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Bacteriological evaluation of *Bacillus* species isolated from food of animal origin.

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ABSTRACT

Genus *Bacillus* is a Gram-positive large bacterial group containing about 305 species; most of them cause serious diseases. The current study aimed to isolate bacillus species from different food sources with an investigation of species causing food poisoning and determine their *in vitro* susceptibility and virulence genes. The study was done on 100 samples of different food sources (raw milk (20), Kareish cheese (10), low salt cheese (10), Ras cheese (Roomy) (10), Rice Kofta (17), Kobiba (17) and beef burger (16)) collected from different sources. The results declared that the most isolated species were *B. cereus* (14/35%), *B. mycoides* (9/22.5%), *B. thuringenesis* (7/17.5%), *B. alcalophilus* (7/17.5%), *B.subtilis* (2/ 5%) and *B. algicola* (1/2.5%) from examined samples. Determination of susceptibility of the isolated *B. cereus* against different antibiotics, showed that all strains were completely resistant to vancomycin (100%) followed by amoxicillin (85.7%), intermediate sensitivity to clindamycin (78.6%) and doxycycline (64.3%), in comparison they were highly susceptible to gentamycin and ciprofloxacin (85.7%) then erythromycin (78.6%) and norfloxacin (71.4%). The molecular examination of virulence genes (*cyt*K, *ces* and *pc-plc*) in the virulent isolates (three isolates of *B. cereus*, two isolates of *B. mycoides*, and two isolates of *B. thuringenesis)* existed presences of *cyt*K and *pc-plc* genes in all examined isolates and absence of *ces* genes. This concluded that several *Bacillus* species may be isolated from dairy and meat products and may have several virulence genes that cause food poisoning, so food hygiene is essential.

1. INTRODUCTION

Bacillus species are big, spore-producing, Gram-positive, aerobic, or facultative anaerobic bacteria that are found throughout nature (soil, water, and the environment) and various food products (Bottone, 2010). It can withstand a variety of environmental conditions, including high temperatures, X-rays, ultraviolet radiation, and acidity. (Vidic et al., 2020). Thus, such thermotolerant spore-former bacteria constitute a considerable risk in keeping the quality of certain foodstuffs like dairy products and meat products (Caamaño-Antelo et al., 2015).

There are many species within the genus bacillus involving; *B. cereus, B. mycoides, B. pseudomycoides, B. anthracis, B. thuringiensis, and B. weihenstephanens is* (Hassan et al., 2019). Bacillus cereus is believed to provide a risk to the food processing industry, due to its capacity to produce diverse toxins, grow and survive at refrigeration temperatures, and produce thermoduric endospores (McKillip, 2000). It may gain entry to food either through contamination of water source or utensils used for food processing (Naguib et al., 2014).

Several food products may act as a vehicle for *B. cereus* infection like meat products and dairy products (Yang et al., 2017). Food poisoning produced by *B. cereus* is characterized by the occurrence of two types of diarrheal and/or emetic type and causes plagues that are often underrated (Papan et al., 2019). The pathogenicity of *B. cereus* is caused by the production of a series of enterotoxins "two protein complexes and two enterotoxin proteins" which are known as non-hemolytic enterotoxin (NHE), hemolysin BL (HBL), cytotoxin K (*cyt*K), and enterotoxin T that able to tolerate heat during the growth of bacteria in the small intestine (Rajkovic, 2014). The emetic form occurred by the emetic toxin, cereulide (Logan, 2012), encoded by the *ces* gene cluster (*ces* HPTABCD), placed on a plasmid in strains belonging to a particular line of *B. cereus* (Økstad and Kolstø, 2011).

The establishment of antibiotic resistance in *B. cereus* strains happens as a result of antibiotic misuse or acquired resistance due to resistant gene transfer, resulting in the failure of antibiotic treatment (Gao et al., 2018). The existing study focused on the isolation of different *Bacillus* species from various food sources, examined the resistance of pathogenic strains to different antibiotics, and determined their virulence genes.

2. MATERIAL AND METHODS

2.1. Samples:

A total of 100 different random food samples (Raw milk (20), 25 ml of each sample, Kareish cheese (10), low salt cheese (10), Ras cheese (Roomy)(10), Rice Kofta (17), Kobiba (17) and beef burger (16), (25 gm or ml of each sample) were collected from October 2022 until January 2023, from different supermarkets at Qalyubia governorate

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and transferred without delay to (Animal Health Research Institute-Banha branch) in a separate container for bacteriological examination. The protocol of this work was approved by the Ethical Committee, Fac. Vet. Med. Benha University (Ethical approval No. BUFVTM12-07-23).

2.2. Preparation of samples occur According to (APHA, 2002)

each sample was weighed to 10 grams and transferred to a sterile plastic bag containing 90 ml of pepton water. The samples were then homogenized using a Stomacher (M A 106402, France, 450 to 640 strokes per minute) for 2 minutes.

2.3. Isolation of Bacillus species according to Rhodehamel and Harmon (2001)

Firstly, the examined samples were cultured into brain heart infusion broth (BHIB,HIMEDIA) and incubated for 24-48 hours at 37°C. After that, a loopful was streaked from enrichment broth into PEMBA (Polymyxin pyruvate egg-yolk mannitol–bromothymol blue agar) plates and incubated for 24 hrs at 37 °C.

2.4. Identification of Bacillus species

This occurs according to different biochemical tests (Oxidase, Catalase, Lysine decarboxylase, Vogeus-proskaur, Citrate utilization, Egg yolk reaction, Triple sugar iron, Urea, Motility) according to De Vos *et al.* (2009 and Markey *et al*. (2013).

2.5. Anti-microbial Susceptibility:

In vitro susceptibility test was done on the pathogenic isolated *Bacillus* species strains (*B. cereus, B. theringesis and B. mycoides*) to examine their susceptibility for different antibiotics (Table1) using the disc-diffusion method according to Koneman *et al* (1997).

Table 1 Antimicrobial discs standardization, concentrations, and interpretation to their effect according to CLSI (2018)

Antimicrobial disks		Disk concentrations	Zone of inhibition (mm) Resistant ≥ mm (R)	Intermediate mm range (IS)	Sensitive ≤ mm (S)
Amoxicillin	AMX/25	25µg	14	15-17	18
Ciprofloxacin	CIP/5	5 µg	15	16-20	21
Doxycycline	Do 30	30 µg	14	15-19	20
Erythromycin	E/15	15 µg	13	14-17	18
Gentamicin	CN/10	10 µg	12	13-14	15
Norfloxacin	NOR/10	10 µg	12	13-16	17
Vancomycin	VA30	30 µg	14	15-16	17
Clindamycin	DA2	2 µg	14	15-20	21

*2.6. Detection of virulence genes (*cytK, ces and Pc-plc*) by using PCR.*

2.6.1. DNA Extraction: Occurred by using QIAamp DNA Mini Kit for DNA extraction and purification.

2.6.2. Preparation of PCR reaction mix: Master mix for PCR reaction prepared as instruction of Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit as follow: 12.5 *µl* mastermix (2x premix) placed into PCR tubes with 5.5 *µl* PCR grade water, 1 *µl* of Forward primer , 1 *µl* of Reverse primer and 5 *µl of* Template DNA. The used primers were tabulated in table (2).

Table 2 Primer sequence and size of Amplified products:

Primer	Sequence	Amplified product	Reference
cytK	ACAGATATCGGICAAAATGC CAAGTIACTTGACCIGTTGC	421 bp	Ehling-Schulz *et al.* (2006)
ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1271 bp	
Pc-plc	GAGTTAGAGAACGGTATTTATGCTGC CTACTGCCGCTCCATGAATCC	411 bp	Martínez-Blanch *et al.* (2009)

The prepared PCR tubes were placed into a thermocycler and then the DNA fragments were analyzed by using Gel electrophoresis as recorded before by (Sambrook *et al*., 1989).

3. RESULTS

3.1. Identification of the isolated strains.

3.1.1. Microscopical appearance: Bacillus cereus *is* a Gram–positive, short rod-shaped, and spore-forming strain.

3.1.2. Colonial appearance: Bacillus cereus grew as crenate, fimbriate, or slightly rhizoid irregular colonies up to 5 mm in diameter, turquoise to peacock blue in color with flat ground glass surface and surrounded by a precipitate from hydrolyzed egg yolk. The colonies of *B. thuringiensis* have a similar appearance to those of *B. cereus* but tend to have slightly more regular edges. B. mycoides has markedly rhizoid or hairy-looking colonies that can have an almost fungal appearance.

3.1.3. Biochemical tests: The used biochemical tests identified the isolated bacillus-like growth colonies (40) into six bacillus species (Table 3).

3.2. Positive samples incidence in the studied food products: The bacteriological examination of the collected products revealed the presence of bacillus-like growth in 40/100 (40%) of examined samples represented as follow: 10/20 (50%) of raw milk samples, 1/10 (10%) from Kareish cheese, 3/10 (30%) from low salt cheese, 4/10 (40%) from Ras cheese (Roomy), 10/17 (58.8%) samples of Rice Kofta, 9/17 (52.9%) from Kobiba and 3/16 (18.75%) from beef burger samples (Table 4).

Table 3 Biochemical tests for isolated strains

Test	*B.Cereus*	*B. mycoides*	*B .thuringenesis*	*B. alcalophilus*	*B. subtilis*	*B. algicola*
Oxidase	-	-	-		D	-
Lysine decarboxylase	-	D	+		-	
Catalase	+	+	+	+	+	W
Voges-proskaur	+	+	+	-	+	
Citrate utilization	+	D	+	-	+	-
Egg yolk reaction	+	D	+	-	-	
TSI	+	D	D	+	+	
Urea	D	D	-	-	-	+
Motility	+	-	+		+	+
No. of isolates	14	9	7	7	2	1

Symbols: (-, negative) , (+, positive) , (D, different strains give different reactions) , (W, weak reaction)

Table 4 Occurrence of Positive samples in the studied food products

Samples	No. of samples	Positive bacillus- like growth NO.	%*	%**	Negative samples NO.	%*	%**
Raw milk	20	10	50.0	10.0	10	50.0	10.0
Kareish cheese	10	1	10.0	1.0	9	90.0	9.0
Low salt cheese	10	3	30.0	3.0	7	70.0	7.0
Ras cheese	10	4	40.0	4.0	6	60.0	6.0
Rice kofta	17	10	58.8	10.0	7	41.2	7.0
Kobiba	17	9	52.9	9.0	8	47.1	8.0
Beef burger	16	3	18.75	3.0	13	81.25	13.0
Total	100	40	40.0	40.0	60	60.0	60.0

%* Percentage related to total No. of each examined sample %** Percentage related to total No. of samples. (n= 100)

3.3. Prevalence of *Bacillus* species isolated from examined samples.

The biochemical examination of bacillus-like growth isolated from raw milk samples 10\20 (50%) showed the identification of the following strains: 4 (40%) *B. cereus*, 3 (30%) *B. mycoides*, 1 (10%) *B. alcalophilus*, 1 (10%) *B. subtilis and* 1 (10%) *B. algicola.* While only *B. mycoides* was isolated from one sample of Kareish cheese. *B. alcalophilus* 2 (66.6) and *B. subtilis*1 (33.4%) were identified in low salt cheese. Otherwise, in Roomy (Ras) cheese there were 2 (50%) *B.cereus*, 1 (25%) *B. mycoides*, and 1 (25%) *B. alcalophilus* (Table 5). While that isolated from meat products; 5 (50%) *B. cereus, 2 (20%) B. mycoides*, 3 (30%) *B. thuringenesis* were representing the 10 isolates from Rice kofta samples. The 9 isolates from Kobiba samples were identified as 3 (33.4%) *B. cereus*, 2 (22.2) *B. mycoides*, 2 (22.2%) *B. thuringenesis, and* 2 (22.2*) B. alcalophilus*. Otherwise, 2 (66.7) *B. thuringenesis and* 1 (33.3%) *B. alcalophilus* were the 3 isolates from the beef burger (Table 5).

3.4. Antibiogram results for *Bacillus* isolates.

The in-vitro sensitivity tests for the isolated *B. cereus* exposed that, the isolates were highly resistant f~~or~~ to vancomycin (100%) and amoxicillin (85.7%). Meanwhile, they were intermediate sensitive to clindamycin (78.6%) and doxycycline (64.3%). Moreover, they were highly susceptible to gentamicin and ciprofloxacin (85.7%) followed by erythromycin (78.6%) then norfloxacin (71.4%) (Table 6)

While *B. mycoides* were highly resistant to amoxicillin and clindamycin (77.8%) followed by erythromycin (66.7%). They were intermediate susceptibility to doxycycline (55.6%). However, they were sensitive to ciprofloxacin (100%), then norfloxacin (88.9%), and gentamycin and vancomycin (66.7%) (Table 7). Otherwise, *B. thuringenesis* was highly resistant to vancomycin (100%), clindamycin (85.7%), and erythromycin (85.7%). Meanwhile, they were intermediate sensitivity to amoxicillin and doxycycline (71.4%). However, they were sensitive to ciprofloxacin (100%), norfloxacin (100%), and gentamycin (85.7%) (Table 8).

Table 6 In-Vitro anti-microbial susceptibility test for isolated *B. cereus* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	0	0.0	2	14.3	12	85.7	R
Erythromycin	15 µg	11	78.6	1	7.1	2	14.3	S
Doxycycline	30 µg	3	21.4	9	64.3	2	14.3	IS
Gentamicin	10 µg	12	85.7	2	14.3	0	0.0	S
Norfloxacin	10 µg	10	71.4	3	21.4	1	7.2	S
Ciprofloxacin	5 µg	12	85.7	2	14.3	0	0.0	S
Vancomycin	30 µg	0	0.0	0	0.0	14	100	R
Clindamycin	2 µg	2	14.3	11	78.6	1	7.2	IS

Table 7 In-Vitro anti-microbial sensitivity test for isolated *B. mycoides* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	0	0.0	2	22.2	7	77.8	R
Erythromycin	15 µg	1	11.1	2	22.2	6	66.7	R
Doxycycline	30 µg	3	33.3	5	55.6	1	11.1	IS
Gentamicin	10 µg	6	66.7	3	33.3	0	0.0	S
Norfloxacin	10 µg	8	88.9	1	11.1	0	0.0	S
Ciprofloxacin	5 µg	9	100	0	0.0	0	0.0	S
Vancomycin	30 µg	6	66.7	3	33.3	0	0.0	S
Clindamycin	2 µg	0	0.0	2	22.2	7	77.8	R

Table 8 In-Vitro anti-microbial sensitivity test for isolated *B. thuringenesis* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	0	0.0	5	71.4	2	28.6	IS
Erythromycin	15 µg	0	0.0	1	14.3	6	85.7	R
Doxycycline	30 µg	2	28.6	5	71.4	0	0.0	IS
Gentamicin	10 µg	6	85.7	1	14.3	0	0.0	S
Norfloxacin	10 µg	7	100	0	0.0	0	0.0	S
Ciprofloxacin	5 µg	7	100	0	0.0	0	0.0	S
Vancomycin	30 µg	0	0.0	0	0.0	7	100	R
Clindamycin	2 µg	0	0.0	1	14.3	6	85.7	R

3.5. Results of PCR amplification of *ces, cyt*K and *pc-plc* genes.

PCR using three sets of primers was used for the detection of three virulence genes. These genes were cytotoxic K gene (*cyt*K), emetic cereulide synthetase gene (*ces*), and a degradative enzyme known as phosphatidyl inositol- and phosphatidylcholine specific phospholipases (pc-plc). It was applied on seven random isolates of (3 *B. cereus, 2 B. theringenesis and 2 B. mycoides)*. PCR results showed that PCR results showed that, *cyt*K and *pc-plc* virulence genes were revealed in all seven studied strains. While *ces* gene was unable to be detected in all strains, (Table 9) (Fig 1).

Table 9 Results of virulence genes amplification for Bacillus species

Bacillus species	Results of virulence genes		
	Pc-plc	*cyt*K	*ces*
B.cereus 1	+	+	-
B.cereus 2	+	+	-
B.cereus 3	+	+	-
*B.theringenesis*1	+	+	-
B.theringenesis 2	+	+	-
B.mycoides 1	+	+	-
B.mycoides 2	+	+	-

4. DISCUSSION

According to several studies, the source of *B. cereus* contamination in food products is recontamination during processing (Svensson et al., 2000).

The existing study examined different *Bacillus* species in different food sources as milk, dairy products, and meat products. Results recorded in table (4) described the prevalence of bacillus-like growth in examined samples in which 40% of examined samples showed the development of bacillus-like growth, this was higher than that reported by Mohamed et al. (2016), who determined the presence of bacillus-like growth in 27.5% examined samples.

The prevalence of *Bacillus* species isolated from each examined sample (Table 5) showed the presence of 40% *B. cereus*, 30% *B. mycoides*, 10% *B. alchophilus*, 10% *B. subtilis* and 10% *B. algicola* in raw milk. This is nearly similar to Elafify et al. (2023) and Abraha et al. (2017) who determined the prevalence of *B. cereus* in raw milk as 37.5%, and 38.8% respectively.

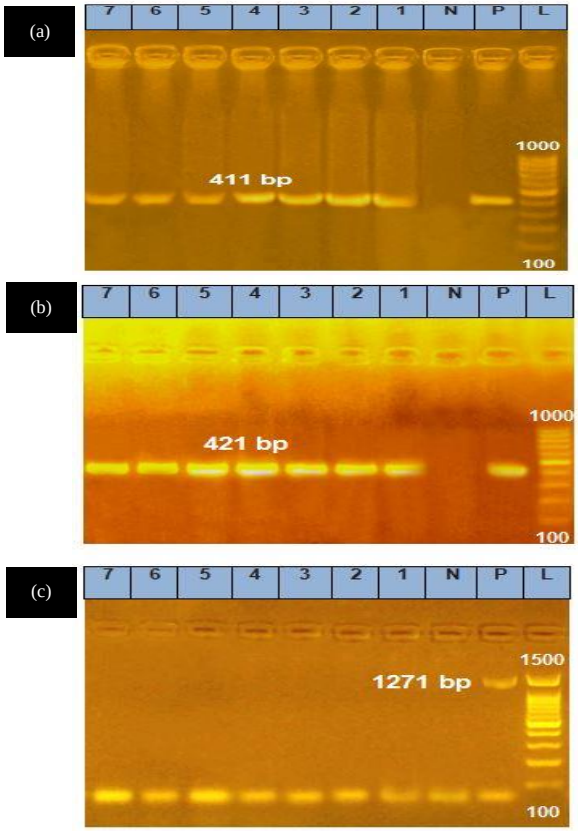

Fig 1 Gel electrophoresis for virulence genes for Bacillus species. (a) PC-PLC gene, (b) cytotoxic K gene (cytK) gene, and (c) cereulide synthetase gene (ces) gene. Lane L: 100-1000 bp. DNA Ladder. Neg.: Negative control. Pos.: Positive control Lane 1 -7: positive for virulence genes

While higher than that reported by Sanaa et al. (2020), who detected the presence of 15.5% of *B. cereus*, 11.1% *B. mycoides*, 4% *B. subtilis*, *B. pantothenticus* (8.8%), *B. licheniformis* (13.3%), *B. coagulans* (37.7%), *B. megaterium* (4.4%) in examined milk samples. But lower than that examined by Osama et al. (2020), who isolated 52% of *B. cereus* from raw milk.

The examined Kareish cheeses showed the presence of only *B. mycoides* in one sample only, while failed to isolate *B. cereus* like that previously stated by Osama et al. (2020) and Ibrahim et al. (2015) also Ahmed et al., (2023) and Elafify et al. (2023) reported the isolation of *B. cereus* 16%, 32.5% respectively in examined Kareish cheese samples. Low salt cheese samples examination showed the presence of 66.6% *B. alcalophilus* and 33.4% *B. subtilis* while unable to isolate *B.cereus* and this came in agreement with Ibrahim et al. (2015) and Heikal et al. (2014).

For Ras cheese, the examination declared the presence of *B. cereus* (50%), *B. mycoides* (25%), and *B. alcalophilus* (25%). Compared with our study a higher percentage of *B. cereus* was also isolated by Nawar (2007), who isolated 48% of *B. cereus* from Ras cheese, while lower levels were detected by Osama et al. (2020) and Abdeen et al. (2020) who detected 16% and 8.5% respectively of *B. cereus* in Ras cheese.

In the case of meat products, the examined Rice kofta samples showed the presence of *B. cereus* (50%), *B. mycoides* (20%), and *B. thuringenesis* (30%), this is higher than that previously reported by Abd El-Tawab et al. (2020a), who isolated *B. cereus* (12%), *B. mycoides* (4%) and failed to isolate *B. thuringenesis* from examined samples of Rice Kofta. The higher level of *B. cereus* (56.7%) was isolated previously from Rice kofta by Hassan et al. (2019). And for Kobiba, the examined samples declared the existence of *B. cereus* (33.4%), *B. mycoides* (22.2%) *B. thuringenesis* (22.2%), and *B. alcalophilus* (22.2%). This came disagree with Abd El-Tawab et al. (2020a) and Abdel-Wahaab et al. (2018). While for Beef burger samples there was unsuccessful isolation of *B. cereus*, but *B. thuringenesis* (66.7%), and *B. alcalophilus* (33.3%) were identified. This opposes the recorded results reported by Hassan et al. (2019) and Abd El-Tawab et al. (2020b). According to the records, rice kofta had the highest concentration of *B. cereus* among meat products. This occurs because of the presence of rice, which is rich in starch and serves as an ideal medium for *B. cereus growth*.

The in-vitro susceptibility of *B. cereus* isolates (Table 6) determined that they were resistant to vancomycin (100%) and amoxicillin (85.7%) and showed intermediate resistance to doxycycline (64.3%) and clindamycin (78.6%) as reported by Elafify et al. (2023). But they were highly susceptibility to gentamycin (85.7%) and ciprofloxacin (85.7%) as determined by Abd El-Tawab et al. (2020b); Elafify et al. (2023) and Ahmed et al. (2023). Whereas *B. mycoides* isolates (Table 7) showed resistance to amoxicillin and clindamycin (77.8%), followed by erythromycin (66.7%) and intermediate susceptibility to doxycycline (55.6%) and highly sensitive to ciprofloxacin (100%) then norfloxacin (88.9%), gentamycin and vancomycin (66.7%). Also, *B. thuringenesis* (Table 8) showed complete resistance to vancomycin (100%), followed by erythromycin and clindamycin (85.7%) and intermediate susceptibility to amoxicillin and doxycycline (71.4%) and highly sensitive to norfloxacin and ciprofloxacin (100%), gentamycin (85.7%). This came nearly similar to the previously recorded by Markey *et al*. (2013) which discovered that chromosomally encoded -lactamases are the reason why *Bacillus cereus* strains frequently show resistance to penicillin and other -lactam drugs. Both *Bacillus thuringiensis* and the majority of *B. mycoides* strains have developed resistance to -lactam antibiotics.

In our study, the results of PCR (Table 9 and Fig. 1) showed amplification of *PC-PLC* and *cyt*K genes in all examined samples at 411 and 421 bp respectively this is agreed with former studies (Ahmed et al., 2023; Al. Habaty et al., 2020). While *ces* gene failed to be amplified in any of the examined samples, which disagreed with Al. Habaty et al. (2020) and Gharib et al. (2020), who determined *ces* gene in all examined strains.

5. CONCLUSIONS

The present study concluded that different bacillus species isolated from dairy and meat products carry many virulence genes and cause food poisoning to consumers. So, strict hygienic measures must be applied during food handling and processing to avoid the contamination of food by bacillus species.

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