Molecular detection of antibiotic resistant bla gene in B. cereus isolated from meat products

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ABSTRACT

In this study the incidence of Bacillus cereus (B. cereus) and antibiotic sensitivity test was undertaken. A total of one hundred random samples of meat products of rice kofta, kobeba, chicken pane and chicken nuggets (25 of each) were collected from different supermarkets at different times in Menofia and Kalyobia governorates, Egypt and tested for occurrence of Bacillus group. Bacillus cereus were detected in 24%, 12%, 20% and 10% of kobiba–shami, rice kofta, chicken pane and chicken nuggets, respectively. Bacillus mycoids failed to be isolated from kobiba–shami and isolated from 4%, 16% and 4% of rice kofta, chicken pane and chicken nuggets, respectively. Also, Bacillus thuringenis failed to be isolated from kobiba–shami and rice kofta while it isolated from 8% of both chicken pane and chicken nuggets. Fourteen strains of B. cereus were examined for antimicrobial susceptibility testing. The foremost common drug resistance was to penicillin, amoxicillin and amoxicillin + clavulanic (100% for each). On the other hand, B. cereus was completely prone to vancomycin and gentamycin (100% for each). Using PCR, all tested B. cereus isolates harbored bla gene including those resistant to many antibiotics, that may make antibiotic treatment to be unsuccessful (Friedman et al., 2016). So, knowing the antibiotic resistance of Bacillus cereus is vital for using the drug of choice for bacterial treatment.

Multiple drug resistant isolates of Bacillus cereus because of production of beta-lactamase have a significant threat to Public Health. Beta-lactamases, being one amongst the potential virulence factors make these strains resistant to penicillin, ampicillin, and even to third generation cephalosporin (Cormian et al., 1998).

PCR has become one of the most important molecular diagnostic methods for detection of foodborne pathogens and is considered to be a valuable alternative to culture-based detection techniques due to its speed, limit of detection (LOD), sensitivity and specificity (Maurer, 2011; Rodríguez-Lázaro et al., 2013). So, the present study was designed for molecular detection of antibiotic resistant bla gene in B. cereus isolated from meat products.

1. INTRODUCTION

B. cereus is the most important foodborne pathogen in the B. cereus group and has been associated with food-poisoning illnesses in humans and other kinds of clinical infections (Bottone, 2010). B. cereus produces two types of toxins- emetic (vomiting) and diarrhoeal-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 diarrhoeal syndrome is caused by diarrhoeal toxins produced during growth of the bacteria in the small intestine (Ehling-Schulz et al., 2006).

Meat and meat products are a suitable media for growth because they are high in moisture, high in nitrogenous compounds (amino acids, peptides, and proteins) and abundantly 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).

B. cereus give purple color by Gram stain, motile (flagellated), sporulated, rod shaped bacterium which belongs to the Bacillus genus (Montville and Matthews 2005).

Program identification of Bacillus cereus is commonly involved of isolation on selective media, showing of motility, hemolysis pattern on blood agar, and production of acid from glucose fermentation (Stenfors Arnesen et al., 2008).

The most effective method for treating bacterial infection was antibiotic treatment, containing those produced by Bacillus cereus; however, the appearance of antibiotic-resistant strains due to the wide usage of antibiotics,
400 Lab Blender (Seward Medical, London, UK) for 30 seconds.

2.3. Isolation of B. cereus
Isolation was conducted following (Shinagawa, 1990); briefly, samples were inoculated into brain heart infusion broth (BHIB) containing polymyxin (100 units/ml). The broth tubes were incubated at 37°C for 24-48 hours. Later a loopful taken from enrichment broth and put on PEMBA (Polymyxin pyruvate egg-yolk mannitol–broth mycolyl blue agar plates and incubated at 37°C for 24 hr. Bacillus cereus grow as crenate, filibrate, or slightly rhizoid colonies up to 5 mm diameter, turquoise to peacock blue in color with flat ground glass surface, and surrounded by a precipitate from hydrolyzed egg yolk.

2.4. Identification of Bacillus cereus:
B. cereus isolates were subjected for various biochemical tests like catalase test, nitrate reduction test, motility test, lecinthinase activity, modified Voges-Proskauer (VP) test, haemolysis on 5% sheep blood agar and various sugar (Inositol, dulcitol, fructose, dextrose, sucrose, mannnitol and salicin) fermentation test were conducted. The suspected isolates were identified morphologically and biochemically according to Koneman et al. (1992). The presumptive isolates showing positive catalase test, Voges-Proskauer test, positive nitrate reduction test, positive for anaerobic utilization of sugars by Bacillus cereus and produce β-haemolytic.

2.5 Antibiotic sensitivity Testing:
Bacillus cereus isolates were tested to their sensitivity to 9 antibiotics (Penicillin (10 g), Oxacillin (10 g), Oxacillin+ Clavulanic acid (30 g), Co trimoxazole (25 g), Vancomycin (30 g), Erythromycin (15 g), Gentamicin (10 g),Ciprofloxacin (5 g) and Kanamycin (30 g)) in line with the standard disc diffusion method (The Clinical and Laboratory Standards Institute (CLSI), 2010 and Yu et al., 2019). Isolates were put on the plate of Mueller-Hinton agar and incubated for 16–18 h at 37°C. The colony afterward was chosen and suspended in 0.85% physiological saline to 0.5 McFarland standards and placed on the surface of a Mueller-Hinton agar plate. After the inoculum was dried, the antibiotic discs were placed on the surface of the plates. The plates of Mueller-Hinton agar were incubated for 16–18 h at 35 ± 2°C, and the zone of inhibition was measured. The isolates were categorized as susceptible (S), intermediate (I), or resistant (R) in line with Clinical and Laboratory Standards Institute (CLSI) guidelines and also measuring the different zones of inhibition and interpreted as said by the interpretation of zone diameter measures for staphylococcus aureus (CLSI 2010).

2.6. Detection of bla gene by Polymerase Chain Reaction
2.6.1. DNA extraction:
Genomic DNA extraction and purification was done using QIAamp DNA Mini Kit. PCR was done to detect bla gene. A positive reference strain of Bacillus 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).

| Gene | Sequence of primer (5’)-3’ | Amplified product length | Reference |
|------|---------------------------|--------------------------|-----------|
| bla  | F 5’CATTCGAAGTGGAACGCAAAA3’ | 680 bp | Chen et al., 2004 |
|      | R 5’TGTCGCCGTAACATTCCAGTC3’ |               |           |

Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit as shown in table (2)

Table 1 Provides details about the primer used

Table 2 Preparation of PCR Master Mix

| Component                  | Volume/reaction |
|----------------------------|-----------------|
| Emerald Amp GT PCR master mix (1X premix) | 12.5 μl |
| PCR grade water            | 4.5 μl         |
| Forward primer (20 pmol)   | 1 μl           |
| Reverse primer (20 pmol)   | 1 μl           |
| Template DNA               | 6 μl           |
| Total                      | 25 μl          |

Table 3 Temperature and time conditions of the primers during PCR

| Gene     | Primary denaturation | Secondary denaturation | Annealing | Extension | No. of cycles | Final extension |
|----------|----------------------|------------------------|-----------|-----------|---------------|----------------|
| bla      | 94°C, 30 sec         | 50°C, 45 sec           | 72°C, 10 min | 10 min    | 35 cycles     |                |

3. RESULTS
It was evident from the result recorded in table (4) that, B. cereus was present in kobeba–shami, rice kofta, chicken pane and chicken nuggets at percentage of 24%, 12%, 20% and 10% of respectively. Bacillus mycoids failed to be isolated from kobba–shami but isolated from rice kofta, chicken pane and chicken nuggets at percentage of 4%, 16 and 4% of respectively. Bacillus thuringenesis is failed to be isolated from kobba–shami and rice kofta while it isolated from both 8% of chicken pane and chicken nuggets.

As shown in table (5), Disc diffusion susceptibility testing revealed that B. cereus isolated strains from meat products were susceptible to Vancomycin 30 g (100%), Gentamycin 10 g (100%) and Ciprofloxacin 5 g (78.5%) and highly resistant to Penicillin 10 g (100%), Amoxicillin 10 g (100%) and Amoxicillin + clavulanic acid 30 g (100%).

Photo (1) declared the results of testing of strains of B. cereus isolated from different meat products using specific primer for bla antibiotics resistance gene. bla gene was found in 100% of isolates of B. cereus.

Table 4 Incidence of Bacillus serotypes isolated from the examined meat products (n=25)

| Products  | B. cereus | B. mycoids | B. thuringenesis |
|-----------|-----------|------------|-----------------|
|           | No %      | No %       | No %            |
| Kobba–shami | 6        | 24         | 0               |
| Rice kofta  | 3         | 12         | 1               |
| Chicken pane | 5        | 20         | 4               |
| Chicken nuggets | 4    | 16         | 1               |

Table 5 Antibiotics sensitivity test of B. cereus isolated from examined samples by disc diffusion method (n=14)

Antimicrobial agents

| Antibiotic agents | Sensitive | Intermediate | Resistant |
|-------------------|-----------|--------------|----------|
|                   | No %      | No %         | No %     |
| Penicillin 10 g   | 0         | 0            | 14       |
| Amoxicillin 10 g  | 0         | 0            | 14       |
| Amoxicillin + clavulanic acid 30 g | 0 | 0 | 14 |
| Cotrimoxazole 25 g | 2 | 14.2 | 5 | 35.7 | 7 | 50 |
| Vancomycin 30 g   | 14        | 100          | 0        | 0       |
| Erythromycin 15 g | 6         | 43           | 2        | 14      |
| Gentamycin 10 g   | 14        | 100          | 0        | 0       |
| Ciprofloxacin 5 g | 11        | 78.5         | 3        | 21.5    | 0       |
| Kanamycin 30 g    | 6         | 43           | 5        | 35.5    | 3       | 21.5 |

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4. DISCUSSION

Strains of food-borne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Kiessling et al., 2002; Roy et al., 2007). Results in table (3) revealed that B. cereus was the predominant isolated serotype from the examined samples of kobeba-shami, rice kofta, chicken pane and chicken nuggets, respectively. On the other hand, B. mycoides failed to be isolated from kobeba-shami and isolated from rice kofta, chicken-pane and chicken nuggets, respectively. Bacillus thuringensis is failed to be isolated from kobeba-shami and rice kofta while it isolated from 8% both chicken pane and chicken nuggets. Abd El Wahab et al. (2018) isolated B. cereus in percentages of 60, 52% from rice kofta and kobeba, respectively. Esraa (2018) found that the incidence of B. cereus in chicken nuggets was 48%, meanwhile, Dowidare (2009) isolated B. cereus from 12% of examined kobeba.

As shown in table (4), 14 strains of B. cereus were tested for antimicrobial susceptibility testing. The most common drug resistance was to penicillin, amoxicillin and amoxicillin+clavulanic. Similar results were obtained by Abd El Tawab et al. (2019) for amoxicillin + clavulanic (100%), Fielder et al. (2019) for penicillin (100%) and amoxicillin +clavulanic (99.3%), Yu et al. (2020) for penicillin (100%) and amoxicillin + clavulanic (97.83%) and Guven et al. (2006), who found that B. cereus isolates from meat and meat products showed a high resistance to amoxicillin.

On the other hand, B. cereus was completely susceptible to vancomycin and gentamycin (100% for each). Similar results were obtained by Shawish and Tarabees (2017) for vancomycin (100%) and Dikbas (2010) (100%) and Yu et al. (2020) (96.47%) for gentamicin. Also B. cereus was susceptible to ciprofloxacin, erythromycin, co-trimoxazole and kanamycin. Nearly similar result obtained by Fielder et al. (2019) (99.3%) and Yu et al. (2020) (78.8%) for ciprofloxacin and (76.36%) and Dikbas 2010 (67.5%) for kanamycin.

In our study all tested B. cereus isolates harbored bla gene which agreed with Abd El Tawab et al. (2019) (100%). These variations in the results were attributed to the quality of raw materials and the hygienic state during preparation and processing of the product.

The high frequency of isolation of B. cereus from meat products may be attributed to processing of minced meat also additives and spices that added to this products, which can increase the number of Bacillus spores. Therefore, it is important to use additives from a trustful source during processing of raw meat and test these additives regularly for the presence of bacillus spore (Shawish and Tarabees, 2017).

Food-poisoning by B. cereus can be produced by either infection or intoxication, which leads to a diarrheal or an emetic type of disease, respectively. The diarrheal syndrome is caused by enterotoxins produced by vegetative cells during the growth in the small intestine after consumption of contaminated foods (Schoeni & Wong, 2005). The emetic syndrome is associated with the production of the cereulide toxin in foods before consumption (Ehling-Schulze et al., 2004; Stenfors Arnesen et al., 2008). Beta-lactamases, being one amongst the potential virulence factors make these strains resistant to penicillin, ampicillin, and even to third generation cephalosporin (Cormian et al., 1998)

5. CONCLUSION

We can conclude that B. cereus was the predominant isolated serotype of Bacillus group, chicken panne and nuggets are the most contaminated products and all tested B. cereus strains were harbored the antibiotic resistant and bla gene.

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