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Incidence and characterization of *Bacillus cereus* in some meat products using PCR

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ABSTRACT

Bacillus cereus (*B. cereus)* is widespread in nature and foods. Several members of this group are recognized as causing food spoilage and/or health issues. This study was designed to determine the prevalence and genetic diversity of *B. cereus* strains isolated from 120 samples of meat products represented by beef burger, sausage, luncheon and rice kofta (30 of each) which were collected from different shops and supermarkets in Gharbia governorate. Samples were examined for the presence of *B. cereus* group after selective plating on MYP agar and enumeration of each sample. The highest incidence of *B. cereus* was recorded in rice kofta samples (56.7%) followed by sausage (46.7%), beef burger (43.3%) and luncheon (26.7%), with a count $4.39\times10^4\pm0.58\times10^4$, $1.12\times10^4\pm0.25\times10^4$, $8.06\times10^3\pm1.69\times10^3$ and $3.73\times10^3\pm0.51\times10^3$, respectively. Further biochemical tests were carried out for identification before being subjected to PCR for diarrheal gene (HBLA gene) which has been found in 37.5% of the tested isolates

1. INTRODUCTION

Meat and meat products are ideal for many organisms to grow because they are high in moisture, rich in nitrogenous compounds (amino acids, peptides and proteins) and plentifully supplied with minerals and accessory growth factors. Furthermore, they have some fermentable carbohydrates, usually glycogen and keep favorable pH for growth of most microorganisms (Galvaz et al., 2010). *Bacillus cereus* (*B. cereus*) is a Gram-positive, motile (flagellated), spore-forming, rod shaped bacterium that belongs to the Bacillus genus. Species within this genus include *B. anthracis, B. cereus, B. mycoides, B. thuringiensis, B. pseudomycoides and B. weihenstephanensis* (Montville and Matthews, 2005). *B. cereus* consider a potential threat to food processing due to its ability to form thermoduric endospore, ability to grow and survive at refrigeration temperature and toxin production (McKillip, 2000), besides the risk of its transmission through processed, pasteurized, sterilized, and heat-treated food products due to its resistance endo spores (Kotiranta et al., 2000).

Bacillus cereus produces two types of toxins; emetic (vomiting) and diarrhea; causing two types of illness. The emetic syndrome is caused by emetic toxin produced by the bacteria during the growth phase in the food. The diarrheal syndrome is caused by diarrheal toxins produced during growth of the bacteria in the small intestine (Ehling-Schulz et al., 2006). Several toxins have been described that may cause two types of food borne diseases. The non-hemolytic enterotoxin complex (NHE) and the hemolytic enterotoxin complex (HBL) as well as a variant of the single cytotoxin K have been linked to the diarrhoeal form of the disease, while the depsipeptide toxin cereulide has been shown to be the causative agent of the emetic form of the disease (Stenfors et al., 2008).

During the last decade, several tools, such as multiplex PCR for toxin gene profiling, and other methods for toxin quantitation in complex food matrices have been developed (Stark et al., 2013), which facilitate and significantly improve *B. cereus* diagnostics (Ehling-Schulz and Messelhäusser, 2013).

The present study was conducted to evaluate the safety of common meat products (luncheon, sausage, beef burger and rice kofta) at Gharbia Governorate by studying the prevalence of *B. cereus* strains and application of PCR for demonstration of virulence factors of the isolated *B. cereus* strains.

2. MATERIAL AND METHODS

One hundred and twenty random samples of meat products represented by luncheon; sausage, beef burger and rice kofta (30 of each) were collected from different supermarkets at different times in Gharbia governorate, Egypt. Each sample kept in a separated sterile plastic bag and transferred to the laboratory under complete aseptic conditions without undue delay for detection of *B. cereus* and application of PCR for demonstration of virulence factors of the isolated *B. cereus* strains.

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2.1. Bacteriological examination:

2.1.1. Preparation of samples: according to ICMSF (1996).

2.1.2. *Enumeration and isolation of B. cereus* (Harrigan and McCane, 1976):

Duplicated plates of Mannitol Egg Yolk Polymyxin Agar (M.Y.P.) were used for Bacillus isolation. Typical colonies of *B. cereus* characterized by pink color and surrounded by a white halo were picked up and spread over the surface of slope nutrient agar slant then incubated at 37 °C for 24 hours after which kept in the refrigerator at 4 °C for further identification of such bacteria.

2.1.3. Identification of Bacillus cereus:

The suspected bacterial isolates were identified morphologically and biochemically according to Koneman et al. (1992).

2.1.3.1. Microscopical identification:

2.1.3.1.1.1. *Staining* (FDA, 2002):

The suspected microorganisms are Gram positive aerobic spore formers.

2.1.3.1.1.2. *Motility test*:

Positive results showed migration of organisms from the stab line and diffuse into the medium causing turbidity. Some strains exhibited fuzzy streaks of growth.

2.1.3.1.2. Biochemical identification (Macfaddin, 2000):

2.1.3.1.2.1. *Catalase activity test*:

2.1.3.1.2.2. *Oxidase*:

The test is positive if the color turns to mauve, violet or deep purple within 10 seconds.

2.1.3.1.2.3. *Indole test:*

The formation of a red ring (surface layer) after 10 minutes was considered a positive reaction.

2.1.3.1.2.4. *Methyl Red test***:**

The development of a red color was considered positive test.

2.1.3.1.2.5. Modified Vogas Proskauer test:

A positive result was indicated by pink color which developed after one hour.

2.1.3.1.2.6. Citrate utilization test:

The development of blue coloration indicated utilization of citrate.

2.1.3.1.2.7. Urease test:

Development of pink color denoted a hydrolysis of urea. Negative tubes were re-examined after further incubation for 24 hours.

2.1.3.1.2.8. Hydrogen sulphide production test:

On Triple Sugar Iron (TSI) agar, hydrogen sulphide production was noted by blacking the medium.

2.1.3.1.2.9.Gelatin hydrolysis test:

Nutrient gelatin stab cultures were grown at room temperature and observed daily after cooling to about 18 °C.

2.1.3.1.2.10. Detection of Arginine decarboxylase (ADH):

Turbidity and violet color indicate a positive ADH.

2.1.3.1.2.11. Bile esculin test:

The test is interpreted as a positive result only if more than half the medium is dark brown or black after incubation.

2.1.3.1.2.12. Egg yolk lecithinase:

Lecithinase producers make opalescent zone. Some organisms (e.g. *B. cereus*) give a wide opalescent zone.

2.1.3.1.2.13. Starch hydrolysis:

The hydrolysis of starch was indicated by absence of dark blue zone surrounding the colonies.

2.1.3.1.2.14. Nitrate reduction test:

Positive result is indicated by orange or pink color. *B. cereus* gives +ve result.

2.1.3.1.2.15. Sugar fermentation:

After incubation at 37°C, the reactions of inoculated tubes were noticed every 24 hrs for 7successive days.

2.1.4. Proteolytic activity of B. cereus (Lecithinase activity):

Detection of Lecithinase activity for *B. cereus* was adopted according to the method recommended by Nabrdalik et al. (2010). Mannitol egg yolk polymyxin agar (MYP) is formulated for detection of proteolytic activity of certain bacteria particularly *B. cereus*

2.1.5. Lipolytic activity (Nabrdalik and Grata, 2011)

A nutrient medium based on Tributyrin (glycerol tributyrate) was used to detect the lipolytic activity of organisms such as *B. cereus*. Production of the enzyme lipase splits tributyrin resulting in lipolytic colonies surrounded by a clear zone in an opaque medium.

2.1.6. Polymerase Chain Reaction (PCR):

DNA ladder (molecular marker): 100 bp (Fermentas, lot No: 00052518). Primer sequences of *B. cereus* used for PCR identification system:

- Application of PCR for identification diarrheal (*HBLA*) genes of *B. cereus* was performed essentially by using primers (Pharmacia Biotech) as shown in table (1).
- Genomic DNA extraction (Sanjoy et al., 2009): Using GeneJET Genomic DNA Purification Kit according to (Fermentas).
- DNA Amplification:

  Amplification of diarrheal gene of *B. cereus* (Hansen and Hendriksen, 2001):

Table 1 Primers for identification diarrheal (*HBLA*) genes of *B.cereus* by PCR

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
HBLA (F)	5′ GTGCAGATGTTGATGCCGAT ′3	320	Banerjee et al. (2011)
HBLA (R)	5′ ATGCCACTGCGTGGACATAT ′3		

2.2. Statistical Analysis:

The obtained results were statistically evaluated by application of ANOVA test according to Feldman et al. (2003).

3. RESULTS

Incidence of Bacillus cereus in examined meat products samples

The results obtained in table (2) revealed that 43% (n=52) isolates of *B. cereus* were isolated from 120 examined meat products samples (luncheon, beef burger, sausage and rice kofta) with incidence 26.7%, 43.3%, 46.7% and 56.7%, respectively.

Total Bacillus cereus count in examined meat products samples

The obtained results in table (3) revealed that the mean of *B. cereus* count in the examined meat products samples (luncheon, beef burger, sausage and rice kofta) were 3.73×10^3, 8.06×10^3, 1.12×10^4 and 4.39×10^4, respectively. Moreover, the statistical results revealed that, the mean values within examined samples of meat products showed high significant differences ($P<0.01$).

The obtained results in table (4) revealed the acceptability of the examined samples of meat products (luncheon, beef burger, sausage and rice kofta) depending on their contamination with *B. cereus* were 73.3%, 56.7%, 53.3% and 43.3%, respectively were accepted for human consumption while 26.7%, 43.3%, 46.7% and 56.7%, respectively were unfit for human consumption according to EOS (2005). Table (5) showed proteolytic and lipolytic activity of *B. cereus* isolated from the examined samples of meat products were 80.8 and 53.8 %, respectively. The proteolytic activity (luncheon, beef burger, sausage and rice kofta) was 75%, 69.2%, 85.7% and 88.2%, respectively, while the lipolytic activity was 25, 53.8, 57.1 and 64.7 % in luncheon, beef burger, sausage and rice kofta respectively.

Genotypic detection of some virulence genes in isolated Bacillus cereus strains using polymerase chain reaction

PCR results showed that *hblC* virulence gene was detected in 6 out of 16 (37%) studied strains (1 Luncheon, 2 Beef burger, 1 sausage and 2 Rice kofta) giving product of 320 bp. as shown in table (6).

4. DISCUSSION

Bacillus cereus has emerged as major foodborne pathogen during the last few decades that causes two types of illness through elaboration of one emetic toxin and three different enterotoxins (Per and Terje, 2006). Regarding to luncheon samples, the obtained results were nearly similar to that recorded by Ghanyem-Hanan *et al.* (2014), who recorded 35%, but higher than those obtained by Samir *et al.* (2012), who recorded lower incidences (20%) and lower than those obtained by El- Soliman (2013), who recorded higher incidence (74.3%). While, for the beef burger, our results were nearly similar to that obtained by Ghanyem-Hanan *et al.* (2014) (35%) and Soleimani et al. (2017) (31.25%), and lower than that obtained by Heikal et al. (2006). In addition, for sausage samples, the obtained results were similar to that recorded by Abd El-Wahaab-Shimaa et al. (2018), who found 40%, and lower than those obtained by Ibrahim-Hemmat et al. (2014), who recorded 72%, but higher than those obtained by Guven et al. (2006), who found 16%. Moreover, concerning rice kofta, the result were nearly similar to Abd El-Wahaab-Shimaa et al. (2018), who recorded 60%, and lower than Ibrahim-Hemmet et al. (2014), who recorded 88%, but higher than Atia (2014), who recorded 44 %.

The mean counts of *B. cereus* in the examined meat products samples (luncheon, beef burger, sausage and rice kofta) were 3.73×10^3, 8.06×10^3, 1.12×10^4 and 4.39×10^4, respectively. Concerning luncheon, the obtained result nearly similar to that obtained Abosrea-Nadia (2005), who recorded 2×10^3, and higher than those obtained by Abdou et al. (2011), who found *B. Cereus* count 4×10^2, and lower than those obtained by Soliman (2013), who recorded 2.25×10^5.

Table 2 Enumeration of *B. cereus* in the examined samples meat products (n=30).

Meat products	Min	Max	Mean ± S.E*
Luncheon	1.0×10^2	8.9×10^3	$3.73\times10^3\pm 0.51\times10^3$
Beef burger	2.0×10^2	1.7×10^4	$8.06\times10^3\pm 1.69\times10^3$
Sausage	5.0×10^2	4.3×10^4	$1.12\times10^4\pm 0.25\times10^4$
Rice kofta	7.0×10^2	9.1×10^4	$4.39\times10^4\pm 0.58\times10^4$

S.E* = Standard error of mean. The results showed high significant differences (P<0.01).

Table 3 Incidence of *B. cereus* in the examined samples of meat products (n=30)

Meat products	Positive samples	
	NO.	%
Luncheon	8	26.7
Beef burger	13	43.3
Sausage	14	46.7
Rice kofta	17	56.7
Total	52	43.3

4 Acceptability of the examined samples of meat products depending on their contamination with *B. cereus* (n=30).

Meat products	*B. cereus* count /25 g*	Accepted samples		Unaccepted samples	
		No.	%	No.	%
Luncheon	Free	22	73.3	8	26.7
Beef burger	Free	17	56.7	13	43.3
Sausage	Free	16	53.3	14	46.7
Rice kofta	Free	13	43.3	17	56.7
Total (120)		68	56.7	52	43.3

* Egyptian Organization for Standardization "EOS" (2005). No 1114-2005 for luncheon. No 1688-2005 for beef burger. No 1972-2005 for sausage. No 1973-2005 for kofta

Table 5 Proteolytic and lipolytic activity of *B. cereus* isolated from the examined samples of meat products.

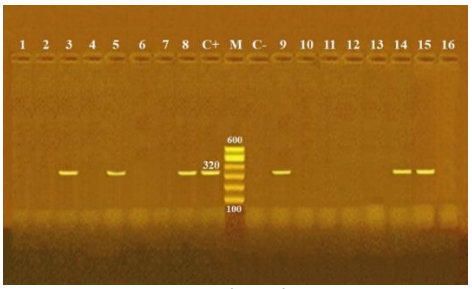
Products	Positive samples		Proteolytic activity *		Lipolytic activity**	
	No.	%	No.	%	No.	%
Luncheon	8	26.7	6	75.0	2	25.0
Beef burger	13	43.3	9	69.2	7	53.8
Sausage	14	46.7	12	85.7	8	57.1
Rice kofta	17	56.7	15	88.2	11	64.7
Total	52	43.3	42	80.8	28	53.8

* MYP Agar Base (Opaque zone around colonies). ** Tributrin Agar Base (Clear zone around colonies)

Table 6 Incidence of diarrheal gene of different *B .cereus* strains isolated from the examined samples of meat products (n= 16 strains).

Meat products	*HBLA* gene	
	NO.	%*
Luncheon	1	25
Beef burger	2	50
Sausage	1	25
Rice kofta	2	50
Total	6	37.5

*The percentage was calculated according to positive samples.



Photograph 1 Agarose gel electrophoresis of PCR of diarrheal "*HBLA*" gene (320 bp) for characterization of *B. cereus*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *B. cereus* for diarrheal gene. Lane C-: Control negative. Lanes 3, 5, 8, 9, 14 & 15: Positive *B. cereus* strains for diarrheal gene. Lanes 1, 2, 4, 6, 7, 10, 11, 12, 13 & 16: Negative *B. cereus* for diarrheal gene.

Meanwhile *B. cereus* count obtained from beef burger samples were 8.06×10^3 which is nearly similar to results obtained by Abd El-Wahaab-Shimaa et al. (2018) 2.35×10^3.

Moreover, *B. cereus* count in sausage samples were 1.12×10^4 which similar to those obtained by Khalifa (1997), who found *B. cereus* count 3×10^4, and slightly lower than those obtained by Guven et al. (2006) which was 1.4×10^5. and higher than those obtained by Abd El-Wahaab-Shimaa et al. (2018) which was 7.82×10^3.

At last rice kofta samples gave count 4.3×10^4 results were nearly similar to that recorded by Ibrahim-Hemmat *et al.* (2014) and Abd El-Wahaab-Shimaa et al. (2018). Improper handling of meat product after cooking allows the spores of *B. cereus* to germinate resulting in vegetative cells to multiply and lead to food poisoning. the lack of sanitary condition during processing, handling and storage may act as the main source of food contamination with *B. cereus*. Also, (Torky-Amal, 2004). Several toxins have been described that may cause two types of food borne diseases. The non-hemolytic enterotoxin complex (NHE) and the hemolytic enterotoxin complex (HBL) as well as a variant of the single cytotoxin K have been linked to the diarrheal form of the disease (Stenfors et al., 2008). For *hblA* (diarrheal gene) the obtained results slightly agreed with that obtained by Torkar and Seme (2009), who estimated *hblC* virulence gene in 31.6 of tested isolates and higher than those obtained by Bekeir-Hanaa et al. (2018) and Abd El-Wahaab-Shimaa et al. (2018), who recoded 10% and 14% of tested isolates positive *hblC* virulence gene, respectively. and lower than results obtained by Rather et al. (2011) and Osman et al. (2018) which were 66.1% and 45.2%, respectively. The false positive may be due to the fact that food matrix significantly affected the expression of these enterotoxin genes who found especially the entFM gene, was lower in a real food matrix than in laboratory broth (Fan *et al.*, 2016).

5. CONCULOSION

The meat products are considered main source of *B. cereus* that can cause disease to consumers. More attempts must be focused on cold-chain maintenance in production, distribution, and storage of meat products. Hygienic slaughter of animals in slaughterhouses could improve the safety of carcasses and raw meat used in meat production.

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