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Molecular studies on antibiotic resistant *Bacillus cereus* isolated from meat products and human in Kaliobia governorate, Egypt

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ABSTRACT

The current study was conducted on 210 random samples of meat products (beef burger, kofta, luncheon, minced meat, sausage) and diarrheic human stool of patients suffering from vomiting and diarrhea (35 for each). The meat products were collected from different shops and hospitals at Kaliobia Governorate, Egypt, for detection of *B. cereus* strains, and their phenotypic characterization as well as antibiotic resistant genes. Bacteriological examination of the collected samples indicated the identification of 51 (24.3%) isolates of *B. cereus* from 210 samples as 11 (31.4%) from kofta 13 (37.1%) from minced meat, 9 (25.7%) from sausage, 7 (20.0%) from beef burger, 6 (17.1%) from luncheon samples, and 5 (14.3%) from human stool specimens. Most of 51 isolated *B. cereus* strains had the ability for biofilm production. The antibiotic sensitivity profiles revealed that the isolated *B. cereus* was highly resistant for Penicillin-G followed by methicillin, ampicillin, oxytetracycline, sulfamethoxazole and cefotaxime. Meanwhile, they were highly sensitive to gentamycin and norfloxacin followed by ciprofloxacin, meropenem and florphenicol. Further, PCR declared that *bla*, *tetA* and *erm* genes were amplified in 9, 7, 6 out of 10 studied *B. cereus* isolates giving products of 680 bp, 502 bp, and 645 bp, respectively. Therefore, one can conclude that *B. cereus*, especially antibiotic resistances ones, is meat-borne pathogens of public health importance and they may be the causative agents in patients suffering from vomiting and diarrhea.

1. INTRODUCTION

Meat products are considered as good sources of high biological value proteins, vitamins specially vitamin B and certain minerals essential for growth and health of human beings. Contamination of meat products with toxigenic *B. cereus* is one of the underestimated foodborne illness worldwide (Ceuppens *et al.*, 2013). In Egypt, there is no accurate surveillance data about the numbers of *B. cereus* induced food poisoning cases. The lack of accurate data may be because of the resemblance of the symptoms with the other foodborne pathogens (Normanno *et al.*, 2007).

This bacterium is a Gram-positive, motile, rod shaped endospore-forming bacteria, aerobic that also grows well anaerobically and characterized as mesophilic or psychotrophic; mesophilic strains have a growth range of 15-55 °C and their spores tend to be more heat resistant. Whereas, psychotrophic ones have a growth range of 4-35 °C and their spores tend to be less heat resistant (Granum and Baird Parker, 2000; Organji *et al.*, 2015). Schedule identification of *B. cereus* is generally comprised isolation on selective media, revealing of motility, hemolysis prototype on blood agar, and acidification of glucose (Stenfors-Arnesen *et al.*, 2008).

The pathogenicity of *B. cereus* could be attributed to large number of secreted cytotoxins that may contribute to diarrhoeal disease, that is elicited by three pore-forming heat-labile enterotoxins; the two enterotoxin-complexes *nhe* (non-hemolytic enterotoxin) and *hbl* (haemolysin BL), each consist of three different protein components, named *nheA*, *nheB*, *nheC* and L2, L1, respectively, beside single-component toxin *cytK* "cytotoxin K" (Stenfors Arnesen *et al.*, 2008; Fagerlund *et al.*, 2010; Pfrunder *et al.*, 2016). In addition to these proteins, *B. cereus* produces degradative enzymes (proteases, sphingomyelinase, phosphatidyl inositol- and phosphatidylcholine-specific phospholipases "PIPLC and PC-PLC") which are either secreted or directed to the cell surface (Abostate *et al.*, 2006; Gohar *et al.*, 2008).

The gastrointestinal manifestations of the disease caused by *B. cereus* is connected to two clinical pictures; diarrhea and emesis. In general, both types of food poisoning are relatively mild and self-limiting, and the symptoms usually disappear within 24 h. Nevertheless, during the last few years, severe forms of both types of disease have occasionally involved hospitalization or even deaths (Dierick *et al.*, 2005). The wide-spread and imprudent use of antibiotics in food animals is thought to be accountable for the emergence and wider spreading of antimicrobial

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resistant (AMR) bacteria in humans (Shea, 2004; Kukanich *et al.*, 2005; Hawkey and Jones, 2009).

Antimicrobial resistance may be intrinsic resistance or acquired resistance. Intrinsic resistance is related to the unique physiological properties of a micro-organism, in which their metabolic activity is substantially unaffected by the presence of an antimicrobial compound. Such resistances are generally chromosomally encoded and are typically responsible for observed differences in resistance observed between genera, species and strains of bacteria. It can be associated with differences in cell wall structures, the ability to pump antimicrobial compounds out of the bacterial cell using efflux pumps, or the production of enzymes capable of inactivating antimicrobial compounds within the bacterial cell (Russell, 2001; Gilbert and McBain, 2003).

Acquired resistance, in which a previously sensitive bacterium becomes resistant, can arise as a result of a mutation in chromosomal DNA of a house-keeping structural or regulatory gene of the organisms (Courvalin and Trieu-Cuot, 2001; Martinez and Baquero, 2002) or through the acquisition of one or more antimicrobial resistance genes as a result of horizontal gene transfer within and between bacterial species, which can occur in the environment (*in vitro*) or during infection (*in vivo*), include conjugation, transduction and transformation, and can involve one or more defined genetic elements including: bacteriophages, plasmids, conjugative transposons and integrons (Carattoli, 2001; D'Costa *et al.*, 2006). The antimicrobial drug resistance of *B. cereus* strains through production of β -lactamases is one of potential virulence factors causing a serious public health concern (Schlegelova, *et al.*, 2003; Tewari *et al.*, 2012).

B. cereus can form biofilms on stainless steel that are more resistant to sanitizers than planktonic and attached single cells (Peng *et al.*, 2002; Schoeni and Wong, 2005). Significant increase in heat resistance is observed in *B. cereus* spores attached to stainless steel surfaces (Simmonds *et al.*, 2003; Schoeni and Wong, 2005).

As *B. cereus* induced food poisoning symptoms in human and the level of contamination of meat products with *B. cereus* constitutes serious problems for consumers, the present study was conducted to throw light over *B. cereus* isolates, specially antibiotic resistant ones, in common meat products beside diarrheic human stool of patients suffering from vomiting and diarrhea beside determination of virulence and their antibacterial resistant genes.

2. MATERIAL AND METHODS

2.1. Samples

A total of 210 random samples of meat products (beef burger, kofta, luncheon, minced meat, sausage) and

diarrheic human stool from patients suffered from vomiting and diarrhea (35 for each), were collected from different shops and hospitals at Kaliobia Governorate, Egypt, for detection the prevalence of enterotoxigenic *B. cereus* strains, beside the phenotypic characterization and detection of some virulence genes in them.

2.2. Bacteriological examination

25 grams of each meat product samples were prepared for bacteriological examination following APHA (2001). Beside two grams of each stool sample was homogenized in 18 mL of sterile pure water then 1 ml was added to a universal bottle containing 9 ml of 0.1% peptone water and incubated at 37 °C for 24 h for primary enrichment. Observation of turbidity in enrichment cultures was considered as a presumptive positive result (Organji *et al.*, 2015).

2.2.1. Isolation and identification of *B. cereus* strains

Typical *B. cereus* colonies (blue colonies surrounded by a blue zone of egg yolk precipitation against greenish yellow background on *B. cereus* agar base with Polymyxin B and Egg yolk supplements and whitish colonies with a zone of precipitation and red media on *B. cereus* medium with Polymyxin B and Egg yolk supplements) were picked up for identification morphologically by Gram stain and biochemical tests following De Vos *et al.* (2009) and Markey *et al.* (2013).

2.2.2. Detection the biofilm formation of isolated *Bacillus cereus* strains

The isolated *B. cereus* strains were examined for the development of biofilm using tube method of Hassan *et al.* (2011).

2.2.3. *In vitro* anti-microbial sensitivity test

The isolated *B. cereus* strains were subjected to sensitivity test against different antibiotics using the disc method of CLSI (2018).

2.2.4. Detection of antibiotic resistant genes of *B. cereus* by PCR

Genotypic detection of three antibiotic resistant genes, Beta-lactamase producing gene (*bla*); tetracycline resistant A gene (*tetA*) and erythromycin resistant gene (*erm*), in 10 random *B. cereus* isolates using polymerase chain reaction, following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara, Japan) and 1. 5% agarose gel electrophoreses (Sambrook *et al.*, 1989) using the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table (1).

Table 1 Primers sequences, target genes, amplicons sizes and cycling conditions required for PCR protocol.

Target gene	Primer sequence (5'-3')	Amplified segment (bp.)	Primary denaturation	Amplification (35 cycles)			Final extension	References
				Secondary denaturation	Annealing	Extension		
<i>bla</i>	F CATTGCAAGTTGAAGCGAAA	680 bp.	72 °C 45 sec.	50 °C 40 sec.	94 °C 30 sec.	94 °C 5 min.	72 °C 10 min.	Chen <i>et al.</i> (2004)
	R TGTCCCGTAACTTCCAGCTC							
<i>tetA</i>	F GGCGGTCTTCTCATCATGC	502 bp.	72 °C 45 sec.	58 °C 40 sec.	94 °C 30 sec.	94 °C 5 min.	72 °C 10 min.	Rather <i>et al.</i> (2012)
	R CGGCAGGCAGAGCAAGTAGA							
<i>erm</i>	F TCTAAAAAGCATGTAAAAGAA	645 bp.	72 °C 45 sec.	52 °C 40 sec.	94 °C 30 sec.	94 °C 5 min.	72 °C 7 min.	Adimpong <i>et al.</i> (2012)
	R TTCGATAGTTTATAATATTAGT							

3. RESULTS AND DISCUSSION

Bacillus cereus is one of the potential spoilage bacteria associated with meat products and the presence of them with high levels indicates a potential risk of producing toxins and food poisoning cases worldwide (Ceuppens *et al.*, 2013).

The recovered *B. cereus* isolates were Gram positive, short rod shaped and motile. They grew well on *B. cereus* agar base and *Bacillus cereus* media showed typical colonies. The results of biochemical identification showed that all isolates had characteristic biochemical reaction to be *B. cereus* where all the isolates (n=51) were positive for catalase, citrate utilization, nitrate reduction, Voges-Proskauer, and starch and gelatin hydrolysis. Meanwhile, they were negative for oxidase, indole and urease tests.

The results of bacteriological examination of examined samples (Table 2) cleared that 51 (24.3%) isolates of *B. cereus* were recovered from 210 samples. They were mostly isolated from kofta samples (13, 37.1%) followed by minced meat (11, 31.4%) then sausage (9, 25.7%); beef burger (7, 20.0%); luncheon (6, 17.1%) and stool samples (5, 14.3%). The results of *B. cereus* isolation from meat products were nearly similar to those obtained by Abd El-Tawab *et al.* (2015), Salim, Dalia *et al.* (2015), Shawish and Tarabees (2017), Soleimani *et al.* (2017), Abd El-Wahaab, Shimaa *et al.* (2018) and El-Shora, Heba (2019). But disagreed with those obtained by Rather *et al.* (2011), Tewari *et al.* (2012), Ibrahim, Hemmat *et al.* (2014) and El-Sayed (2019), who isolated *B. cereus* from meat products with lower incidence, and with those of Ghanaym (2014), who recorded higher incidence.

Regarding the results of *B. cereus* isolation from human stool, they conceded those recorded by Azemi *et al.* (2013), Martinelli *et al.* (2013) and Organji *et al.* (2015), who isolated *B. cereus* strains from stool samples of diarrheic patients. Moreover, the surveillance systems for foodborne disease differ between countries, and so it is difficult to compare data and obtain true incidence estimates. Several factors contribute to underreporting of most outbreaks of foodborne *B. cereus* disease. The clinical course is generally short and mild, so patients rarely seek medical attention, and when diagnosed, the disease is not always reportable (Stenfors Arnesen *et al.*, 2008; Al-Abri *et al.*, 2011; Organji *et al.*, 2015). Actually, the diagnosis of diarrhea stool samples in clinical laboratories in Egypt is solely based on the detection of *Salmonella*, *Shigella*, *E. coli* and *Entamoeba*. Thus, diarrhea caused by other pathogens such as *Campylobacter* and *B. cereus* may not be reported and usually if stool cultures were negative for the sought-after pathogens, then the diarrhea case could be reported as "unknown etiology" and/or "viral infection". So, detection of *B. cereus* must be considered in these cases.

The colonial appearance and the biochemical profile of *B. cereus* isolated was similar to those previously reported by Abd El-Tawab *et al.* (2015), Savic *et al.* (2015), Bashir *et al.* (2017) and El-Sayed (2019). In addition, most 51 isolated *B. cereus* strains had the ability for biofilm production that was clearly marked by a visible film lined the wall and the bottom of the tube where 47 *B. cereus* isolated strains (92.2%) produce biofilm, 38 (74.5%) strains showed strong biofilm, 9 (17.7%) strains showed moderate one and 4 (7.8%) isolates failed to produce biofilms. These results came in harmony with those of Jee-Hoon and Beuchat (2005), Wijman *et al.* (2007), and Ozdemir and Arslan (2019). The ability of *B. cereus* to

exist in biofilms is most probably important for its persistence in food industry equipment (Wijman *et al.*, 2007), as the biofilm protects spores and vegetative cells from inactivation by sanitizers (Ryu and Beuchat, 2005). The results of *in-vitro* sensitivity tests for isolated *B. cereus* (Table 3) revealed that the isolated *B. cereus* were highly resistant to Penicillin-G (92.2%) followed by methicillin (90.2%), ampicillin (88.2%), oxytetracycline (82.3%), Sulfa-trimethoprim (80.4%) and cefotaxime (54.9%). Meanwhile, they were intermediate sensitive to streptomycin (62.8%), erythromycin (58.8%) and neomycin (56.9%). Moreover, they were highly sensitive to gentamycin and norfloxacin (82.3% for each) followed by ciprofloxacin (74.5%), meropenem (72.5%) and florphenicol (62.8%). Nearly similar were recorded by Park *et al.* (2009), Banerjee *et al.* (2011), Rather *et al.* (2012), Merzougui *et al.* (2014), Savic *et al.* (2015), Bashir *et al.* (2017), El-Sayed (2019) and Solanki *et al.* (2019). Moreover, the results proved that, multiple antibiotic resistances are widely spread among isolated strains of *B. cereus* and decided the fact of Shalini and Rameshwar (2005) that the food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human populations.

Table 2 Prevalence of *B. cereus* strains isolated from examined samples of meat products and human stools.

Samples	Number of sample	Negative samples		Positive samples	
		No.	%	No.	%
Beef burger	35	28	80.0	7	20.0
kofta	35	22	62.9	13	37.1
luncheon	35	29	82.9	6	17.1
Minced meat	35	24	68.6	11	31.4
Sausage	35	26	74.3	9	25.7
Stool	35	30	85.7	5	14.3
Total	210	159	75.7	51	24.3

Percentage in relation to total number of each sample in each row (35 for each sample and 210 for total).

Table 3 In-Vitro anti-microbial sensitivity test for isolated *B. cereus* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		AA
		No.	%	No.	%	No.	%	
Penicillin-G	10 u	0	0.0	4	7.8	47	92.2	R
Methicillin	5 µg	1	2.0	4	7.8	46	90.2	R
Ampicillin	20 µg	1	2.0	5	9.8	45	88.2	R
Oxytetracycline	30 µg	1	2.0	8	15.7	42	82.3	R
Sulfa-trimethoprim	TMP5	2	3.9	8	15.7	41	80.4	R
Cefotaxime	30 µg	6	11.8	17	33.3	28	54.9	R
Ciprofloxacin	5 µg	38	74.5	0	0.0	13	25.5	S
Streptomycin	S/10	5	9.8	32	62.8	14	27.4	IS
Erythromycin	15 µg	10	19.6	30	58.8	11	21.6	IS
Neomycin	30 µg	12	23.5	29	56.9	10	19.6	IS
Gentamicin	10 µg	42	82.3	6	11.8	3	5.9	S
Norfloxacin	10 µg	42	82.3	8	15.7	1	2.0	S
Meropenem	10 µg	37	72.5	13	25.5	1	2.0	S
Florphenicol	30 µg	32	62.8	7	13.7	12	23.5	S

No.: Number of isolates. AA: Antibiogram activity. %: Percentage in relation to total number of isolates (51)

In addition, the results are of serious concern as these drugs, especially β -lactam antibiotics, are still considered the most recommended for the treatment of gastrointestinal diseases caused by bacterial infections in animals and human; however their efficiency has greatly deteriorated due to the production of β -lactamases by resistant bacterial strains (Bottone, 2010). So, it should be avoided as clinical treatment to gastroenteritis caused by *B. cereus* (Savic *et al.*, 2016).

PCR technique is capable of identifying the antibiotic resistant genes in pathogenic *B. cereus* isolates (Park *et al.*, 2009; Avsar *et al.*, 2017). The results of PCR for amplification of *bla* gene in *B. cereus* isolates (Fig. 1) showed that it was amplified in 9 out of 10 studied *B. cereus* isolates giving product of 680 bp. Similar detection of *bla* gene in *B. cereus* strains were recorded by Chen *et al.* (2004). Park *et al.* (2009), Tahmasebi *et al.* (2014), Kohneshahri *et al.* (2016), Savi *et al.* (2016), Avsar *et al.* (2017), El-Sayed (2019) and El-Shora, Heba (2019). Meanwhile, *tetA* gene was amplified in 7 out of 10 studied *B. cereus* isolates giving product of 502 bp (Fig. 2). Similar detection of this gene in *B. cereus* strains were recorded by Park *et al.* (2009), Rather *et al.* (2012), Avsar *et al.* (2017), El-Sayed (2019) and El-Shora, Heba (2019). Moreover, *erm* gene (Fig. 3) it was amplified in 6 out of 10 studied *B. cereus* isolates giving product of 645 bp. Similar detection of *erm* gene in *B. cereus* strains were recorded by Park *et al.* (2009), Adimpong *et al.* (2012) and El-Sayed (2019). But these data disagreed with results of El-Shora, Heba (2019), who failed to detect *erm* gene in *B. cereus* isolates.

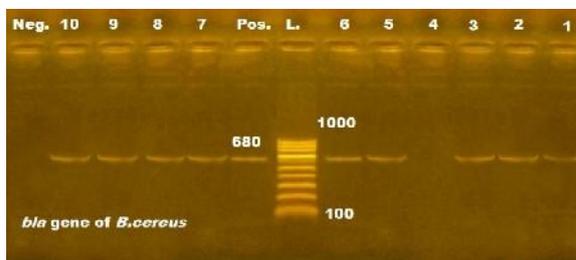


Fig. 1 Beta-lactamase producing (*bla*) gene, Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*E. coli* AJ413986). Pos.: Positive control (*B. cereus* form Ahri. at 680 bp.). Lane 1-3 and 5-10: *B. cereus* (Positive at 680 bp.). Lane 4: *B. cereus* (Negative)

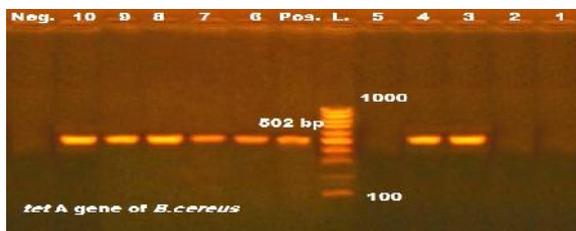


Fig. 2 Tetracycline resistant A (*tetA*) gene, Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*E. coli* AJ413986). Pos.: Positive control (*B. cereus* form Ahri. at 502 bp.). Lane 3; 4 and 6-10: *B. cereus* (Positive at 502 bp.). Lane 1; 2 and 5: *B. cereus* (Negative)

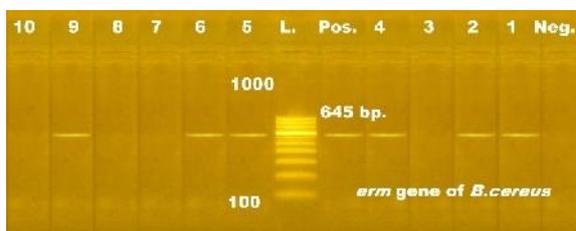


Fig. 3 Erythromycin resistant (*erm*) gene, Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control (*E. coli* AJ413986). Pos.: Positive control (*B. cereus* form Ahri. at 645 bp.). Lane 1; 2; 4-6 and 9: *B. cereus* (Positive at 645 bp.). Lane 3; 7; 8 and 10: *B. cereus* (Negative)

4. CONCLUSION

The present study concluded that *B. cereus* strains are enterotoxigenic ones with multiple antibiotic resistances. They are meat-borne pathogens of public health importance and they may be the causative agents in patients suffering from vomiting and diarrhea, so, detection of *B. cereus* must be considered in these cases and schedule antibiotic

susceptibility testing of *B. cereus* isolates recovered from meat products and human stool of patients will guide choosing the appropriate antibiotic. Moreover, the obtained data authenticate the significance of *B. cereus* isolation in disease control and prevention programs, and in regular clinical and food quality control laboratories in Egypt.

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