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Prevalence and bacteriological investigation of *Bacillus cereus* isolated from meat and milk products in El-Gharbia governorate, Egypt.

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ABSTRACT

Bacillus cereus is a food-borne pathogen that can cause emesis, diarrhea and spoilage of different food products. For this, 125 random samples of meat products (luncheon and sausage) and milk products (ras or romy cheese, karish cheese and rice pudding), 25 of each, were taken from various shops and marketplaces in El-Gharbia governorate, Egypt to be examined for prevalence, isolation and identification of *Bacillus cereus.* Additionally, the significance of *Bacillus cereus* for public health was covered. The bacteriological examination of collected samples showed that a total of 40/125 (32%) *Bacillus cereus* strains were obtained; mostly they were isolated from Ras cheese 14/25 (56%) followed by Luncheon 11/25 (44%), Rice pudding 8/25 (32%) then from Sausage 7/25 (28%). There was no isolated *Bacillus cereus* from Karish cheese 0/25 (0%). Polymerase chain reaction for five random isolated studied strains showed that *gro*EL (diagnostic and marker gene of *Bacillus cereus*) gene was detected in all five studied strains. The anti-microbial sensitivity test for 20 *Bacillus cereus* strains revealed that they were completely sensitive to ciprofloxacin, norfloxacin and levofloxacin (100%) followed by gentamycin (80%), doxycycline (60%), chloramphenicol (50%), erythromycin (30%) then clindamycin (20%), whereas they were completely resistant to ampicillin, cefipime, aztreonam and amoxicillin-clavulanic acid. In conclusion, *Bacillus cereus* is a serious pathogens could contaminate foods as meat and milk products. So, hygiene procedures should be followed from the beginning of foods industries until reaching the consumer for controlling *Bacillus cereus* prevalence.

1. INTRODUCTION

Bacillus species are a widely spread bacteria in nature. They are frequently linked to food poisoning outbreaks (Camele et al., 2019).

Bacillus cereus is a Gram-positive bacterium that is a member of the *Bacillus* genus. Among the species in this genus: *Bacillus anthracis, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis, Bacillus cytotoxicus, and Bacillus toyonensis* (Liu et al., 2015).

Bacillus cereus is motile with flagella, rod shape, spore-forming bacterium, aerobic that can also grow anaerobically and is classified as mesophilic or psychotrophic, mesophilic strains can grow in temperatures ranging from 15 to 55 ºC and their spores are typically more resistant to heat. While psychotrophic ones can grow in temperatures ranging from 4 to 35 ºC and their spores are typically less resistant to heat (Organji et al., 2015).

Bacillus cereus contamination depends on the production of multiple enzymes including phospholipases, proteases and hemolysins, and also have the biofilm formation capability, as well as they have toxin-encoding genes that play a crucial role in the pathogenesis (Tirloni et al., 2020).

Several selective media as polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) and mannitol egg yolk phenol red polymyxin agar (MYP) were used for *Bacillus cereus* isolation from food. The selectivity of these media depends on the hydrolysis of egg yolk lecithin and the absence of the use of mannitol by *Bacillus cereus* as well as the presence of selective compounds such as polymyxin (Hendriksen and Hansen, 2011). Polymerase chain reaction (PCR) has emerged as one of the most significant diagnostic techniques for detecting food-borne pathogens and is regarded as a valuable substitute to the culture-based detection methods, because of its quickness, sensitivity, specificity and detection limit, (Rodríguez-Lázaro et al., 2013). The *gro*EL gene possesses the potential to be a phylogenetic marker for *Bacillus cereus* strains detection and identification (Chang et al., 2003).

Antibiotics commonly used to treat *Bacillus cereus* infection include vancomycin, linezolid, gentamicin, levofloxacin and clindamycin (Aygun et al., 2016). Anti-microbial drug resistance of *Bacillus cereus* is caused by the production of β-lactamases, one of the potential virulence factors, leading to a serious threat to public health (Tewari et al., 2012).

The current study aimed to investigate the prevalence and bacteriological characterization of *Bacillus cereus* isolated from meat and milk products in El-Gharbia governorate, Egypt and determine the sensitivity of antibiotics for isolates.

2. MATERIAL AND METHODS

Whole experimental technique was accepted by Benha University, Animal Ethical Committee of Faculty of

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Veterinary Medicine with ethical approval number (BUFVTM 12-06-23).

2.1. Sample collection:

A total of 125 random samples of meat products (luncheon and sausage) and milk products (ras cheese, karish cheese and rice pudding), 25 of each, were collected to be examined for prevalence of *Bacillus cereus*. The samples were taken from various shops and marketplaces in El-Gharbia governorate between the periods from March to July 2022. Each sample (25 grams) was taken individually in sterile plastic bags and directly transported in an ice box to Animal Health Research Laboratory, Tanta branch for examination.

2.2. Bacteriological examination:

2.2.1. Preparation of the samples (APHA, 2001):

Under aseptic conditions, 25 grams of each sample were placed in a sterile stomacher bag containing 225 ml sterile 0.1% peptone water (Oxoid) (for food-borne pathogens isolation). At stomacher, the content was homogenized for 3 minutes then the mixture was allowed to stand at room temperature for 5 minutes.

2.2.2. Isolation and identification of Bacillus cereus (Tallent et al.*, 2012):*

One ml of the ready prepared sample was injected into 9 ml Tryptone soya broth (Oxoid) and aerobically incubated at 37°C for 24 h. Then a loopful of incubated Tryptone soya broth was streaked onto the following media: PEMBA agar plate (Oxoid) and MYP agar plate (Oxoid) then incubated at 37°C for 24 h and examined to check for the presence of *Bacillus cereus* like colonies. The positive suspected colonies were retained in semi-solid nutrient agar and incubated at 37°C for 24 h.

The isolates were tested for motility and were identified morphologically by Gram-stain in accordance with Quinn et al. (2011) and biochemically by Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Urease test, Catalase test, Oxidase test, Nitrate reduction test and Gelatin hydrolysis test in accordance with Markey et al. (2013).

2.3. Virulence tests of Bacillus cereus strains:

The hemolytic activity of *Bacillus cereus* isolates was detected according to Tallent et al. (2012). The amylase activity (starch hydrolysis test) of *Bacillus cereus* isolates were tested using the method reported by Ashwini et al. (2011). The proteolytic (caseinase), the lecithinase activity and the lipolytic activity of *Bacillus cereus* strains were tested using the method reported by Yang and Fang (2003) and *Bacillus cereus* isolates were examined for biofilm production using the tube method reported by Hassan et al. (2011).

2.4. Genotypic detection of Bacillus cereus isolates using polymerase chain reaction (PCR):

For Genotypic identification of five random isolated *Bacillus cereus* isolates, the PCR-technique using primer for *gro*EL as shown in table (1) and the cycling conditions of the primers during cPCR detailed in table (2). Following QIAamp® DNA Mini Kit instructions (Catalogue no.51304), Emerald Amp GT PCR master mix (Takara, Japan) with Code No. RR310A and 1.5% agarose gel electrophoreses (Sambrook et al., 1989).

Table 1 Oligonucleotide primer sequences of *gro*EL gene in *Bacillus cereus* strain

Primer	Sequence	Amplified product	Reference
*gro*EL	F-TGCAACTGTATTAGCACAAGC T R-TACCACGAAGTTTGTTCACTACT	553 bp	Das et al. (2013)

Table 2 Cycling conditions of the primer during cPCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
*gro*EL	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

2.5. Anti-microbial Sensitivity test for Bacillus cereus strains (CLSI, 2021):

The anti-microbial sensitivity test was done on twenty *Bacillus cereus* strains to study their antimicrobial resistance or sensitivity to twelve antimicrobial discs (Oxoid): ciprofloxacin (CIP 30 µg), norfloxacin (NOR 10 µg), levofloxacin (LEV 5 µg), ampicillin (AMP 10 µg), cefipime (FEP 30 µg), aztreonam (ATM 30 µg), amoxicillin-clavulanic acid (AMC 30 µg), gentamycin (CN 10 µg), doxycycline (DO 30 µg), chloramphenicol (C 30 µg), erythromycin (E 15 µg) and clindamycin (DA 2 µg) using disc diffusion technique of koneman et al. (1997) according to recommendation of the Clinical Laboratory Standard Institute (CLSI, 2021).

3. RESULTS

The recovered *Bacillus cereus* isolates were mannitol negative and hydrolyze lecithin, they grew well and produced characteristic turquoise to pea cook blue colonies (as the media contain Bromothymol blue indicator) whose diameter is approximately 5mm and encircled by an egg yolk precipitation zone of the same color on the PEMBA agar plate as shown in figure (1). And they produce whitish pink colonies (as the media contain Phenol red indicator) surrounded by a white precipitation zone on the MYP agar plate. All recovered *Bacillus cereus* isolates were Gram-positive and rods-shaped bacilli, all isolates are motile as shown in figure (2).

The result of biochemical reactions showed that all isolated strains had all distinguishing biochemical characteristics as that of *Bacillus cereus*, all *Bacillus cereus* isolates were positive Voges Proskauer test, citrate utilization test on Simmons`s citrate agar medium as shown in figure (3), catalase test as shown in figure (4), nitrate reduction test and gelatin hydrolysis test. While were negative indole test, methyl red test, urease test (urea was not hydrolyzed) as shown in figure (5) and oxidase test. In addition, all *Bacillus cereus* isolates have the capability for production of biofilm, as evidenced by a visible film that coated the tube's wall and bottom. Also, all *Bacillus cereus* isolates have proteolytic and lipolytic activity.

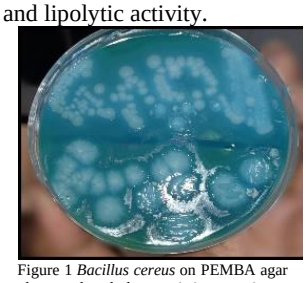

Figure 1 *Bacillus cereus* on PEMBA agar plate produced characteristic turquoise to pea cook blue colonies whose diameter is approximately 5mm and encircled by an egg yolk precipitation zone of the same color on the agar plate.

Figure 2 Motility test. *Bacillus cereus* was motile. A cloudy medium was observed as motile bacteria were seen to spread from point of stabbing on semi-solid agar medium.

Figure 3 Citrate utilization test. *Bacillus cereus* was positive. Change in the color of Simmons`s citrate agar medium from green to blue.

Figure 4 Catalase test. *Bacillus cereus* was positive immediate bubble formation (effervescence)

Figure 5 Urease test. *Bacillus cereus* was negative (urea was not hydrolyzed). The culture medium remained yellowish color.

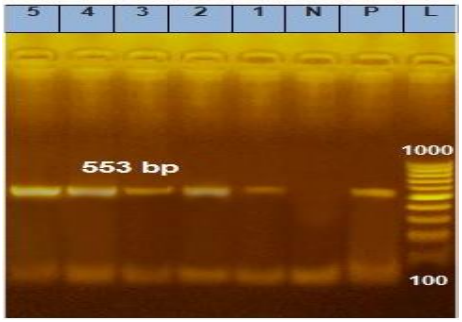

Figure 6 Agarose gel electrophoresis for an amplified product of *gro*EL gene. Electrophoretic pattern of PCR products of positive *gro*EL (diagnostic and phylogenetic marker gene of *Bacillus cereus*) amplified by *gro*EL primer in 1% gel and stained by ethidium bromide showing product of 553 bp. Lane (L): 100-1000 bp Ladder. Lane (N): Negative control. Lane (P): Positive control (at 553 bp). Lanes from 1 to 5: *Bacillus cereus* strains were positive for *gro*EL gene at 553 bp.
*Positive and negative controls were represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Dokki, Giza, Egypt.

The bacteriological examination of collected samples (Table 3) showed that, 40 *Bacillus cereus* isolated strains were obtained from 125 samples 40/125 (32%); mostly they were isolated from ras cheese 14/25 (56%) followed by luncheon 11/25 (44%), rice pudding 8/25 (32%) then from sausage 7/25 (28%) and there is no isolated *Bacillus cereus* from karish cheese samples 0/25 (0%).

Table 3 Prevalence of *Bacillus cereus* isolated from meat and milk products:

Samples	No. of samples	No. of positive samples	Positive percentage (%)
Ras cheese	25	14	56%
Luncheon	25	11	44%
Rice pudding	25	8	32%
Sausage	25	7	28%
Quraish cheese	25	0	0%
Total	125	40	32%

Over-all *Bacillus cereus* prevalence in all meat products samples were 18/50 (36%), these results are higher than *Bacillus cereus* prevalence in all milk products which was 22/75 (29.33%).
The polymerase chain reaction for five *Bacillus cereus* strains (Figure 6) showed that *gro*EL gene (diagnostic and phylogenetic marker gene of *Bacillus cereus*) was detected in all five isolated studied strains (100%). The *gro*EL gene was amplified at 553 bp for the five isolates of *Bacillus cereus*.
The anti-microbial sensitivity test for 20 *Bacillus cereus* strains (Table 4) showed that they were completely sensitive to ciprofloxacin, norfloxacin and levofloxacin 20/20 (100%) followed by gentamycin 16/20 (80%), doxycycline 12/20 (60%), chloramphenicol 10/20 (50%), erythromycin 6/20 (30%) then clindamycin 4/20 (20%), whereas they were completely resistant to ampicillin, cefipime, aztreonam and amoxicillin-clavulanic acid 0/20 (0%).

Table 4 Anti-microbial sensitivity test for *Bacillus cereus* isolated strains: (N=20)

Antimicrobial Family	Antimicrobial disc	Sensitive		Intermediate		Resistant	
		No. of isolates	%	No. of isolates	%	No. of isolates	%
Fluoroquinolones	Ciprofloxacin (CIP 30 µg)	20	100	0	0	0	0
	Norfloxacin (NOR 10 µg)	20	100	0	0	0	0
	Levofloxacin (LEV 5 µg)	20	100	0	0	0	0
β -lactam	Ampicillin (AMP 10 µg)	0	0	1	5	19	95
	Cefipime (FEP 30 µg)	0	0	1	5	19	95
	Aztreonam (ATM 30 µg)	0	0	0	0	20	100
	Amoxicillin-clavulanic acid (AMC 30 µg)	0	0	0	0	20	100
Aminoglycosides	Gentamycin (CN 10 µg)	16	80	2	10	2	10
Tetracycline	Doxycycline (DO 30 µg)	12	60	4	20	4	20
Phenicol	Chloramphenicol (C 30 µg)	10	50	10	50	0	0
Macrolides	Erythromycin (E 15 µg)	6	30	10	50	4	20
Glycopeptide	Clindamycin (DA 2 µg)	4	20	8	40	8	40

4. DISCUSSION

Bacillus cereus can cause emesis, diarrhea, fatal meningitis and food deterioration (Evreux et al., 2007).
The recovered *Bacillus cereus* isolates grew well and produced characteristic turquoise to pea cook blue colonies, whose diameter is approximately 5mm and encircled by an egg yolk precipitation zone of the same color on the PEMBA agar plate. Which is comparable to the earlier reports by Tallent et al. (2012) and Abd El-Tawab et al. (2015); and they produce whitish pink colonies surrounded by a white precipitation zone on the MYP agar plate. This is comparable to the earlier reports by Tallent et al. (2012), Abd El-Tawab et al. (2019) and Tharwat et al. (2020).
The biochemical identification findings revealed a characteristically identical biochemical reaction to be *Bacillus cereus* which is comparable to the earlier finding reported by Abd El-Tawab et al. (2015).
The ability of *Bacillus cereus* to produce biofilms plays a significant role in its capability to persist in equipment used in the food industry (Wijman et al., 2007). The biofilm guards spores and vegetative cells from sanitizers inactivation (Ryu and Beuchat, 2005).
In this study, a total of 40/125 (32%) *Bacillus cereus* strains were isolated from 125 random samples of meat products (luncheon and sausage) and milk products (ras cheese, karish cheese and rice pudding), 25 of each.
Bacillus cereus incidence was 56% (14/25) in the examined ras cheese samples. This result is higher than that obtained by Berthold-Pluta et al. (2019), who found that the prevalence was (43.4%) and much higher than that obtained by Abdeen et al. (2020) who recorded (8.5%).
The incidence of *Bacillus cereus* was 44% (11/25) in the examined luncheon samples. This result is similar to that reported by Tharwat et al. (2020), who reported 44%, but higher than that reported by Abdel-Tawab et al. (2015), who reported lower incidences (20%) and Shawish and Tarabees (2017), who recorded 15%, while this incidence was lower than that reported by Soliman (2013), who reported a higher incidence (74.3%).
The incidence of *Bacillus cereus* was 32% (8/25) in the examined rice pudding samples. This result is similar to that

reported by Reyes et al. (2007) who reported 35%. However, this incidence was higher than that of Hussein et al. (2015) who recorded 15%. This result is considered low when compared with that reported by other studies such as 76.6% by El-Zamkan et al. (2017) and 60% by Sadek et al. (2006). The incidence of *Bacillus cereus* was 28% (7/25) in the examined sausage samples. This result is nearly similar to that obtained by Shawish and Tarabees (2017), who recorded 25%. While this result was lower than those of Hemmat et al. (2014), who reported 72% and Abdel-Tawab et al. (2015), who reported 40% , but it was higher than that of Güven et al. (2006), who reported 16%.

In the present study, there is no isolated *Bacillus cereus* from karish cheese samples 0/25 (0%). This result is similar to other results reported by Ibrahim et al. (2015) and Osama et al. (2020). Clavel et al. (2004) reported that the acidity of kariesh cheese may be explaining this absence of *Bacillus cereus* in samples. In contrast, other results were reported at 28% and 10% by Sadek et al. (2006) and El Sayed et al. (2011) respectively.

These differences in the results might be related to seasonal, geographical and methodological variances. Furthermore, the level of contamination of samples are dependent on the safeguards used during processing.

Polymerase chain reaction has emerged as a quick and reliable technique for confirmation of toxigenic *Bacillus cereus* (Ombui et al., 2008).

The results of polymerase chain reaction for five *Bacillus cereus* isolated strains showed that *gro*EL gene was detected in all five studied strains (100%) and it was amplified at 553 bp for the five isolates of *Bacillus cereus*. So, all five studied strains were *Bacillus cereus.* This result is similar to those reported by Yushan et al. (2010) and Kim et al. (2013).

The anti-microbial sensitivity test for 20 *Bacillus cereus* strains showed that they were completely sensitive to ciprofloxacin, norfloxacin and levofloxacin 20/20 (100%) followed by gentamycin 16/20 (80%), doxycycline 12/20 (60%), chloromphenicol 10/20 (50%), erythromycin 6/20 (30%) then clindamycin 4/20 (20%), whereas they were completely resistant to ampicillin, cefipime, aztreonam and amoxicillin-clavulanic acid 0/20 (0%). This result is nearly similar to that reported by Abd El-Tawab et al. (2019).

In this study, the most common drug resistance was to β-lactam antibiotics. This result is similar to that reported by Abd El-Tawab et al (2019) for amoxicillin- clavulanic (100%), Fielder et al. (2019) for penicillin (100%) and amoxicillin-clavulanic (99.3%). In contrast, *Bacillus cereus* was completely sensitive to ciprofloxacin, norfloxacin and levofloxacin (100%). This result is similar to that reported by Fielder et al. (2019) (99.3%) for ciprofloxacin.

5. CONCLUSIONS

In this study, *Bacillus cereus* mostly was isolated from ras cheese followed by luncheon, rice pudding then from sausage and there is no isolated *Bacillus cereus* from karish cheese. Finally, based on the results, *Bacillus cereus* is a serious pathogen could contaminate foods as meat and milk products in various shops and marketplaces in El-Gharbia governorate, Egypt. So, hygiene procedures should be followed from the beginning of foods industries until reaching the consumer for controlling *Bacillus cereus* prevalence.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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