Prevalence, Antibigram Pattern and Virulence Genes Profile of *Bacillus cereus* Isolated from Buffalo Milk

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ABSTRACT

In Egypt, the buffalo's milk is greatly consumed on a large population scale, due to its nutrition quality and palatability, so there is a need to ensure its microbiological quality especially for food borne pathogens to be safe for human consumption. The current study was conducted on a total of 85 solitary collected raw buffalo's milk samples at El- Giza Governorate. The cultural examination revealed that *Bacillus cereus* was isolated in 11 samples by (12.94%). The biochemical examination of the 11 isolates exhibited predominant two different biotypes 2 and 5. The antibiogram manner exposed that all isolates were sensitive to gentamicin, neomycin and ciprofloxacin, less sensitive to tetracycline (87.5%) vancomycin (81.25%) and erythromycin (81.25%), intermediate to ampicillin (56.25%) and polymyxin (43.75%) while the eleven isolates were resistant to both amoxicillin and penicillin G (100%). The multiplex PCR was carried out to assess conservative gene 16S rDNA gene, *ces* (cereulide encoding gene) and *entFM* (enterotoxin-encoding gene) among the eleven isolates. The procured data demonstrated that only ten isolates amplified the targeted 16S rDNA product 964 bp (90.9%). On the other side, there were six isolates showed amplicons of average molecular size 486 bp (54.54%) while two isolates amplified 1271 bp (18.18%), which targeted *entFM* and *ces* respectively. The obtained data proposed that raw buffalo's milk may be considered a source of public health concern toxigenic *B. cereus* which may be implemented in food-borne illness.

Key words: *Bacillus cereus*, Toxigenic & Resistance genes, Buffalo's Milk, PCR, Antibiotic Susceptibility

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INTRODUCTION

Bacillus cereus is an aerobic spore-forming, rod-shaped and motile bacterium, positive to Gram stain, commonly implemented in the dairy products deterioration. The microorganism possesses a property of adherence to the dairy stainless-steel equipment as well as the ability to produce various types of toxins; cytotoxic, vomiting inducer and enterotoxins. So, it constitutes a significant risk to health as regarding food poisoning (Lucking et al. 2013; Majed et al. 2016; Hussain et al. 2018). Even if unsuitable environment exists, the organism can produce spores waiting the conditions become favorable; germination of spores during cooling process of dairy products boosting the bacteria to grow and secrete their toxins (Liu et al. 2020). Moreover, because of the spore-forming and the psychotropic leaning of the organism; survival and multiplication of *B. cereus* during milk processing, resulting in usual reported food poisoning cases (Kumari and Sarkar 2016).

Generally, *B. cereus* food infection and intoxication occupied a leading rank among the organisms causing foodborne outbreaks that reported worldwide, (Haque et al. 2021), but unfortunately; in Egypt, there were scarce reports and data about the presence of *B. cereus* among the dairy foodstuff (Abdou et al. 2011; Sadek et al. 2018; Al-Gamal et al. 2019).

There are various species of genus *Bacillus*, of which many of them may similar in morphological, cultural and biochemical appearance. So, their differentiation may rely on the molecular structural genes profile; 16S rDNA is considered an important conservative gene implemented in differential molecular diagnosis of bacterial species (Giffel et al. 1997; Mousa and Marwan 2019).

B. cereus is considered an opportunistic organism commonly result in two different conditions of food poisoning in human being; the first is manifested by nausea and vomiting, while the other is associated with abdominal pain and diarrhea (Osimani et al. 2018).

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Interestingly, the *B. cereus* food poisoning is unique as both the organism and its toxins able to induce the disease. Cereulide is a greatly stable toxin; resist heat, drastic pH conditions and protease activities. Once performed in food, this toxin cannot be inactivated either during processing or in alimentary tract; so, it can induce serious food intoxication illness even when the organism is not present or killed (Kranzler et al. 2016).

Otherwise, the diarrheal form is a food infection; induced by ingestion of food contaminated by viable vegetative bacterial cells not their preformed toxins. When, the organism reaches small intestine, it can secrete many types of enterotoxins, leads to induction of diarrhea (Jeßberger et al. 2017).

The previous illness conditions are attributed to the virulence genes subsequently virulence toxins produced by *B. cereus*. The vomiting form is induced by heat stable toxin named 'cereulide', which is encoded by cereulide synthetase (*ces*) gene. On the other hand, the diarrheal status is persuaded via production of many enterotoxins, one of them is called enterotoxin FM which encoded by *entFM* gene (Forghani et al. 2016).

Taking in consideration the above circumstances, the study targeted the prevalence, identification, antibiogram, and spotlighted important virulence genes of *B. cereus* among raw buffalo milk in Giza city.

MATERIALS AND METHODS

Sampling

An overall 85 raw buffalo's milk samples were collected from farmers and markets at El-Giza Governorate during 2019. The obtained samples were transmitted to the microbiology laboratory in cooled, insulated sterile plastic bags under aseptic condition.

Isolation and Biochemical Identification of *B. Cereus*

A loopful from each milk sample was streaked on plates contained *B. cereus* mannitol yolk polymyxin B agar (Himedia, India). Plates were incubated for 24h, at 30°C and monitored for growth; the suspected colonies were picked up, Gram's stained and inspected microscopically before being streaked into semisolid and slope agar for preservation and further biochemical identification. Biochemical identification of *B. cereus* was performed according to Bacteriological Analytical Manual; via TSI test, catalase, urease and nitrate reduction tests (Rhodehamel and Harmon 2001).

Biotyping

The obtained isolates were biotyped relying on their capability to ferment ammonium salt sugars (ASS), namely, cellobiose, salicin and xylose, as per the scheme planned by Jha and Narayan (1995).

Antimicrobials Susceptibility Testing

Sensitivity versus antibiotics' resistance profile of *B. cereus* isolates were checked via Mueller-Hinton agar (Himedia) using disc diffusion method proposed by the Clinical and Laboratory Standards Institute guidelines (CLSI 2015). The following antibiotic discs (Sigma) (indicate manufacturer and reference) were used; penicillin G (10µg), amoxicillin (10µg), ampicillin

(10µg), erythromycin (15µg), vancomycin (3µg), gentamycin (10µg), neomycin (30µg) ciprofloxacin (5µg), polymyxin (10µg) and tetracycline (30µg). Susceptibility categorization was carried out according to standards guiding antimicrobial disc sensitivity tests (CLSI 2018).

Genomic DNA Extraction and PCR Assay DNA Extraction

The standard strains *Bacillus cereus* (ATCC 14579) and *Staphylococcus aureus* (ATCC 25923) were used for positive and negative standard respectively. The mentioned strains were purchased from the reference laboratory of the Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain-Shams University.

DNA was extracted from *B. cereus* isolates and the standard strains using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) after amendments manufacturer's guidelines. Briefly, 200µl of the sample suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer at 56°C for 10min. After incubation, 200µl of 100% ethanol was added to the lysate. After that the sample was washed and centrifuged keeping the manufacturer's recommendations and nucleic acid was eluted with 100µl of elution buffer provided in the kit.

PCR Detection of Structural and Virulence Genes

PCR screening was performed to determine the existence of a conservative gene (16S rDNA gene) and two other virulence genes; (*ces*) which are responsible for production of emetic cereulide toxin and (*entFM*) an enterotoxin-encoding gene.

The multiplex PCR of the three genes was carried out in a 50µl, PCR reaction containing 25µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1µl of each primer of 20pmol concentrations, 9µl of water, and 10µl of DNA template. The used primers were supplied from Metabion (Germany) and the amplification process was conducted in (Biometra, Germany) thermal cycler (Wehrle et al. 2009; Dzieciol et al. 2013) as described in the Table 2.

The PCR reaction mixtures were analyzed by electrophoresis on a 1.5% (w/v) agarose gel in the presence of 100bp DNA ladder (Fermentas Life Science, EU) according to Sambrook et al. (1989).

RESULTS

Among 85 raw buffalo's milk samples solitary collected from farmers and markets at El- Giza Governorate, the cultural criteria revealed a number of 11 presumptive isolates with a rate of 12.94%. Suspected *B. cereus* colonies exhibited pink look (mannitol-negative) surrounded by a precipitation zone (lecithinase positive).

The suspected *B. cereus* isolates produced catalase, reduced nitrate and ferment glucose, while the production of urease was achieved in seven isolates. The biotyping identification of *B. cereus* isolates declared that the predominating biotypes were 2 (7 isolates) and 5 (3 isolates) as shown in Table 1.

The antibiotic susceptibility manner to ten antimicrobials exposed that the *B. cereus* isolates were highly sensitive to gentamicin (100%), neomycin (100%), Ciprofloxacin (100%), less sensitive to tetracycline (87.5%),

Fig. 1: diagram showing the sensitivity pattern of *Bacillus cereus* isolates to tested antibiotics; the green represented 100% sensitivity, light brown represented lesser sensitivity, dark brown represented intermediate sensitivity, while the red represented 100% resistance.

Fig. 2: Multiplex polymerase chain reaction; detection of structural and virulence genes in *Bacillus cereus* isolates. L: 100 bp DNA ladder. Lanes 1-11: *Bacillus cereus* isolates. Lane Pos: Positive control; amplification of 964 bp represented 16S rDNA, 486 bp represented *entFM*, and 1271 bp represented *ces*. Lane Neg: Negative control.

Table 1: Biotyping scheme of *Bacillus* group as established by Jha and Narayan (1995)

Biotype	Xylose	Salicin	Cellulose
1	-	-	-
2	+	-	+
3	-	+	-
4	+	-	-
5	+	+	+
6	-	-	+
7	-	+	+
8	+	+	-

vancomycin (81.25%), and erythromycin (81.25%) intermediate to ampicillin (56.25%) and polymyxin (43.75%). On the other side, the isolates showed a great resistance against both penicillin G (100%), and amoxicillin (100%) as displayed in Fig. 1.

The multiplex PCR criteria for conservative 16S rDNA gene, *ces* (cereulide encoding gene) and *entFM* (enterotoxin-encoding gene) was carried out on the eleven suspected *B. cereus* isolates.

The obtained data exhibited that only ten isolates amplified the targeted 16S rDNA product 964 bp (90.9%). On the other side, there were six isolates showed amplicons of average molecular size 486 bp (54.54%) while two isolates amplified 1271 bp (18.18%), which targeted *entFM* and *ces* respectively as shown in Fig. 1.

DISCUSSION

Bacillus cereus is one of aerobic spore forming bacteria present everywhere, so can contaminate various food stuff resulting in food poisoning through existence of vegetative form or via production of toxins (Jessberger et al. 2019). The disease pathogenesis is influenced by different interacting factors; some are related to the pathogen itself and the other affected by the consumed foodstuffs as well as the intestinal microbiota (Jessberger et al. 2020).

There are abundant virulence factors produced by *B. cereus* strains resulting in the known two different forms which frequently self-limiting, but in some other conditions become serious and grave (EFSA 2005). These virulence factors are controlled and expressed by a group of master keys; Pleiotropic regulator (PlcR), a regulator of at least 45 genes responsible for virulence toxins and biofilm formation, Spo0A which a sporulation regulator, and CodY which is considered a key regulator of growth and survival of the organism (Ehling-Schulz et al. 2019).

In Egypt, the raw buffalo's milk is consumed on a broad scope, so there is a great concern to ensure its microbial quality for public health impact. This study was conducted to survey the subsistence of *B. cereus* among 85 buffalo's milk samples and declared eleven isolates with incidence of 12.94%. Previous surveys exposed variation in isolation incidence; 4.6% (Shehata et al. 1983), 66.6 % (Sharma et al. 2003) and 26.66% (Yusuf et al. 2018).

This high incidence may be attributed to many reasons comprising the widespread existence in the soil so be able to contaminate the used utensils or animal pasture and bedding as well as the possibility of milk adulteration by various cereal powders (Eneroth et al. 2001; Vidic et al. 2020). Moreover, the survival of spores for prolonged time in the intense processing of dehydrated foods and thus contaminate assorted foodstuffs through dehydrated ingredients (EFSA 2016).

The phenotypic characterization of obtained isolates was greatly similar to the picture of standard *B. cereus* strain. Commonly, there are variations in biochemical reactions and sugar fermentation response of *B. cereus* raw milk isolates. Our study revealed exposure of two predominant biotypes 2 (7 isolates) and 5 (3 isolates), these data was somewhat near other investigations (Hafeez et al. 2012; Yusuf et al. 2018).

The antibiotic susceptibility pattern of *B. cereus* isolates revealed that there was 100% resistance to penicillin G and amoxicillin. Conversely, all isolates were sensitive to gentamicin, neomycin and ciprofloxacin (100%). The isolates response to other antibiotics was variable; less sensitive to tetracycline (87.5%) vancomycin (81.25%) and erythromycin (81.25%), while showed intermediate sensitivity to both ampicillin (56.25%) and polymyxin (43.75%). These data coincided to those reported in many previous investigations; (Owusu-Kwarteng et al. 2017; Rana et al. 2020; Zhao et al. 2020).

Table 2: Multiplex PCR: Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (30 cycles)			Final extension	References
				2ry denaturation	Annealing	Extension		
16S rDNA	GGATTAAGAGCTTGCTCTTAT GATTGAGAGCTTGCTCTCAA	964	94°C 5min	94°C 1min	50°C 1min	72°C 1min	72°C 10min	Wehrle et al. (2009);
entFM	GTTCGTTTCAGGTGCTGGTAC AGCTGGGCCTGTACGTACTT	486						Dzieciol et al. (2013)
Ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271						

The resistance of *B. cereus* group species to β -lactam antibiotics is well established and is attributed to synthesis of β -lactamase enzyme, in the same time the variable resistance degrees of the organism to other antibiotics were mentioned (Abraha et al. 2017; Yu et al. 2019). The multidrug resistance phenomenon is put in mind and considered as public health significance due to the issues of therapy failure as well as control of foodborne illnesses (EFSA 2016).

The multiplex PCR analysis of structural 16S rDNA was performed on the eleven isolates and the data revealed that only ten isolates amplified the targeted molecular weight of 964 bp. This given result may be attributed to that the negative eleventh isolate is seemed to be belonged to *Bacillus thuringiensis*; another member of *B. cereus* group which may show similarity in morphological and biochemical criteria but can be differentiated via molecular identification (Chen and Tsen 2002; Caamaño-Antelo et al. 2015; Sonmez and Erbas 2017; Fei et al. 2019).

Foodborne illnesses induced by *B. cereus* can be assorted into diarrheal and emetic syndromes (Stenfor Arnesen et al. 2008), which in majority of cases, the disease course is mild and self-limiting. Occasionally, the illness behavior goes badly resulting in more severe conditions and may lead to deaths (Naranjo et al. 2011). The *B. cereus* food poisoning (vomiting and/or diarrhea) is attributed to presence of various types of toxins expressed by certain genes.

The multiplex PCR was conducted on *B. cereus* isolates for screening of two genes encoding virulence's toxins; emetic toxin 'cereulide' (*ces*) as well as an enterotoxin (*entFM*). Our results revealed that six isolates carried *entFM* (54.54%) while only two isolates harbored *ces* gene (18.18%) (Fig. 2). Similar data of high prevalence of *entFM* gene and low incidence of *ces* among *B. cereus* isolates from dairy products have previously been mentioned in numerous investigations (Kim et al. 2010; Chaves et al. 2012; Yim et al. 2015 and Zhao et al. 2020).

Conclusion

Contracting from this investigation, the raw buffalo's milk declared a significant isolation ratio of *B. cereus* organism which showed a pattern of multidrug resistance and harbored some toxigenic virulence genes. Consequently, it was proposed that raw buffalo's milk may be a source of *B. cereus* food-borne public health concern. The discrimination of pathogenic *B. cereus* from other morphologically and biochemically similar non-pathogenic *Bacillus* species can be achieved through molecular base.

Authors Contributions

HAA has made the experimental design, collected the milk samples, and prepared the initial draft of this manuscript. EAK and HSM isolated the organism and performed the biochemical identification. DDK performed the antibiotic susceptibility test. All authors participated in DNA extraction and PCR run. All authors have approved final version of the manuscript.

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