



Detection of Virulence Genes in *Bacillus cereus* isolated from Meat Products Using PCR

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ABSTRACT

Bacillus cereus is an opportunistic pathogen that can cause food poisoning in humans as a result of consuming foods containing toxins or bacteria. In this study, the incidence of *B. cereus* and its virulence genes in meat products was investigated. Isolation of *B. cereus* was performed using selective PEMBA media and confirmed by morphological and biochemical tests and Vitek2 compact system. The incidence of *B. cereus* strains in beef and chicken meat products was 28%. The incidence of *Bacillus cereus* in frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets was 16%, 24%, 28%, and 44%, respectively. Moreover, the result of multiplex PCR of virulence genes of *groEL* gene (533bp), *Hbl* gene (1091 bp), *Nhe* gene (766 bp) and *CytK* gene (421bp) indicated that *groEL* gene, *Nhe* gene, *CytK* gene was found in 100% of *B. cereus* isolated from different meat products, while *Hbl* gene was detected in 10% of isolates. The results demonstrate that meat products represent a threat to public health through the transmission of *Bacillus cereus*.

Key words: *Bacillus cereus*, Beef meat, Chicken meat, PCR, Virulence genes, VITEK2

INTRODUCTION

The genus *Bacillus* includes harmless environmental and pathogenic species. The *B. cereus* group is known as pathogens or opportunistic pathogens to humans (Logan, 2012). The *B. cereus* is associated with food poisoning as a result of the consumption of food containing pre-formed toxins or bacteria producing toxins in the human gut (McKillip, 2000).

Genus *Bacillus* are Gram-positive rods able to produce endospores resistant to unfavorable external conditions (Logan and Devos, 2009) that can be distinguished from other spore-formers (*Sporolactobacillus*, *Clostridium*, *Desulfotomaculum*, *Sporosarcina*, and *Thermoactinomyces*) due to their aerobic character (strict or facultative), rod-shaped cells and catalase production (Slepecky and Hemphill, 2006). Schedule identification of *B. cereus* is generally combined with isolation on selective media, illuminating of motility, hemolysis pattern on blood agar, and acidification of glucose (Stenfors et al., 2008).

The pathogenesis of *B. cereus*-induced food poisoning is mostly still unclear. The microorganism transmits an expansive number of potentially toxic

components, including hemolysins, phospholipases, and proteases (Beecher, 2001) nevertheless, the accurate role of some toxins is still unclear. The emetic and the diarrheal syndromes are still the foremost concerns for the public health apprehension and the full appreciative of their pathogenesis is imperative. These syndromes are mainly revealed via the release of two core toxins, a heat-labile diarrheal enterotoxin, and heat-stable emetic enterotoxin (Stenfors et al., 2008).

The diarrheal syndrome revealed 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* is a three-component 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 important for the activity of toxin (Lindback and Granum, 2006).

The objective of this study was to conduct bacteriological and molecular studies on *B. cereus* isolated from frozen rice kofta, frozen kobiba-shami, chicken pane, and chicken nuggets.

MATERIALS AND METHODS

Collection of Samples

A total of one hundred random samples of meat products which including frozen rice kofta, frozen kobibashami, chicken pane and chicken nuggets (25 of each) were collected from different shops, supermarkets in different localities in Menoufia and Kalyobia governorates. Samples conveyed to the laboratory following aseptic and safety precautions.

Isolation and identification of *Bacillus cereus* group

A stomacher was used to homogenize 10 g of each sample in 90 mL of buffered peptone water (BPW) for 2 min. Heat treatment of all samples at 70 °C for 15 min was used to eliminate vegetative cells and allow the isolation of spores (Al- Allaf, 2011). The pasteurized samples were immediately positioned in ice to prevent spore germination. An amount of 100 µl was spread on Polymyxin-pyruvate-Egg yolk-Mannitol-Bromothymol blue agar (PEMBA) media plates and incubated at 37 °C for 24-hr both aerobically and anaerobically. The plates were examined and the presumptive *B. cereus* group was confirmed based on microscopy of Gram-stained preparations and biochemical tests (FDA, 2015). A number of colonies were randomly collected and analyzed by cell morphology under the microscope, Gram staining, ability to form endospores, growth in the presence of sodium chloride, anaerobic growth, catalase and oxidase activity, Voges-Proskauer test and growth at pH 5.7. The ability to ferment carbohydrates, starch hydrolysis, use of citrate as a carbon source, lecithinase activity, and growth inhibition by lysozyme were applied (Al- Allaf, 2011 and FDA, 2015).

Identification of *Bacillus cereus* using VITEK2 BCL Card

Bacterial suspensions were arranged in 3.0 mL of sterile saline and accustomed to a McFarland standard of 1.80-2.20 using the VITEK2 DensiChek (bioMérieux). BCL cards were packed automatically in the VITEK vacuum chamber, sealed, incubated at 35.5°C and read automatically every 15 min for 14 hours. Data were investigated automatically using the VITEK2 database.

PCR detection of virulence *Bacillus cereus* isolates DNA extraction

The isolates of *B. cereus* isolates from different meat products were grown in 5 mL nutrient broth with shaking for 18 h at 30 °C and collected at 5,000 g for 5 min. QIAamp DNA Mini Kit was used for genomic DNA extraction and purification. PCR was achieved to detect *groEL* gene and three enterotoxigenic encoding endotoxins genes *Nhe*, *hbl* and *cytK* genes. A positive reference strain of *B. cereus* ATCC 14579 and sterile MilliQ water as a negative control was used in PCR analysis (Ehling-Schulz et al., 2006; Das et al., 2013). Table 1 provides details about the primers used.

Preparation of PCR master mix

occurred according to Emerald Amp GT PCR mastermi (Takara) CodeNo.RR310A kit as shown in table 2.

Cycling conditions of the primers during PCR

PCR conditions are shown in table 3. Gel electrophoresis was used to analyze PCR fragments for presence and correct size compared to positive control (Sambrook et al., 1989). PCR runs where a negative control displayed amplification or positive control did not amplify were overlooked and repeated.

RESULTS

Prevalence of *Bacillus cereus* in meat products

The prevalence of *B. cereus* in meat products (frozen rice kofta, frozen kobibashami, chicken pane, and chicken nuggets) was 16%, 24%, 44%, and 28%, respectively. Out of 28 *Bacillus* isolates, 18 (36%) isolates obtained from chicken product samples and 10 (20%) isolates were recovered from beef product samples. The incidence of *B. cereus* group in the different meat products shown in table 4.

PCR results

The result obtained using agarose gel electrophoresis of multiplex PCR of virulence genes, *groEL* gene (533bp), *Hbl* gene (1091 bp), *Nhe* (766 bp) and *CytK* gene (421bp) for characterization of virulence genes of *B. cereus* isolated from different meat products showed that *groEL* gene (Figure 1), *Nhe* gene (Figure 3) and *CytK* gene (Figure 4) were found in 100% of tested isolates of *B. cereus* and the *Hbl* gene was detected in 10% of tested *B. cereus* isolates (Figure 4).

Table 1. Oligonucleotide primers sequences used in this study to detect *Bacillus cereus*

Gene	Primer sequence	Length of amplified product (base pair)	Reference
<i>groEL</i>	F: 5'- TGCAACTGTATTAGCACAAGC T -3' R: 5'-TACCACGAAGTTTGTTCACTACT-3'	533	Das et al. (2013)
<i>Nhe</i>	F: 5'-AAG CIG CTC TTC GIA TTC-3' R: 5'-ITI GTT GAA ATA AGC TGT GG-3'	766	Ehling-Schulz et al. (2006)
<i>cytK</i>	F: 5'-ACA GAT ATC GGI CAA AAT GC-3' R: 5'-CAA GTI ACT TGA CCI GTT GC-3'	421	Ehling-Schulz et al. (2006)
<i>Hbl</i>	F: 5'-GTA AAT TAI GAT GAI CAA TTTC-3' R: 5'-AGA ATA GGC ATT CAT AGA TT-3'	1091	Ehling-Schulz et al. (2006)

F: forward, R: reverse

Table 2. PCR master mix component used for PCR reaction for detection of virulence genes of *Bacillus cereus*

Component	Volume
Emerald Amp GT PCR mastermix (2x premix)	12.5µl
PCR grade water	4.5µl
Forward primer(20 pmol)	1µl
Reverse primer (20 pmol)	1µl
Template DNA	6µl
Total	25µl

Table 3. Temperature and time conditions used during PCR assay

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
<i>groEL</i>	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	35	72°C 10 min.
<i>Nhe, hbl, cytK</i>	94°C 5 min.	94°C 30 sec.	49°C 40 sec.	72°C 1 min.	35	72°C 10 min.

Table 4. Incidence of *Bacillus cereus* isolated from examined meat products

Products	Kobiba–shami (n=25)	Rice kofta (n=25)	Total beef products (n=50)	Chicken pane (n=25)	Chicken nuggets (n=25)	Total chicken products (n=50)
No of positive samples	6	4	10	11	7	18
Percentage of positive samples	24%	16%	20%	44%	28%	36%

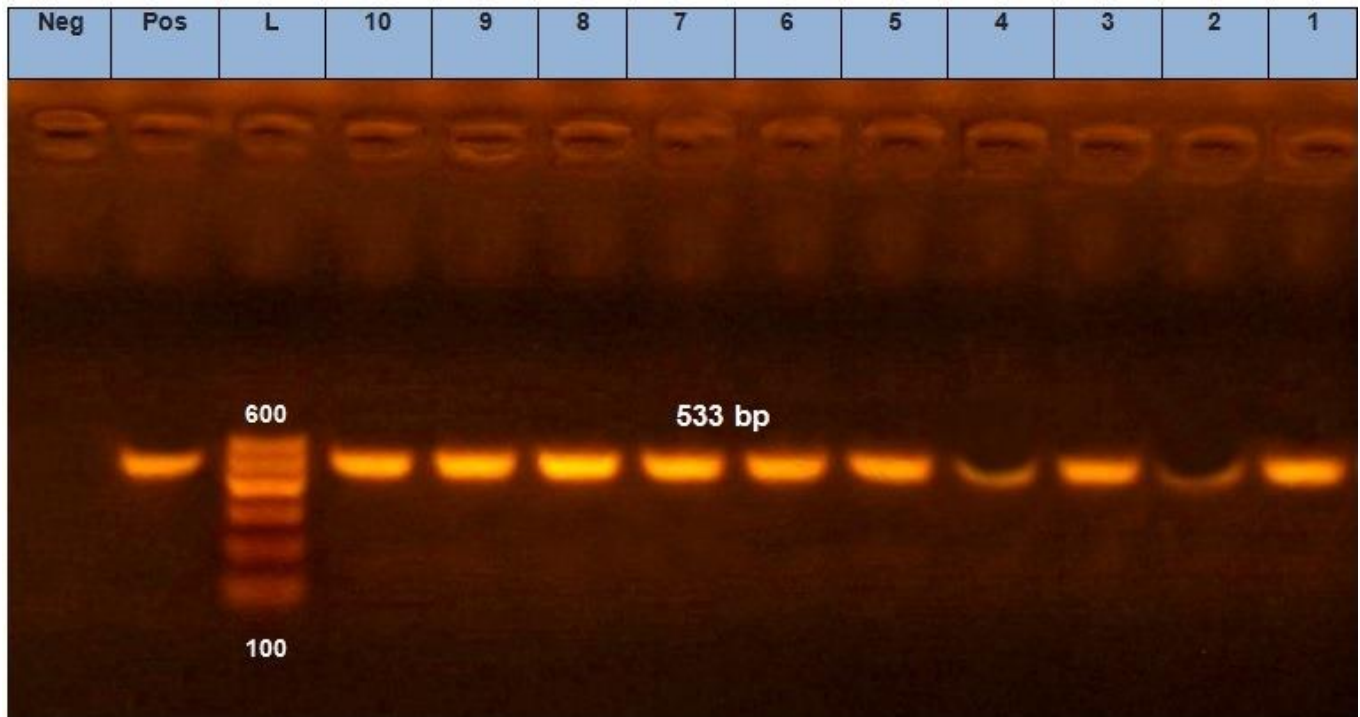


Figure 1. Results of PCR amplification of *groEl* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-600 bp DNA ladder, Lane 1-10: positive samples at 533 bp

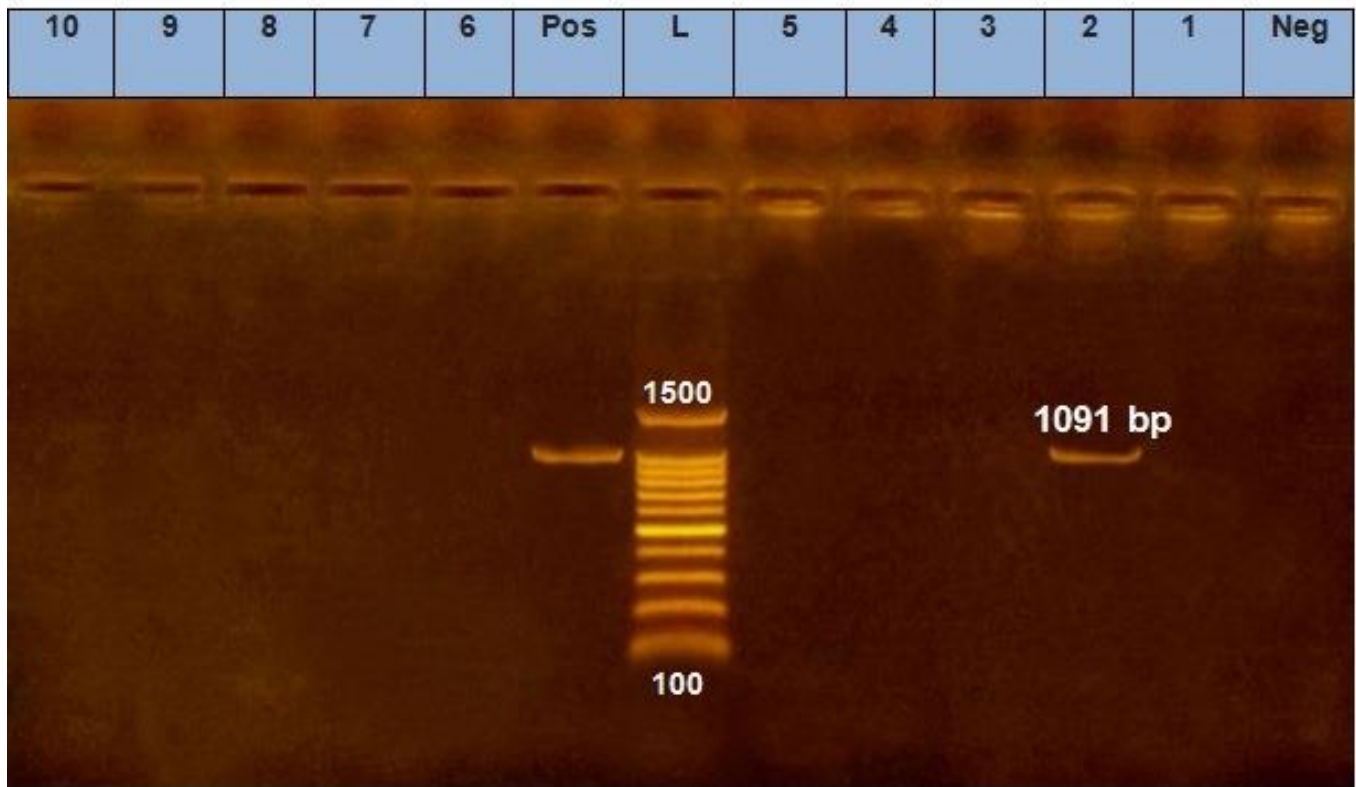


Figure 2. Results of PCR amplification of *hbl* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-1500bp DNA ladder, Lane 2: positive sample at 1091 bp. Lane 1, 3, 4, 5, 6, 7, 8, 9, and 10: negative samples.

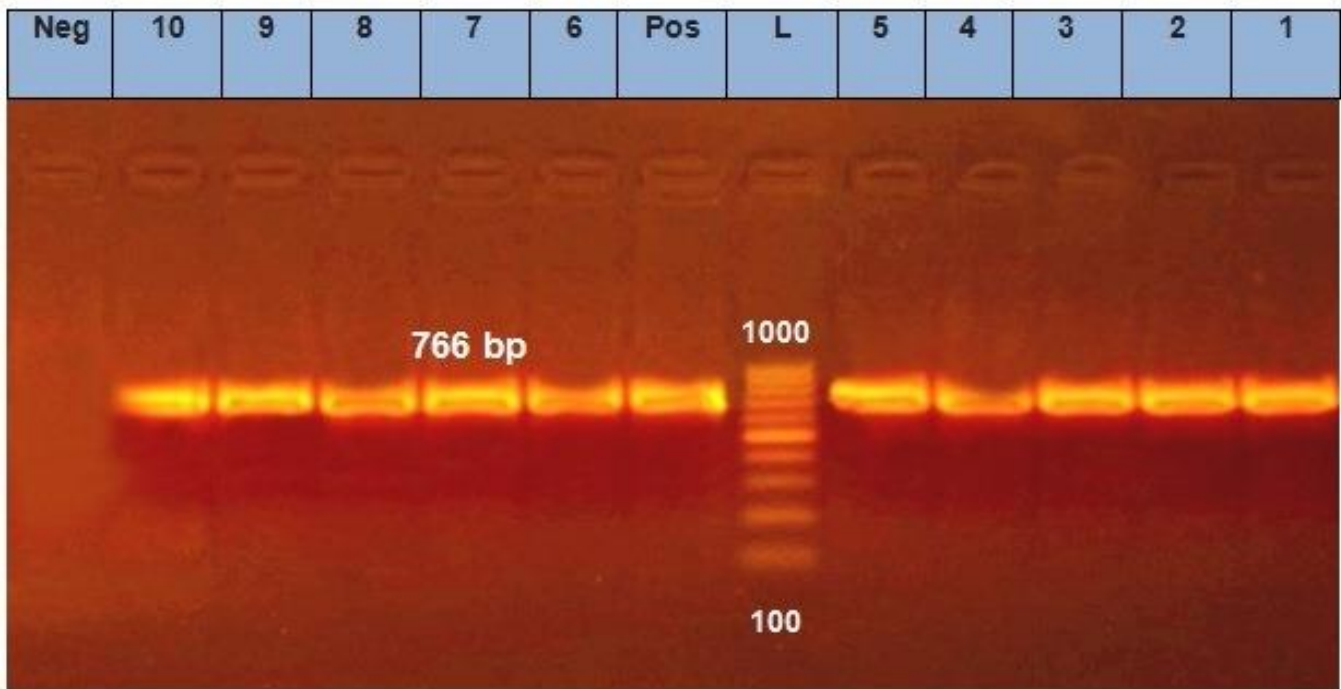


Figure 3. Results of PCR amplification of *Nhe* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-1000bp DNA ladder, Lane 1-10: positive samples at 766 bp.

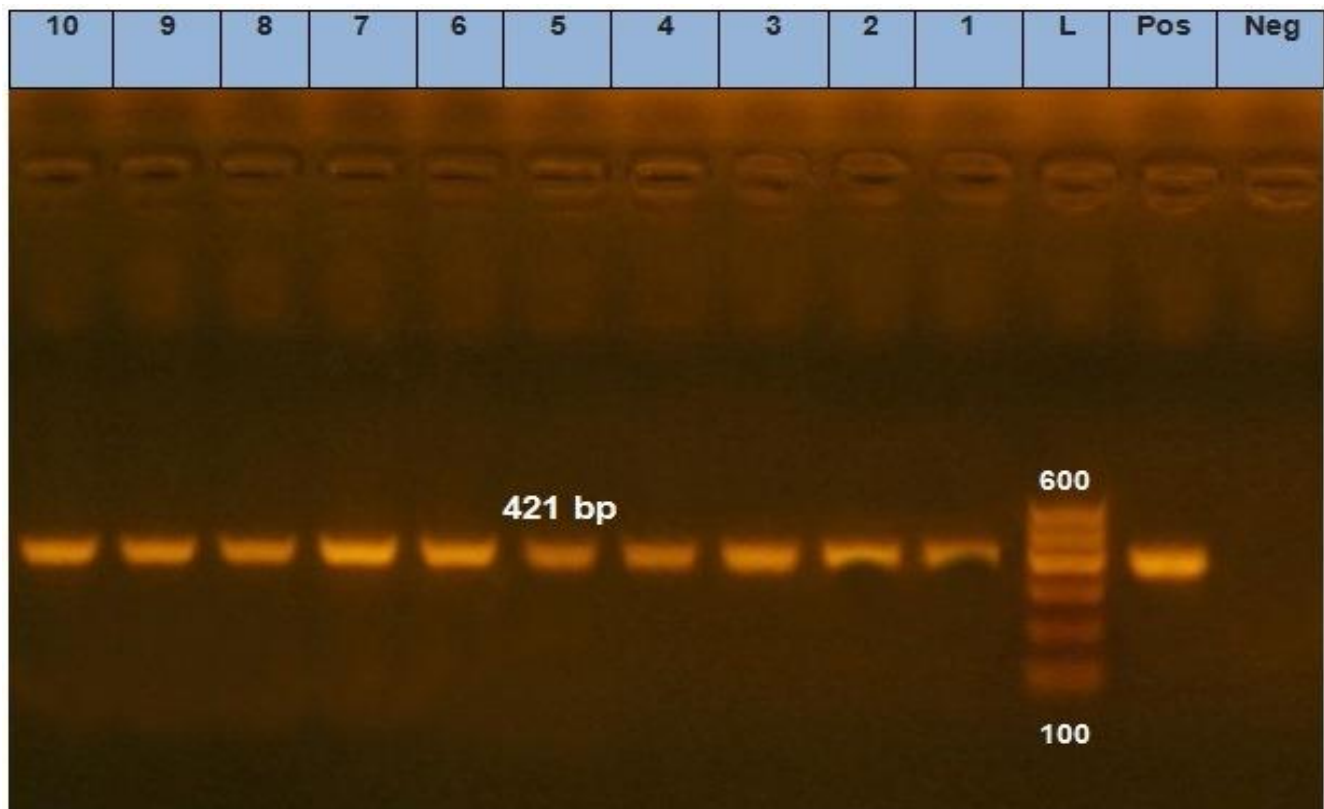


Figure 4. Results of PCR for amplification of *cytK* gene of *Bacillus cereus* isolated from different meat and chicken products. Neg: negative control, Pos: positive control, Lane L: 100-600bp DNA ladder, Lane 1-10: positive samples at 421 bp.

DISCUSSION

Food-borne diseases are reported to be a serious hazard to public health all over the world. Among the organisms responsible for causing foodborne diseases, *B. cereus* has emerged as a major foodborne pathogen during the last few decades and causes two types of illness through the elaboration of enterotoxins (Jay, 2005).

In this study, the incidence of *B. cereus* in meat products was 28%. These results were nearly similar to that obtained by Tewari et al. (2015), who isolated *B. cereus* from 35% of meat products. The results of the present study were higher than those obtained by Ashraf et al. (2019), who isolated *B. cereus* at a percentage of 11.24, while the results were lower than those obtained by Shimaa et al. (2018) who isolated *B. cereus* at a percentage of 47%.

The incidence of *B. cereus* in beef meat products was 20% that was lower than that obtained by Hesham et al. (2018), where the incidence of *B. cereus* was 38.2%. The incidence of *B. cereus* in chicken meat products was 36% nearly similar to results obtained by Hesham et al. (2018), who isolated *B. cereus* from chicken meat products at a rate of 48%.

The incidence of *B. cereus* in Kobeba-shami was 24%. This result was lower than that obtained by Shimaa et al. (2018) where the incidence of *B. cereus* was 52% and Hemmat et al. (2014) who isolated *B. cereus* from 84% of the examined kobeba-shami samples. The incidence of *B. cereus* in Rice kofta was 16%. This result was lower than that obtained by Shimaa et al. (2018) who isolated *B. cereus* at a rate of 60%. The incidence of *B. cereus* in nuggets was 28%. This result was lower than that obtained by Smith et al. (2004) who isolated *B. cereus* at a percentage of 91.6%. The incidence of *B. cereus* in chicken pane was 44% that was higher than that obtained by Smith et al. (2004) who failed to isolate *B. cereus* from examined chicken products.

These variations in the results were attributed to the quality of raw materials and the hygienic state during the preparation and processing of the product. The high frequency of isolation of *B. cereus* from meat products may be attributed to the processing of minced meat also additives and spices added to these products, which can increase the number of *Bacillus* spores. Therefore it is important to use additives from a trustful source during the processing of raw meat and test these additives regularly for the presence of *Bacillus* spore (Shawish and Tarabees,

2017). VITEK2 BCL Card is a highly advanced method for the identification of *B. cereus* (Halket et al., 2010).

In this study, 100% of tested *B. cereus* isolates harbored *Nhe* gene that this result is in accordance with that presented by Anderson et al. (2001) and Ashraf et al. (2019), while *Hbl* gene was found in 10% of the tested isolates, which is similar to findings reported by Ashraf et al. (2019). Also, 100% of tested *B. cereus* were found to harbor *cytK* gene and this result approved with Kamelia et al. (2018) (81.5%) and Ngamwongsatit et al. (2008). Also, *groEL* gene was present in 100% of tested *B. cereus* which is a valuable target for phylogenetic studies to detect the *B. cereus* (Chang et al., 2003) and has already been used in PCR assay to detect the *B. cereus* (Taylor et al., 2005; Chang et al., 2003).

DECLARATIONS

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Competing interests

No competing interest exists

Authors' contributions

All authors contributed equally to this work.

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