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Prevalence and antimicrobial resistance of *Bacillus cereus* isolated from beef products in Egypt

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Abstract
Foodborne pathogens have the main concern in public health and food safety. *Bacillus cereus* food poisoning is one of the most important foodborne pathogens worldwide. In the present study, a total of 200 random beef product samples were collected from different supermarkets located at Menofia and Cairo governorates were examined for the presence of *B. cereus*. In addition, the presence of some virulence encoding genes was evaluated using Multiplex PCR. Finally, the antibiogram testing was conveyed to illustrate the resistance pattern of the confirmed *B. cereus.* The data showed that *B. cereus* was recovered from 22.5%, 30%, 25%, 37.5% and 15% of the minced meat, burger, sausage, kofta, and luncheon respectively. Among the 20 examined isolates 18/20 (90%) were harbor *hblC* enterotoxin encoding gene compared with 20/20 (100) were have *cytK* enterotoxin encoding gene. The isolated strains of *B. cereus* were resistant to penicillin G and sensitive to oxacillin, clindamycin, vancomycin, erythromycin, gentamicin, ciprofloxacin, and ceftriaxone. In all, the obtained data showed the importance of emerging *B. cereus* in disease control and prevention programs, and in regular clinical and food quality control laboratories in Egypt.
Keywords: Antimicrobial susceptibility, *Bacillus cereus*, Beef products, Multiplex PCR, Virulence genes.

Introduction

Processed beef products such as minced meat, kofta, sausage, burger, and luncheon are gaining common popularity as easily quick prepared meat meals that can solve the problem of the high price fresh meat shortage which is not within the reach of large numbers of low-income families. The contamination of these beef products with the foodborne pathogens is still the main worry for public health, amongst contamination with *B. cereus* is one of the most important foodborne pathogens causing food poisoning among the food consumers all-inclusive.

B. cereus is an aerobic spore-forming Gram-positive bacterium normally disseminated in the environment. It is usually isolated from the soil, plant materials, raw meat and processed meat products (Carlin F *et al*., 2010; Ceuppens *et al*., 2013). 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).

Although *B. cereus* is implicated in many foodborne illness outbreaks in many countries worldwide, however only a few cases are reported because the symptoms are mostly similar to *Staphylococcus aureus* and *Clostridium perfringens* food poisoning (Stenfors Arnesen *et al*., 2008; Bottone, 2010; Bennett *et al*., 2013). *B. cereus* has been incriminated as a cause of two types of food poisoning, emetic and diarrheal syndromes (Drobniewski, 1993).

The pathogenesis of *B. cereus*-induced food poisoning is mostly still indistinct. The microorganism conveys an expansive number of potentially toxic components, including hemolysins, phospholipases, and proteases (Drobniewski, 1993; Beecher, 2001) nevertheless, the precise role of some is still ambiguous. The emetic and the diarrheal syndromes are still the foremost worries for the public health apprehension and the full appreciative of their pathogenesis is imperative. These syndromes are mainly manifested via the release of two core toxins, a heat-labile diarrheal enterotoxin, and heat- stable emetic enterotoxin (Stenfors Arnesen *et al*., 2008).

The diarrheal syndrome manifested via the release of one or three diarrheal enterotoxins: the tripartite toxins hemolysin BL (HBL) and non-hemolytic enterotoxin (Nhe), the two forms of cytotoxin K (cytK-1 and cytK-2) and possibly enterotoxin T and enterotoxin FM (Moravek *et al*., 2006). HBL, a three-components toxin, that is encoded by *hblD* and *hblC* genes respectively, and a binding component B encoded by *hblA* gene. The presence of all three components is necessary for the toxin activity (Lindback and Granum, 2006).

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The deceptive of *B. cereus* induced food poisoning symptoms and the lack of the clear-cut surveillance statistics in Egypt. This makes the indulgent of the pathogenesis of *B. cereus* food poisoning confusing. Therefore, the current study was undertaken to estimate the incidence of toxigenic *B. cereus* in some beef products collected at the retail level in Egypt using PCR. Additionally, the antibiotic resistance pattern of 20 *B. cereus* isolates was assessed using disc infusion method.

Material and Methods

Sampling

A total of 200 beef product samples (40 each of minced meat, burger, sausage, kofta, and luncheon) were collected from different supermarkets located at Menofia and Cairo governorates and examined bacteriologically. The presence of toxigenic *B. cereus* was confirmed using PCR based on the presence of virulence encoding genes.

Preparation of samples

The collected samples were transferred instantly under full aseptic conditions for bacteriological isolation and identification of *B. cereus*. Briefly, 25 grams of each product were transferred to 225 ml of 0.1% sterile buffered peptone water (Oxoid, UK), then stomached for 2 minutes to provide a homogenate. The homogenate was heat-treated at 80ºC for 10 minutes to kill all the vegetative bacteria and recover of the *Bacillus* spores (Rahimi *et al*., 2013). One ml of the original dilution transferred to a sterile tube containing 9 ml of sterile buffered peptone and incubated at 34ºC for 24 hrs as a primary enrichment.

Isolation and characterization of Bacillus cereus

The bottles showed turbidity as an indication of *B. cereus* growth were streaked over a dry surface of *Bacillus cereus* selective agar medium (Oxoid, UK) by a bent glass rod and the plates were incubated at 30ºC for 24-48 hrs. Suspected typical colonies were later picked up onto sheep blood agar (Oxoid, UK) and incubated at 34ºC for 24 hrs to observe hemolysis (Tallent *et al*., 2012).

Typical colonies of *B. cereus* that showed β hemolysis were further identified based on the biochemical activities (Holbook and Anderson, 1980; Bottone, 2010; Tallent *et al*., 2012).

Genotypic characterization of B. cereus enterotoxins genes hblC and cytK

The multiplex PCR was carried out according to Ngamwongsatit *et al*. (2008). The PCR reactions containing 12.5 µl PCR Master Mix, 1 µl of each primer (0.4 µM *hlb*C and 0.2 µM *cyt*K as final concentration), of 5 µl of DNA templates and RNase-free water was added to a final volume of 25 µl. The PCR conditions were, 94 °C/ 5 min; 30 cycles of (94ºC for 45 sec, annealing at 54-56ºC for 1 min in case of *hbl*C and at 58ºC in case of *cyt*K, elongation at 72ºC for 2 min) followed by 72ºC for 5 min. 94ºC for 45 sec, annealing at 54 and 56ºC for 1 min in case of *hbl*C and at 58ºC in case of *cyt*K, elongation at 72ºC for 2 min and final extension at 72ºC for 5 min. The products of PCR were separated by electrophoresis on 1.5% agarose gel (AppliChem, Germany) to determine the fragment sizes. The nucleotides sequences of the primers are shown in the Table 1.

Antimicrobial susceptibility test

The antibiotic susceptibility testing was performed using the disc diffusion method (Chon *et al*., 2012). All the isolates were grown in brain heart infusion broth (Oxoid) for 18 hrs at 34ºC and then spread on Mueller-Hinton agar (Oxoid, UK) and left for 15 minutes. Then, eight commercial antibiotic discs (Oxoid, UK) were used: penicillin (10 units), oxacillin (1.0 mg/ml) vancomycin (30 mg/m), clindamycin (2.0 mg/ml), erythromycin (15 mg/ml), gentamicin (10 mg/ml), Ciprofloxacin (5µg) and Ceftriaxone (30 µg), and the plates were then incubated at 37 ˚C for 18–24 hrs (Chon *et al*., 2012).

Results

Prevalence of B. cereus in the examined beef products

The data presented in Table 2 showed the prevalence rate of *B. cereus* in the examined beef products. Among the examined beef products, only 52 out of 200 (26%) were positive for *B. cereus*. The highest prevalence rate was recorded in case of beef kofta 15/40 (37.5%), while the lowest rate was in case of beef luncheon 6/40 (15%).

Genotypic characterization of enterotoxigenic genes using Multiplex PCR

The data obtained in Table 3 demonstrated the incidence rate of the *hblC* and *cytK* enterotoxigenic genes in the examined *B. cereus* isolates. Among the examined isolates, 18/20 (90%) were harbor *hblC* enterotoxin encoding gene compared with 20/20 (100%) were found to have *cytK* enterotoxin encoding gene and exhibited a specific band size (Fig. 1).

Antibiotic sensitivity testing

The data presented in Table 4 showed the antibiotic resistance pattern of the examined *B. cereus* isolates. A total of 51 isolates were tested for their antibiotic sensitivity prototype against 8 commercial antibiotic discs. The data demonstrated that all the isolates (51/51) were resistant to penicillin G (100%) and sensitive to other antibiotics 51/51 (100%).

Discussion

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).

Table 1. Primers nucleotides sequences used for multiplex PCR amplification of *B. cereus* entrotoxins genes.

Target gene	Primer	Size in bp	Primer sequence (5'—3')	TºC	Product size in bp	conc(µM)
*hbl*C	FHblC	19	CCTATCAATACTCTCGCAA	54	695	0.4
	RHblC	20	TTTCCTTTGTTATACGCTGC	56		
*cyt*K	FCytK	20	CGACGTCACAAGTTGTAACA	58	565	0.2
	R2CytK	20	CGTGTGTAAATACCCCAGTT	58		

Table 2. Prevalence rate of *B. cereus* in the examined beef products (40 of each).

Beef products	Positive sample		Negative sample	
	No	%	No	%
Minced meat	9	22.5	31	77.5
Beef burger	12	30	28	70
Beef sausage	10	25	30	75
Beef kofta	15	37.5	25	62.5
Beef luncheon	6	15	34	85
Total	52	26	148	74
Minced meat	No	%	No	%

Table 3. Molecular detection of enterotoxigenic genes of *B. cereus* isolated from examined samples.

Target gene	No of examined isolate	Positive isolate	%
*hbl*C	20	18	90
*cyt*K	20	20	100

Table 4. Antibiotics resistant of *B. cereus* isolated beef products (*n*=51).

Antibiotic tested	Resistant	Intermediate	Sensitive
Penicillin G	51(100.0)	0 (0.0)	0 (0.0)
Oxacillin	0 (0.0)	0 (0.0)	51(100.0)
Vancomycin	0 (0.0)	0 (0.0)	51(100.0)
Clindamycin	0 (0.0)	0 (0.0)	51(100.0)
Erythromycin	0 (0.0)	0 (0.0)	51(100.0)
Gentamicin	0 (0.0)	0 (0.0)	51(100.0)
Ciprofloxacin	0 (0.0)	0 (0.0)	51(100.0)
Ceftriaxone	0 (0.0)	0 (0.0)	51(100.0)

The contamination of beef products probably occurred during handling and preparation or post-processing contamination. In addition, keeping the products unrefrigerated for several hours enhances the multiplication of *B. cereus* and hence the liberation of enterotoxin. The study herein was aimed to estimate the accurate incidence rate of toxigenic *B. cereus* and its antibiotic susceptibility pattern in some beef products collected from different localities in Egypt. The data obtained will probably highlight the emergence of *B. cereus* as a serious underestimated cause of foodborne illness and will help in understanding its pathogenesis.

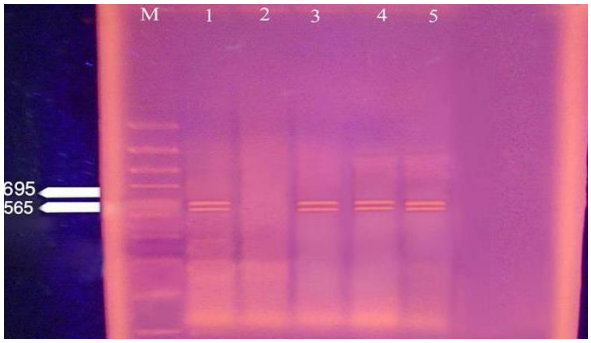

Fig. 1. Genotypic characterization of enterotoxins encoding genes *hhl*C and *cyt*K using specific primers sets. Lane (M): DNA Ladder (10bp); Lane (1): positive control; Lane (2): negative control; Lanes (3-5) *Bacillus cereus* exhibited a specific band size of 595 bp and 565 bp representing *hbl*C and *cyt*K enterotoxin-encoding genes respectively.

The data obtained in the Table 2 demonstrated that the highest incidence rate was recorded in beef kofta and the lowest was in the case of minced meat. This outcome is similar to that obtained by Mohamed and Ghanyem (2015), and higher compared with that obtained by Heikal *et al*. (2006). Conversely, this result is lower than the result obtained by Eid *et al*. (2008).

Processed ready to eat beef products are considered the main source of infection with *B. cereus* and more caution need to be taken in order to minimize the contamination of such products. The selection of fresh and clean flesh, decontamination of the mincing machine, grinders, equipment and knives used in the processing of such products will auspiciously decrease the incidence of *B cereus* foodborne illness cases among the consumers (FDA, 2012; Torky, 1995, 2004). The higher incidence rate of the *B. cereus* in kofta and minced meat in comparison with luncheon can be explained as luncheon during the processing steps the product was subjected to a high temperature that significantly decreases the number of *Bacillus* spores (Torky, 1995).

Additionally, during the processing of minced meat and kofta, additives, seasoning, and spices were added, these additives are considered a potential risk factor can increase the number of *Bacillus* spores and hence magnitude the incidence of food poisoning. Therefore more consideration should be taken during processing of raw meat and kofta, and only use additives from a trustful source. Moreover, these additives should be regularly tested for the presence of *Bacillus* spores.

Schedule examination of beef products for the presence of *Bacillus* spores is requisite. Isolation and identification of *Bacillus* using traditional methods (culturing on selective media and biochemical testing of the confirmed isolates) is still the key element for the confirmation of the infection. The severity of infection with *Bacillus* is conveyed via the liberation of an array of virulence encoding genes.

Multiplex PCR has emerged as the fast and reliable technique for the confirmation of enterotoxigenic *B. cereus* (Guinebretiere *et al*., 2006; Ombui *et al*., 2008). Recently, Ngamwongsatit *et al*. (2008) have developed and evaluated a group of newly efficient primers used for detection of the genes encoding enterotoxin production in 100% of the tested *B. cereus* and *B. thuringensis* strains assuming that, the presence of either gene is an indication for the presence of the whole operon (Ngamwongsatit *et al*., 2008).

In the current work, the existence of the enterotoxin-encoding genes *hbl*C and *cyt*K was assessed in 20 *B. cereus* isolates using specific primers sets that previously approved by Ngamwongsatit *et al*. (2008). The data presented in Table 3 and Figure 1 demonstrated that 18 isolates (90%) and 20 isolates (100%) were positive for *hbl*C and *cyt*K gene, respectively. This outcome is in accordance with that obtained previously obtained by Awny *et al*. (2010). Collectively, emerging of the multiplex PCR as a rapid technique for the affirmation of toxigenic *B. cereus* in food will probably command the pathogenesis of *B. cereus* induced-food poisoning in Egypt.

A total of 51 *B. cereus* isolates were further tested for their antimicrobial susceptibility (Table 4). All the tested isolates were resistant to penicillin G, whereas sensitive to oxacillin, clindamycin, vancomycin, erythromycin, gentamicin, ciprofloxacin, and ceftriaxone. The data obtained herein with the others (Fenselau *et al*., 2008; Organji *et al*., 2015; Jawad *et al*., 2016) showed that *B. cereus* has a broad range of antibiotic susceptibility and validate the resistance to penicillin G by comparing to susceptibility to clindamycin, vancomycin, and erythromycin.

Conclusion

From the obtained data, many conclusions could be drawn, contamination of beef products with *B. cereus* increase the potential of foodborne infections among the consumers. The cleanliness of the equipment, processing machines, knives, and only use additives from trustful sources are measures significantly will minimize the infection with *Bacillus* spores. Schedule antibiotic susceptibility testing of *B. cereus* isolates recovered from beef products will guide choosing the appropriate antibiotic. Also, the data authenticate the significance of counting *B. cereus* in disease control and prevention programs, and in regular clinical and food quality control laboratories in Egypt.

Conflict of interest

The authors declare that there is no conflict of interest.

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