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Molecular profile of some *Bacillus cereus* strains isolated from milk with reference to its susceptibility to lysozyme

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Abstract

The present study aimed to clarify the molecular diversity of *B. cereus* isolated from milk and its susceptibility to lysozyme. A total of 153 individual raw milk samples were randomly collected. Bacteriological examination was carried out according to standard methods. In vitro susceptibility of *Bacillus* species to lysozyme was done for all isolates (No=153). ERIC-PCR fingerprinting was done for 10 *B. cereus* strains. It was found that 61 (39.9%) of milk samples were positive for *B. cereus*. Susceptibility to lysozyme was the highest in *B. cereus* (47.5%) among other *Bacillus* species. Molecular fingerprinting produced patterns of 11 major bands. Dendrogram revealed 3 main clusters. The highest degree of similarity was noticed among isolates in different date of isolation. It could be concluded from this study that the isolated *B. cereus* clones had diversity in terms of molecular profile and sensitivity to lysozyme.

Keywords: *Bacillus cereus,* bacteria, ERIC-PCR, lysozyme

Introduction

Bacillus cereus is the common virulent *Bacillus* species in raw milk and may have a public health hazard[16,22)]. Emetic and diarrheal syndromes are two types of food borne affections caused by *B. cereus*[13)]. Moreover, *B. cereus* could spoil the milk[6)]. The true occurrence of *B. cereus* in Egypt is not clearly understood[17)]. Endolysin is an N-acetylmuramoyl-L-alanine amidase and cleaves the cell wall of *several Bacillus* species when applied exogenously[12)]. Lysozyme is an enzyme that degrades the bacterial cell wall by catalyzing the hydrolysis of β-(1,4)-glycosidic linkages between N-acetylmuramic acid and N-acetyl glucosamine found in the peptidoglycan layer, which is the major component of the cell wall of both gram-positive and gram-negative bacteria[20)]. Repetitive element sequence-based PCR genomic profiling has also been applied for characterization among many of bacterial genera and species, and to match bacterial DNA diversity due to its ability to discriminate several parts of the bacterial genome[24,25)]. The aim of this study was to clarify the diversity of *Bacillus* species in individual raw milk samples and its susceptibility to lysozyme.

Materials and Methods

Sampling and cultivation:

A total of 153 individual raw milk samples

(100 ml, each) were randomly collected from the Educational Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, Egypt, then were heated at 80 °C for 10 min in a water bath [21]. One ml of each heat- treated sample was added to 9.0 ml of nutrient broth (Oxoid, UK). Culture homogenate were incubated at 34 °C for 24 h. Turbid culture homogenate was cultured onto a selec¬tive medium (MYP agar, Oxoid). The streaked plates were incubated at 34 °C for 24–48 h[2]. B. cereus suspected isolates were identified[3,9,28]. The suspected colonies of *B. cereus* were confirmed using the API 50 CHB system (BioMeriéux).

Lysis test of isolated Bacillus species:

A loopful of the tested strain was streaked onto Nutrient agar and incubated overnight at 37 °C. The bacterial suspension was divided in 2x 1.5 ml Eppendorf, 350 μl each, then lysozyme (Sigma-Aldrich) was added. Briefly, a freshly prepared solution of 10 mg/ml in 10 mM Tris-HCl (pH 8.0) was used. Twenty five μl of this stock solution will typically lyse bacterial cells from >1ml of culture media cell pellet re-suspended in 350 μl 10 mM Tris-HCl, pH 8.0, with 0.1 M NaCl, 1 mM EDTA, and 5% [w/v] TRITON X-100.Typical incubation conditions for lysis were 30 min at 37 °C. The lysed suspensions were centrifuged at 13000 x g for 5 min.

Determination of lysis using bicinchoninic acid (BCA) protein assay:

Each positive reaction for lysis will give more free protein than negative one. Detection of the difference in protein content using BCA–Reagent kits (Pierce) was done following the manufacturer instructions. Briefly, 100 μl of each standard and unknown sample replicate was pipetted into a micro plate well. Add 200 μl of working reagent was added to each well and mixed plate thoroughly on a plate shaker for 30 sec. Plate was covered and incubated at 37°C for 30 min. The absorbance was measured at 450 nm on a plate reader[4].

DNA extraction and PCR conditions:

Genomic DNA from ten *B. cereus* isolates (No 1 to 10) was extracted and purified[15]. ERIC-PCR was carried out [23,24]. The dendrogram was designed by un-weighted pair group method with arithmetic average (UPGMA) using SPSS version 16 program.

Table 1. Occurrence of *B. cereus* and related species in 153 individual raw milk samples

Bacillus species	Positive	
	No	%
B. cereus	61	39.9
B. alvei	30	19.6
B. polymyxa	25	16.3
B. laterosporus	14	9.2
B. megaterium	13	8.5
B. mycoides	10	6.5
Total	153	100

Table 2. In vitro susceptibility of isolated *Bacillus* species to lysozyme

Bacillus species	No of tested strains	Positive for lysis test	
		No	%
B. cereus	61	29	47.5
B. alvei	30	2	6.7
B. polymyxa	25	1	4.0
B. laterosporus	14	1	4.0
B. megaterium	13	0	0.0
B. mycoides	10	0	0.0
Total	153	33	21.6

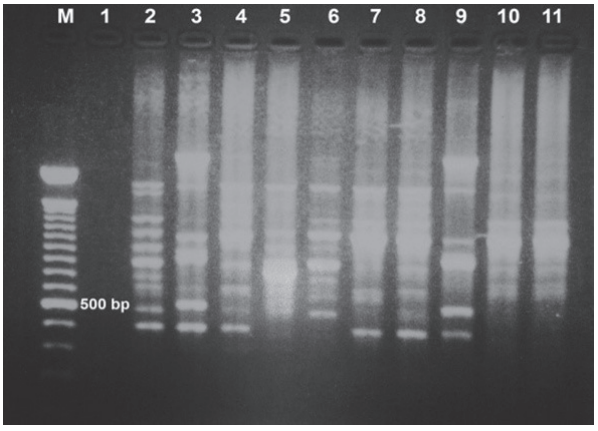

Fig.1. ERIC-PCR fingerprint patterns of *B. cereus* obtained with the ERIC primer pair. Lanes (number of isolate): M (100-bp marker); 1 (negative control); 2-11 (strains of *B. cereus*).

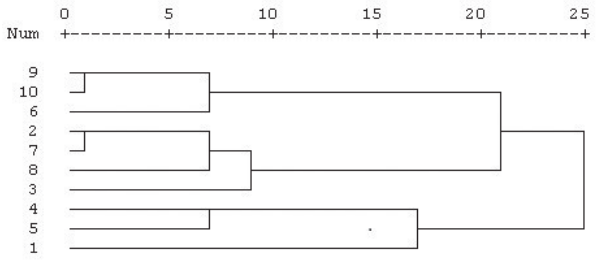

Fig. 2. Dendrogram of *B. cereus* fingerprint patterns obtained with ERIC-PCR (strains 1-10). The number of each isolate (1-10) represented the consequent week of isolation.

Results

Fingerprinting using ERIC-PCR (Fig. 1) produced patterns of 11 major bands between 400 bp and 1.6 kbp. The comparison analysis was carried out according to those major bands. To demonstrate the similarity among the isolates with different molecular patterns, the summarized data obtained by the banding profile was analyzed statistically. Fig. 2 showed analysis of ERIC-PCR products by peak comparison grouped the isolates into 3 main clusters. First cluster includes strains No 9, 10 and 6 with high degree of similarity between strains No 9 and 10. The second cluster consists of 4 strains with high degree of similarity between strains No 2 and 7. The 3rdcluster comprises isolates No 4, 5 and 1 with high degree of similarity between isolates No 4 and 5.

Discussion

Due to the public health significance of *B. cereus*, the current study was designed to isolate different clones from milk. The obtained results gave data about the occurrence of different *Bacillus* species in milk. The diversity of isolated *B. cereus* strains will clarify the potential source of infection and circulating strains in the examined niche. *B. cereus* is important underestimated milk borne bacterium[5]. In this study, *B. cereus, B. alvei, B. polymyxa, B. laterosporus, B. megaterium* and *B. mycoides* were found in the collected milk samples with percentages of 39.9, 19.6, 16.3, 9.2, 8.5 and 6.5, respectively. A previous study found relatively lower percentage[17,2]. Other investigations reported nearly similar results of *B. cereus* contamination in raw milk[18,26]. The occurrence of *B. cereus* in the examined milk may indicate potential risk to the public health. Moreover, *B. cereus* had been isolated from pasteurized milk with different percentages varying according to season of collection[29].

Studying the sensitivity of isolated *Bacillus* species to lysozyme may help in biocontrol strategies. Lysozyme can act as a natural antibiotic[27]. Table 2 shows that 29 *B. cereus* strains out of 61(47.5%) were sensitive to lysozyme under research condition. Bio-preservation rationally exploits the antimicrobial potential of lysozyme in food with a long history of safe use[27].

It was previously found that lysozyme could inhibit *B. subtilis, B. licheniformis, B. megaterium, B. mycoides, B. pumilus, B. coagulans, B. amyloliquefaciens, B. polymexa and B. macerans,* completely. However, *B. cereus* and *B. stearothermophilus* showed a slightly higher resistance[1]. It was found that lysozyme is capable of lysing *B. cereus* cells effectively in vitro in 47.5% (29/61) of isolates. From the obtained results, the lysozyme had lytic effects against other species ranged from zero to 6.7%. The enzymatic degradation of *B. cereus* cell wall material was highly nonlinear as the cell wall represents a highly heterogenous substrate[14]. The lytic effect against *B. cereus* was attributable to their genetic similarity to *B. anthracis*[8]. The killing activity against *B. cereus* is likely due to the difference betweencell wall structure and that of *B. anthracis*[11]. Lysozyme has lytic effect against some gram positive bacteria[10]. The lysozyme bactericidal potency is due to its muramidase, cationic and hydrophobic properties[19]. Identification of genetic profile of tested *B. cereus* strains is often important to success of epidemiological interpretations.

In order to understand the potential source

of transmission of milk borne *B. cereus*, it is important to study the diversity or similarity among the isolates in term of genetic profile. These tested strains had the typical colony morphology, biochemical character and clustered by ERIC-PCR. The genetic diversity was investigated in the present study using ERIC primer. Genomic diversity of *B. cereus* group was investigated by genotyping with rep based PCR using the primer set ERIC2 and reflected high intra-species difference[7]. The used ERIC-PCR was reproducible and discriminative among the tested clones. ERIC-PCR analysis has been carried out for *B. cereus* strains and produced 23 different genotypes [23]. The representative 10 tested strains in the current study produced well detectable and distinguished PCR product bands. As the time of strain isolation is indicated to consequent week of sample collection, the degree of similarity among some isolates may reveal multiple sources of infection by *B. cereus* during the study period (10 weeks). The high degree of similarity among isolates No 9 with No 10, No 2 with No7 and No 4 and 5 was noticed in the present study. It was found that when ERIC–PCR is used, the generated profiles are not related to the subsequent time of isolation (week each). Further studies are needed to confirm the relationship between genetic similarity of milk borne *B. cereus* and its geographic distribution. It could be concluded that *B. cereus* circulates in milk sources with different molecular profiles. Lysozyme could be an effective antibacterial agent. Further studies are needed to study relationship between genotypic profile and geographic region.

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