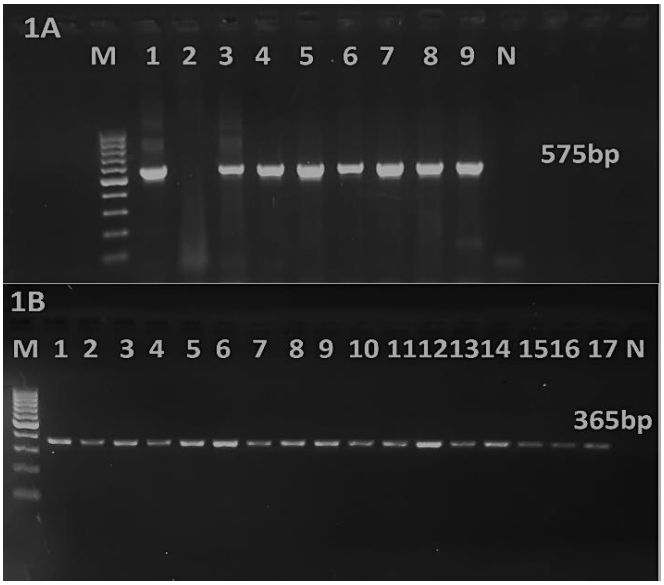

Fig.1 (A) Positive 575 bp PCR products of *motB* gene of *B. cereus s.l.* group. Lane M: 100 bp ladder DNA marker, Lane 1, 3-9: representative *B. cereus positive isolates.* Lanes 2: negative isolate, Lane N: negative control. (B) Positive 365bp of *gyrB* gene of *B. cereus s.s*. specific isolates. Lane M: 100 bp ladder DNA marker, Lanes 1-17: representative *B. cereus s.s.* positive isolates, Lane N: negative control.

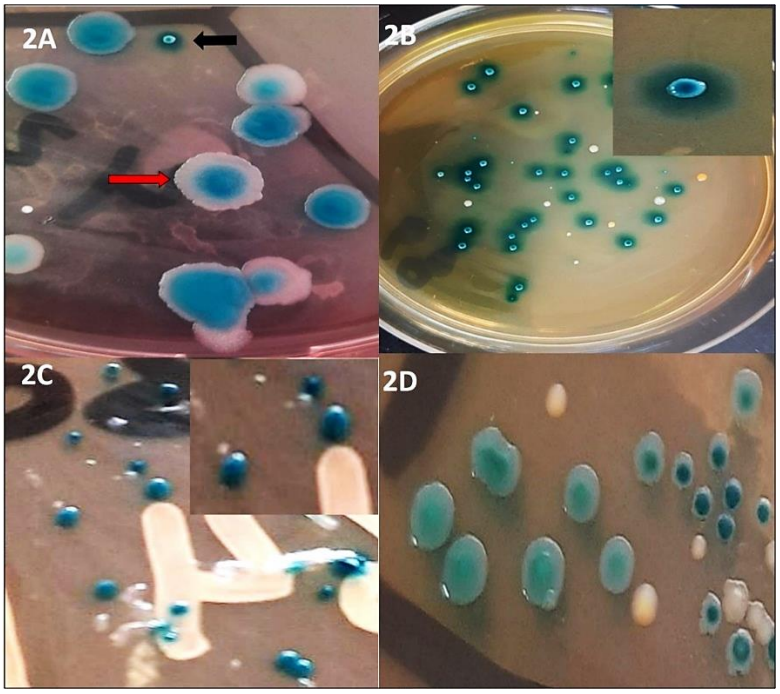

Fig.2 Phenotypic morphological diversity of *B. cereus s.s.* on HiCrome *Bacillus* agar medium (A) Typical large flat (red arrow) and small pinpoint colonies (black arrow) of *Bacillus cereus s. l.* (B) Atypical type a showing small bluish-green colonies. (C) Atypical type b showing small blue to bluish-green colonies. (D) Atypical type C showing large light greenish with smaller bluish-green colonies in between.

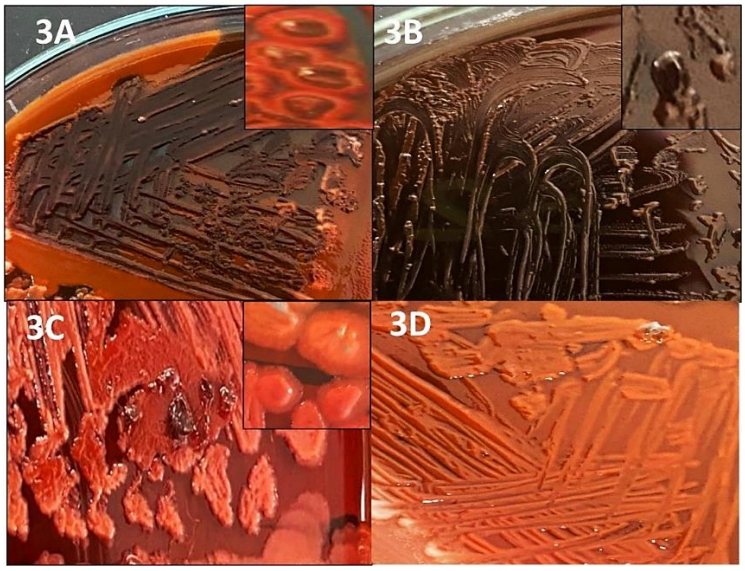

Fig.3 Screening of *B. cereus s.s.* slime producers using Congo red agar plate method. (A) Rough dry black colonies; (B) smooth black colonies; (C) dry red colonies; (D) smooth red colonies.

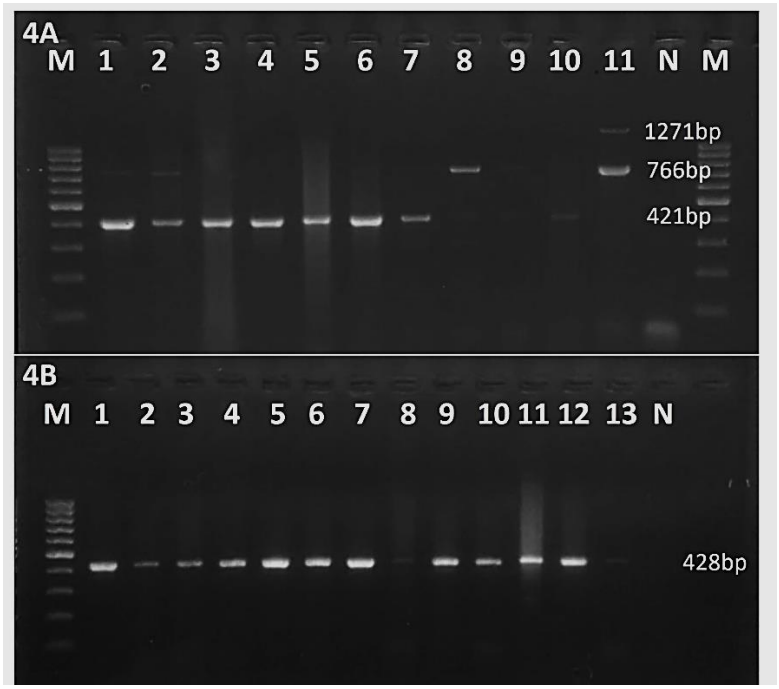

Fig. 4 (A): Multiplex PCR assay detecting the 1271bp, 766bp and 421 bp PCR products of *B. cereus s.s.* toxins *hbl, nhe, and cytK* genes, respectively. Lane M: 100 bp ladder DNA marker, lanes 1-11: representative *B. cereus s.s.* isolates positive for some toxins, Lane N: negative control. (B) Positive 428bp PCR products of *bceT gene.* Lane M: 100 bp ladder DNA marker, Lanes 1-13: *bceT* gene positive *B. cereus s.s.* isolates, Lane N: negative control.

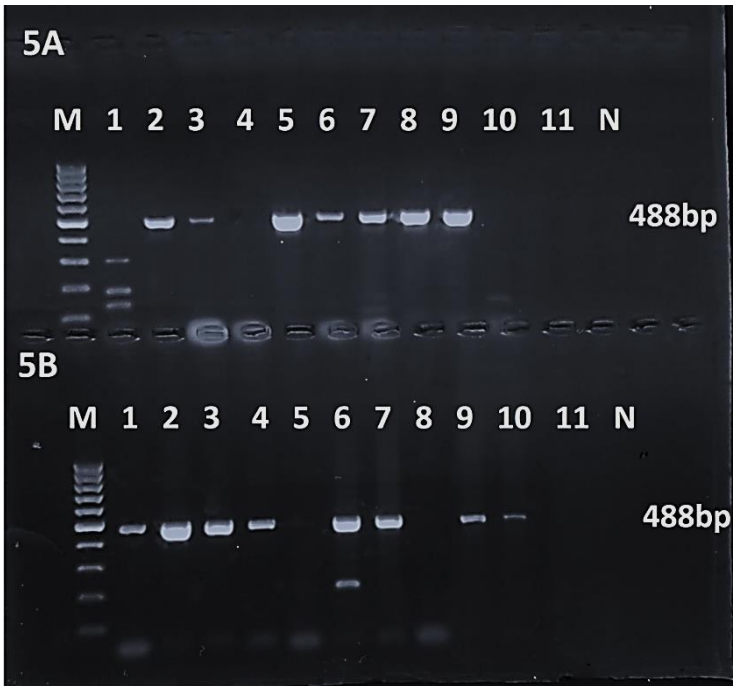

Fig. 5 (A): Positive 488bp PCR products of *tasA* gene in *B. cereus s.s.* isolates. M: 100bp ladder DNA marker, Lanes 2, 3,5-9: *tasA* gene positive isolates, Lanes 1, 4, 10, 11: *tasA* gene negative isolates, N: negative control. (B) Positive 488 bp PCR products of *sipW* gene in *B. cereus s.s.* isolates. M: 100bp ladder DNA marker, Lanes 1-4, 6, 7, 9, 10: *sipW* gene positive isolates, Lanes 5, 8, 11: sipW negative isolates, N: negative control.

	Correlations	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*bceT*	1.												
2	*ces*	.06	1.											
3	*nhe*	.1	.14	1.										
4	*CytK*	.22	.06	.01	1.									
5	*tasA*	.08	.12	.18	.45*	1.								
6	*swip*	.23	.13	.17	.42*	.87*	1.							
7	*tasA* and *sipW*	.2	.13	.19	.44*	.94*	.98*	1.						
8	Hemolysis	-.07	-.29*	-.3*	-.07	-.07	.03	-.01	1.					
9	Biofilm formation	.06	-.01	-.14	-.12	-.23	-.12	-.16	.16	1.				
10	Slime formation	-.03	.13	.06	.37*	.04	.02	.02	-.19	-.06	1.			
11	Total biofilm positive	.02	.14	-.11	-.08	-.13	.	-.05	.12	.9*	-.04	1.		
12	Total slime positive	-.05	.06	.19	.22	.17	.14	.15	-.07	-.01	.76*	.02	1.	
13	Morphology	-.32*	.11	-.14	.03	.25	.14	.17	-.15	-.13	.25	-.09	.26*	1.

-1. -.8 -.6 -.4 -.2 . .2 .4 .6 .8 1.

Fig. 6: Correlation matrix of different genotypes and phenotypes investigated in this study. The values of Pearson's correlation coefficient are represented by shades of colors (blue for positive correlation, red for negative correlation). The intensity of the color represents the coefficient's value. Significant correlation at p-value 0.05 represented by *.

DISCUSSION

B. cereus is a typical pathogenic and spoilage bacteria in raw milk. It can be isolated from the whole chain of the dairy industry. The presence and dispersion of *B. cereus* and its spores are correlated with the contamination of the dairy environment. This includes the farm soil, bedding, dust, air, feces, and fodder. Also, dirty teats and milking equipment are included. Also, *B. cereus*-contaminated raw milk could be one of the

causes of its widespread in the farm environment. *B. cereus* spores can convey to milk at milking time. Moreover, spores can endure the pasteurization process, acting as a potential activator of their germination, rather than being an effective barrier. Under favorable environmental conditions, the vegetative forms can replicate in dairy products, impacting their quality and safety, and potentially leading to spoilage of the product. Moreover, *B. cereus* is diverse from raw milk to the final product and sometimes constitutes a consumer's risk (Stenfors Arnesen *et al*., 2008; Liang *et al*., 2022; Tirloni *et al*., 2022; Savaşan *et al*., 2023). This study aims to screen milk samples for the presence of *B. cereus* group. Therefore, 189 milk samples, including 67 clinically mastitic milk and 122 BTM, were submitted for bacterial isolation. Overall, 88/189 samples yielded *B. cereus*-like colonies, representing a total of 46.6% *B. cereus*-like isolates. Their bacterial counts in positive BTM samples were estimated to vary from 20 to 200 cfu/ml. Previously, Berthold-Pluta *et al*. (2019) mentioned that the taste and flavor defects in pasteurized milk could occur when *B. cereus* count increased above 5.0 cfu/ml. Likewise, when the incidence of *B. cereus* increased, the products showed more defects, as a result of the high proteolytic activity and lecithinase production, which led to sweet curd and bitty cream. 0'Connell *et al*. (2013) found that the mean *B. cereus* count for all milk samples was 40 cfu/ml; they attributed this count to many factors. Housing cows and water testing were positively associated with the *B. cereus* count in BTM while the frequency of fresh grass allocation was negatively associated with the *B. cereus* count in BTM. They added that the associations between dry wiping teats, the feeding of silage, the efficacy of the cleaning wash and *B. cereus* indicated the potential adoption of such managerial factors to reduce the count of *B. cereus* in BTM. Moreover, the severity of *B. cereus* is attributed to its ability to endure in dairy products as spores after being processed during manufacturing (Kwon *et al.*, 2022). Therefore, both safety and quality can be affected.

As *B. cereus* is a complex species characterized by high phenotypic and genotypic similarity (Owusu-Kwarteng *et al*., 2017), a molecular-based technique was used as a more confirmatory test to stand on the species level of the B. *cereus* group. Based on the obtained PCR results, 78 of 88 *B. cereus*-like isolates were confirmed to be *B. cereus s.l.* with a prevalence of 41.3%. In contrast, the other 10 isolates (5.3%) could not be confirmed as the *B. cereus s.l.* Moreover, 60 isolates of the 78 were confirmed to be *B. cereus s.s.* with a prevalence of 31.7%. The remaining 18 isolates (9.5%) did not amplify the specific PCR product of *B. cereus s.s.*, so they were assumed to belong to the B. *cereus s.l.* group, but not the *sensu stricto*. Chen and Tsen (2002) declared that the discrimination between *B. cereus* and *B. thrungensis* is difficult. They also reported the error for identification of *B. thrungensis* as *B. cereus*, using the *gyrB*-based primers BC1/BC2r reported by Yamada *et al.* (1999), which were used in this study, to be 92%. Therefore, to exclude any misidentification, all isolates identified as *B. cereus s.s.* were screened for the presence of the *cry* gene using the *cry* gene-specific primers of *B. thrungensis*. All isolates were negative and so confirmed to be *B. cereus s.s*.

Many literatures were concerned with the percentage of *B. cereus s.l.* in raw milk as a group species. The current study could be one of the first to focus in detail on *B. cereus s.s.*, which may be one of the important causes of both bovine mastitis and raw BTM contamination. Owusu-Kwarteng *et al*. (2017) reported a slightly higher percentage of *B. cereus s.l.* (46.6%) in raw milk samples. A higher percentage (61.11%) of *B. cereus s.l.* isolates in raw milk samples was demonstrated by Meng *et al*. (2022), while lower incidence rates of *B. cereus s.l.* in raw milk (33%) and (12.5%) were mentioned by McAuley *et al*. (2014) and Ben-Akacha *et al*. (2024), respectively. Eid *et al*. (2023)

illustrated that *B. cereus* is one of the most important causes of bovine mastitis. Some *Bacillus spp*. can cause gangrenous mastitis, and the contaminated udders can lead to the shedding of *B. cereus* in raw milk. These previous results agreed with the present results, as 36/67 (53.7%) isolates from clinically mastitic milk samples were PCR-confirmed to be *B. cereus s.l.*; from which 31 isolates were PCR-confirmed as *B. cereus s.s.* with a percentage of 46.3%. Meng *et al*. (2022) reported that *B. cereus s.l.* isolates in raw milk might originate from the feces, teat skin, teat cups, and dip cups. In our opinion, these may be strong sources of infection of the udder, and may not only cause mastitis, but also spread infection with *B. cereus* between the lactating animals during milking.

This study was concerned with the details of morphological, phenotypic and genotypic characteristics of *B. cereus s.s.* as this species was one of the most prevalent species of *B. cereus s.l.* group isolated in this investigation from mastitic and bulk tank milk samples collected from different Egyptian dairy farms in many governorates.

In this statement, 69 isolates were obtained by Rossi *et al.* (2018) from the dairy production chain, but only 6 isolates were confirmed phenotypically to be *B. cereus s.s.* were isolated from raw milk samples. This *B. cereus s.s.* is also a serious human foodborne pathogen that has caused outbreaks in many countries (Sornchuer *et al*., 2024).

It is worth mentioning that *B. cereus s.s.* PCR-confirmed isolates showed both typical and atypical morphological characteristics on HiCrome Bacillus agar. The typical colony morphology of *B. cereus s.s.* on HiCrome Bacillus agar medium appeared as large flat and small atypical pinpoint colonies with blue centers. The blue centers' colonies are the result of β-D-glucosidase reaction. Precipitation zones surrounding typical colonies are caused by phospholipase C reaction (PLC), while lack of mannitol fermentation resulted in a pink background of the medium. The diversity in the atypical colony's features may be attributed to many chemical enzymatic reactions produced by *B. cereus s.s.* strains and the chrome agar medium components, giving each strain its characteristics on the same medium. The atypical type (a) shows small bluish-green colonies. This is the result of β-D-glucosidase reaction with weak PLC activity on HiCrome *Bacillus* agar that could be misidentified as presumptive *B. cereus* due to their atypical pin-point growth and fermentation of mannitol, which resulted in a yellow background. The atypical type (b) shows small blue to bluish-green colonies, which can be attributed to β-D-glucosidase reactions with a lack of PLC activity on HiCrome *Bacillus* agar, and therefore could be misidentified as presumptive *B. cereus* due to their atypical pin-point growth and lack of fermentation of mannitol, which resulted in a pink background. The atypical type (c) shows large light greenish colonies with smaller bluish-green colonies in between. This is due to a weakly β-D-glucosidase positive reaction and PLC-negative reaction, lacking the distinctive halo surrounding, with a lack of mannitol fermentation in the background. So that it could be misidentified as presumptive *B. cereus* due to its atypical growth. The explanations of the atypical morphologies were supported by Fuchs *et al.* (2022), who also observed these atypical morphological manners of *B. cereus s.l.* on chromogenic media. They noted that atypical colony morphologies may still occur. The highest rates of atypical β-D-glucosidase negative colonies were observed on BRI (12.7%) and HI (6.4%). They attributed the atypical morphologies of *B. cereus s.l.* to either a milk-derived or soil-derived panC type/toxin profile combination. This explanation was near our expectation, as most of the isolates with atypical colony morphology originated from BTM samples (20/29). Prior studies reported ß-D-glucosidase-negative *B. cereus s.l.* colonies on chromogenic *B. cereus* agar media. This was a worry for a proper valuation (Fricker *et al.*, 2008; Hendriksen and Hansen, 2011; Tallent *et al*., 2012).

In this study, *B. cereus s.s.* isolates were screened for different virulence characteristics, including hemolytic activities, biofilm-forming ability, and slime-producing ability. Based on hemolytic activities, 31 (51.7%) isolates showed α hemolysis, while 25 (41.7%) showed β-hemolysis. Only 4 (6.6%) isolates were non-hemolytic. Meanwhile, Didouh *et al*. (2023) found that all recovered *B. cereus s.l.* group isolates displayed β-hemolytic activity. Ben-Akacha *et al*. (2024) reported that 81.4% of their studied isolates showed β-hemolysis on blood agar plates.

Radmehr (2023) reported the importance of investigating the biofilm-forming ability of dairy pathogens for their behavior and characterization studies. The *B. cereus* ability to stick to surfaces and form biofilms is alarming for manufacturers, where biofilm can be a continuous source of contamination during different production stages in dairy plants (Kumari and Sarker, 2016). Although *B.cereus* biofilm is composed mainly of vegetative cells, sporulation can occur in the biofilm. So, biofilm can be an important source of spore contamination (Faille *et al.,* 2014).

Considering biofilm-forming abilities, 28 isolates produced biofilm with different grades (46.7%). *B. cereus s.s.* isolates were strong, moderate, and weak biofilm producers (25, 11.7 and 10%, respectively), while 32 (53.3%) were non-biofilm producers. The percentage of biofilm-positive isolates was lower than that mentioned by Radmehr (2023) who reported that out of their *B. cereus* group isolates 53.7% could form a biofilm. However, most of these isolates had weak biofilm formation ability, and only 4.9% had a strong ability to produce biofilm. In the study of Alonso *et al.* (2021), all the isolates of *B. cereus* were 100% biofilm, using the microtiter plate method.

Concerning the slime production on CRA medium, 26 and 25 isolates were strong and moderate slime producers (51/60), with a total positive percentage of 85%, while 9 isolates were non-slime producers. In 2023, Savaşan *et al.* reported that 40% of their *B. cereus* isolates had slime-producing activity. The danger of the biofilm-forming ability of *Bacillus* strains was noted by Faille *et al*. (2014), who stated that it can form biofilms in the piping and milk tanks of dairy plants, making them highly resistant to heat and cleaning-in-place (CIP) systems. Similarly to the biofilm of other species, biofilm produced by *B. cereus* may contain other bacterial species and can contribute to the community's flexibility and extension (Majed *et al*., 2016). These data highlighted that the dairy industry needs to adopt control measures on the initial quality of raw materials and in CIP cleaning applications (Alonso *et al*., 2021). Moreover, serious control points like transport and storage tanks, production equipment, places, and personnel from the stage of raw milk to the final product, and the implementation of sanitation practices at these points are important against *B. cereus* contamination (Savaşan *et al*., 2023).

B. cereus can produce heat-resistant enterotoxins. These enterotoxins can tolerate food processing and represent consumers' hazards. Moreover, it is a typical psychrophilic bacterium that can secrete extracellular heat-resistant proteases and lipases in decreased temperature conditions. These enzymes can induce a bitter or sour milk taste, or the gelation of milk. Also, it can shorten the shelf-life of milk products (Samarzija *et al.*, 2012; Matéos *et al.*, 2015; Zeighami *et al.*, 2020; Liang *et al*., 2022).

Concerning safety, *B. cereus* can produce both diarrhea and vomiting toxins. Diarrhea toxins include nonhemolytic enterotoxin and hemolysin BL. These two toxins are heat-sensitive (Ceuppens *et al.*, 2013). On the contrary, the vomiting toxin 'cereulide' is a heat-stable toxin and cannot be inactivated even if exposed to 121 °C for 90 min (Agata *et al.*, 2002). Moreover, *B. cereus* can produce heat-resistant spores. These spores

can endure in processed dairy products, and consequently, constitute a risk to the safety of the dairy industry (Liang *et al*., 2022). Therefore, it is critically essential to establish a method to detect *B. cereus s.s.* enterotoxins to guarantee and improve the quality of raw milk.

All PCR-confirmed *B. cereus s.s.* isolates were screened for the presence of five toxin genes. The result revealed that none of the isolates harbored the *hbl* gene, while the *cytK* and *bceT* and *nhe* genes are the most prevalent, 86.7, 85 and 46.7%, respectively, with higher rates in the strains isolated from individual mastitic milk versus those from BTM. Only one isolate from the BTM sample harbored the *ces* gene (1.7%), which induces the emetic syndrome. Recently, different percentages of enterotoxigenic genes were described by Ben Akacha *et al*. (2024). They showed that 8.5% and 67.8% of *B.cereus* isolates carried *hbl* and *nhe*, respectively, and the detection rates of *cytk*, *bceT*, and *ces* genes were 72.9%, 64.4%, and 5.1%, respectively. Radmehr (2023), identified virulence genes *nheA*, *nheB*, *nheC* and *hblA* in most isolates, and *cytk* gene only in 46% of their *B. cereus* isolates, indicating that many potential diarrheal strains existed in the collected samples, meanwhile, no isolates contained the *ces* gene. On the other side, Owusu-Kwarteng *et al*. (2017) recorded the prevalence of *cytk* and the emetic gene *ces* among *B. cereus s.l.* isolates as 75 and 9%, respectively. They detected the emetic *B. cereus s.l.* gene from only milk and milk products, but not from soil samples, this notification is accepted with the obtained result as *ces* gene was detected only in BTM sample, not in mastitic milk.

In the current study, all *B. cereus s.s.* isolates were screened for the presence of two biofilm-related genes: *tasA* and *sipW* genes. As depicted, 53.3% and 50 % of isolates harbored the *tasA* and *sipW* genes, respectively. In this concern, veterinary practice lacks studies about the genes related to the biofilm-forming ability of *B. cereus s.s.*. However, Bianco *et al*. (2021) isolated *B. cereus s.s.* from patients' blood. They verified the presence of biofilm-associated genes in these isolates, although their strains were not screened for their ability to form biofilm. A high prevalence of both *tasA* and *sipW* genes was reported by Caro-Astorga *et al*. (2015). The isolates carried *calY*, *tasA*, and *sipW* genes. They proposed that the presence of *calY*, *tasA*, and *sipW* genes be sufficient for biofilm production. Interestingly, the identified virulence factors seem to be regularly distributed among *B. cereus s.s.* isolates. Therefore, screening for virulence factors is essential to describe the pathogenic power of the *B. cereus* group strains regardless of their correct species identification.

Lately, Sornchuer *et al*. (2024) isolated 12 *B. cereus s.s.* strains from different foodstuffs. These strains harbored both *tasA* and *sipW* genes.

One of the interesting findings in this study is the phenotypic diversity of PCR-confirmed *B. cereus s.s.* isolates on HiCrome Bacillus agar. Therefore, the distribution of virulence characteristics among these isolates with diverse morphological features was important to be studied. When comparing the isolates with typical colony morphology with those of atypical colony morphology, it was noticed that 100% of typical isolates were hemolytic, versus 85.7% of the total atypical isolates. Also, 53.1% and 78.1% of the typical isolates were biofilm and slime producers, versus 39.3% and 92.9% of the atypical isolates, respectively. These results declared that the atypical isolates of *B. cereus s.s.* have virulence characteristics, so they have the same danger as typical forms as well. No earlier researchers studied this diversity, and according to the available knowledge, this study may be the first that focuses on such diversity in detail.

Furthermore, the distribution of PCR-positive toxins and biofilm-related genes among *B. cereus s.s.* isolates with typical and atypical

colony morphology was declared in this study. The recorded results illustrated that the percentages of toxins and biofilm-related genes in typical isolates were 53.1, 84.4, 93.8, 0, 46.9, and 46.9 % for *nhe*, *cytK*, *bceT*, *ces*, *tasA*, and *sipW* genes versus 39.3, 89.3, 75, 3.6, 60.7 and 53.57% in total atypical isolates, respectively. These results indicated that the atypical forms of *B. cereus s.s*. also owned virulence genes with various percentages like the typical form. Moreover, one of them harbored the emetic gene, which is considered a potential hazard in dairy products (*ces* gene; 3.8%) that was not detected in the morphologically typical strains. Additionally, the atypical *B. cereus s.s*. isolates possessed biofilm-related genes *tasA*, and *sipW* genes higher than typical form (60.7 and 53.57% versus 46.9 and 46.9 %, respectively). No previous literature discussed the relation between phenotypic and genotypic diversity of *B. cereus s.s.* strains. However, Das *et al*. (2013) isolated two atypical enterotoxigenic *B. cereus* isolates from white shrimp with a negative VP reaction. Both isolates harbored the enterotoxin-producing genes, *hblA*, and *entB*.

By studying the toxin gene profiles of *B. cereus s.s.* isolates, 58/60 *B. cereus s.s.* isolates harbored at least one toxin gene. According to the positive toxin genes, nine toxin gene profiles were found and named A (included 4 different toxin genes; *bceT*, *ces*, *cytK* and *nhe*), B (included 3 different toxin genes; *bceT*, *cytK* and *nhe*), C (included 2 different toxin genes; *bceT* and *cytK*), D (included 2 different toxin genes; *bceT* and *nhe*), E (included 2 different toxin genes; *nhe* and *cytK*), F (included only one toxin gene; *bceT*), G (included only one toxin gene; *nhe*), H (included only one toxin gene; *cytK*) and profile I (all toxin genes are negative). Moreover, we displayed the specific toxin gene profiles of *B. cereus s.s.* isolates of both typical and atypical colony morphology. Totally, both B (3 different enterotoxin genes) and C (2 different enterotoxin genes) profiles were the most common profiles with percentages of 35 and 40%, respectively. While profiles A (4 different enterotoxin genes) and G (*nhe* gene only) were the lowest profiles (1.67%). Toxin profile C (*bceT* and *cytK* genes) was the dominant profile among both typical and atypical isolates with percentages of 43.8 and 35.7%, respectively. Only 2 (3.33%) of *B. cereus s.s.* isolates did not contain any toxin genes (profile I). The high rate of different toxigenic profiles in the atypical form of *B. cereus s.s.* indicated that these morphological forms are not less dangerous than the typical form.

In agreement with these results, Rossi *et al.* (2018) found 95.65% of their isolates carried at least one toxin gene. Lately, Meng *et al*. (2022) detected 14 different virulence patterns. They reported that the diarrheal strains were the most common among isolates from raw milk and dairy farm environmental samples. They found only one isolate with no virulence gene (2.13%). Furthermore, Cruz-Facundoa *et al*. (2023) identified 8 different entero-toxigenic profiles in *B. cereus* group isolated from foods. The common profile was the one had positive 3 enterotoxin genes (*nhe*, *hbl*, and c*ytK*) with a rate of 27.9%.

Sornchuer *et al*. (2024) reported that the cytotoxicity assays and molecular detection of virulence determinants of *B. cereus* group isolates could offer additional information about their potential to affect human health. However, molecular detection of toxin genes in the *B. cereus* group isolate is necessarily not consistent with its pathogenic potential (Miller *et al*., 2018). The current study statistically analyzed the correlation between all screened phenotypes and genotypes, using Pearson's correlation analysis of the presence/absence data of all assays. The most observed association was between *tasA* and *sipW* genes. Their correlation coefficient was 0.87, where a strong congruence was found with a statistically significant dependence at p-value≤ 0.001. This association may be attributed to the localization of *tasA* gene downstream of the locus of signal peptidase gene *sipW*, in the *sipW-tasA* operon (Fagerlund *et al*., 2014; Caro-Astorga *et al*.,

2015). The correlation between *tasA* and/or *sipW* genes and biofilm formation was surprising. Although these genes were reported as biofilm-related genes, the correlation between them was negative and slight. In 2006, Minnaard *et al*. highlighted positive and negative correlations for the multiple virulence characteristics of the *B. cereus* group. A correlation between ribotype, presence of toxin genes and the biological activity of the strains was shown. Generally, there is a shortage of data to discuss this point. Moreover, studying the correlation of more isolates in the future could resolve other correlations, especially those with marginal or lower significance. We hope that this work will be the beginning of more work to cover this point soon.

In conclusion, this study focused on *B. cereus s.s.* and its phenotypic and genotypic virulence characteristics. Also, the diversity of both phenotypes and genotypes was studied. It was concluded that the atypical forms were not less virulent than their typical form. Therefore, efforts should be adopted to prevent misidentification of the atypical forms of *B. cereus s.s.* in the veterinary practice, especially in dairy plants. Additionally, *B. cereus* s.s. showed wide diversity in raw BTM and is considered one of the important causes of mastitis in dairy farms in Egypt.

The presence of potentially pathogenic *B. cereus s.s*. strains that carry many toxin-associated genes in dairy farms require the implementation of strict hygienic practices to avoid raw milk contamination to improve food safety and decrease human hazards.

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الخصائص المظهرية ، القدرة المسببة للضراوة والتنوع الجيني لميكروبات الباسيلس سيريس المعزولة من حليب اللأبقار الخام لبعض مزارع الألبان المصرية

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هدفت هذه الدراسة إلى معرفة معدل الإصابة والخصائص الظاهرية والضراوة والتنوع الجيني لمجموعة B. cereus sensu lato في حليب الخزانات الخام (BTM) ولبن البقر المصاب بالتهاب الضرع الظاهرى مع الاهتمام بشكل خاص بميكروب B. cereus sensu stricto. تم فحص عينات لبن تشمل ١٢٢ عينة من لبن التنكات المجمعة و ٦٧ عينة من لبن ابقار مصابة بالتهاب الضرع الظاهرى. وقد تم عزل البكتريا العصوية *Bacillus cereus sensu stricto* وتاكيدها باختبار تفاعل البلمرة المتسلسل وبلغت نسبتها 31.7% . واظهرت ٣٢ معزولة شكلا ظاهريا نموذجيا بينما اظهرت الـ ٢٨ معزولة الاخرى اشكالا ظاهرية غير نموذجية. بين المعزولات ٩٣,٤ و ٤٦,٧ و ٨٥ % كانت تسبب انحلال للدم ومنتجة للاغشية الخلوية والمخاط على الترتيب. وكانت نسبة وجود جينات الـ *cytk* و *bcetT* و *nhe* هى الاكثر انتشارا بنسب ٨٦,٧ و٨٥ و٤٦,٧% على التوالي كما ان عزلة واحدة فقط احتوت على جين الـ ces بنسبة ١,٧%. كما احتوت ٥٠ و ٥٣,٣ % من المعزولات على جينى الـ tasA و الـ sipW المرتبطان بتكوين الغشاء الحلوى.

عند مقارنة المعزولات النموذجية بالمعزولات الغير النموذجية من هذا الميكروب كانت ١٠٠% من المعزولات النموذجية تسبب انحلال للدم مقابل ٨٥,٧% من المعزولات غير النموذجية، وكانت ٥٣,١% و٧٨,١% من المعزولات النموذجية منتجة للأغشية الخلوية والمخاط مقابل ٣٩,٣% و ٩٢,٩% من المعزولات غير النموذجية. وتم العثور على وجود ٩ أنماط مختلفة لجينات السموم.

وكانت نسب انتشار الجينات المرتبطة بالسموم والأغشية الخلوية في المعزولات النموذجية ٥٣,١ و٨٤,٤ و٩٣,٨ و٠ و٤٦,٩ و٤٦,٩% لجينات ال *nhe* و *cyt K* و *bceT* و *ces* و *tasA* و *sipW* علي التوالي مقارنة بـ ٣٩,٤ و ٨٩,٣ و ٧٥و ٣,٦ و ٦٠,٧ و ٥٣,٥٧% فى المعزولات غير نموذجية وكشفت الدراسة عن وجود ميكروبات الـ *B. cereus sensu stricto* المرضية فى اللبن كما ثبت ان المعزولات الغير نموذجية منها لاتقل فى الضراوة عن المعزولات النموذجية لذلك يجب بذل الجهود من اجل منع التشخيص الخاطىء لها كما يجب فرض الممارسات الصحية الصارمة فى مزارع الالبان لتحسين سلامة اللبن وتقليل المخاطر على المستهلكين.