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Phenotypic and molecular detection of Beta-Lactamase enzyme produced by *Bacillus cereus* isolated from pasteurized and raw milk

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ABSTRACT

Background: In a survey of milk samples obtained from individual farms and pasteurized milk plants in Ardabil city, 38% of raw milk samples and 22% of pasteurized milk samples were found to be contaminated with *Bacillus cereus*. In total 200 milk samples,100 of raw milk and 100 of pasteurized milk, were tested for the presence of *B. cereus* contamination.

Methods: The milk samples tested positive for *B. cereus* were used to isolate the DNA from the and subsequently used in molecular genetic analysis. Statistically, 38% of raw milk samples and 22 % of pasteurized milk samples were found contaminated with *B. cereus*. The results of the present study confirms that processed milk also carries a very high probability of *B. cereus* contamination .In the present study, three different primer sets were used to amplify DNA obtained from *B. cereus* isolates and the gene expression profile of beta-lactamase producing genes (*SHV*,*TEM*,*CTX-M*) were analyzed using PCR.

Results: *TEM* types of beta-lactamases exhibited maximum percentage frequency in raw milk isolates whereas pasteurized milk isolates had the most frequency of *CTX-M* type of beta-lactamases. It was observed that both raw and pasteurized milk are a source of *B. cereus* as evident by pure isolates obtained on agar plates.

***Conclusion*:** The gene expression profile shows that milk samples are frequently contaminated with antibiotic resistant strains of *B. cereus*.

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Introduction

Bacillus cereus is a gram positive and spore forming bacteria which shows rod shape morphology and grows aerobically. It is one of the most common contaminants in dairy products, meat and eggs. *Bacillus* species are common in pasteurized milk samples they have been also found in milk powders and infant formulas (1). In a recent study Lima et al. have found the presence of *Bacillus cereus* in human donor milk samples even after holder pasteurization was performed (2). A recent study from Jammu region in India studied the prevalence and antibiotic sensitivity profile of *Bacillus cereus* in milk and milk products and found that 28.37% samples were tested positive for *B. cereus* contamination. It was observed that ice cream had the highest prevalence of *B. cereus*. Moreover, the antibiogram reveals that isolates were highly sensitive for gentamicin (100%) while resistant to penicillin G (100%) (3). Such studies show that *B. cereus* is one of the most common and important microorganism which causes food-borne disorders in humans. The entry of *B. cereus* through contaminated food causes gastrointestinal disturbance leading to diarrhea (4). The various toxins such as hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), cytotoxin K (CytK) produced by *B. cereus* are the principle cause behind the pathogenicity shown by this bacterium (5). It was shown by Li et al. that expression of *cytK*, *hblD*, *nheA*, and *entFM* enterotoxin genes varies between the strains and depends on food matrix may influence the expression profile of these genes (6).

Antibiotics are extensively used to treat the infections caused by *B. cereus*. However, the wide spread misuse of antibiotics has led to emergence of antibiotic resistant *B. cereus* strains. Moreover, the antibiotic resistant strains of *B. cereus* have also emerged due to horizontal gene transfer of resistant genes from other bacterial species (7). In another study by Shhlegelova et al, it has been shown that *B. cereus* strains showed low sensitivity to ampicillin, cephalothin, and oxacillin while resistance to streptomycin. It was also observed that the same sample was contaminated with two different subpopulations of *B. cereus* with different levels of sensitivity towards streptomycin (8). The results of the above studies reflect a growing trend of antibiotic resistance in *B. cereus* strains and so, it requires an urgent attention due to its major impact on human health. This presents an urgent need to obtain the antibiotic sensitivity profile of *B. cereus* strains found in common food items such as milk. Milk is one of the most common dairy product frequently found contaminated with *Bacillus cereus* spores because the pasteurization process using low-temperature for sterilization does not eliminate the spores completely (9,10). Contamination of milk samples with *B. cereus* spores is a very important public health issue due to the fact that milk is one of the most widely consumed dairy products in the world. Thus, the aim of the present study is to evaluate the prevalence of *B. cereus* in milk samples obtained from common markets. Moreover, the present study also focused on analysing the antibiotic sensitivity profile, both by at phenotypic and genetic levels, of the *B. cereus* strains found in the milk samples.

Materials and Methods

Milk Sample collection

Raw sterile milk samples were collected in bottles aseptically from milk markets of different regions in Ardabil city. Before collection, the milk from bulk was shacked to get homogenized. Then, 10 ml of milk sample was taken in the universal bottle. This universal bottle was labeled with a waterproof ink pen. Label included date, day, place of milk collection and whether raw or pressurized milk. Finally, collected samples were refrigerated and stored for further use. 100 samples were of raw milk and 100 of pasteurized milk were analyzed in the present study (11, 12).

Bacillus cereus identification and isolation of colonies

Bacillus cereus vegetative cells were isolated on agar base (5% sheep blood agar) from milk samples, followed by inoculation of the isolates on the selective mannitol egg yolk polymyxin agar (MYP) (HiMedia, India). After 24 hours of incubation at 37 °C in aerobic conditions, the growth of *Bacillus cereus* was visualized using colonial morphology, precipitated hydrolyzed lecithin across colonies and also a failure of *Bacillus cereus* to use mannitol sugar. 5 mm diameter colonies appeared and had specific distinctive turquoise to pink color bounded by egg yolk drizzle precipitates of the same color were well-thought-out as *Bacillus cereus* (13). Further, isolates were confirmed by microscopic examination using Gram's staining and specific biochemical tests.

Antibiotic susceptibility Testing

Isolates of *Bacillus cereus* from milk were tested for antimicrobial susceptibility using disc diffusion method of CCLI guidelines (14). Padtan Teb (Tehran, Iran) provided the discs of cefotaxime (30µg), cefteriaxon (30µg), ceftizoxime (30µg), gentamicin (10µg), ciprofloxacin, (10µg), amoxicillin (30µg), amikacin (30µg), ceftazidime (30µg) and nitrofurantoin (30µg). Also, minimum inhibitory concentration was also evaluated for five antibiotics with method of broth microdilution and CCLI guidelines (14).

Screening for ESBL

All isolates from 200 different milk samples were screened for production of ESBL underwent as per CLSI guidelines. This was carried out by a routin susceptibility test. Two discs were applied for ceftazidime (30 µg) and cefotaxim (30µg). An inhibition zone less than 27 and 22 mm for cefotaxim and ceftazidime respectively indicated for Extended Spectrum Beta Lactamase (ESBL) production.

Phenotypic confirmatory test for ESBL production

For phenotypic confirmation Combination disk test (CDT) was carried out using ceftazidime (30 µg), ceftazidime-clavulanic acid (30/10 µg), cefotaxim (30µg) and cefotaxime-clavulanic acid (30/10 µg) The isolates were tested by antibiotic specific discs using Mueller–Hinton agar medium as per recommended by CLSI [14]. If the inhibition zone diameter was ≥5 mm larger for clavulanic acid than without, it was interpreted as phenotypic confirming of ESBL production.

DNA Extraction

To prepare genomic DNA, one colony of each *Bacillus cereus* sample was taken that was already grown on LB broth overnight. Each colony was boiled in 100µl water for 10 min to release the DNA from the bacterial cells. Chloroform: isoamyl alcohol in ratio of 24:1 was added to the lysate, mixed and centrifuged at 8000 x g for 15 min. The upper layer containing DNA was used for PCR reaction (15).

Amplification and PCR product analysis

ESBL genes were amplified using *blah*, *TEM*, *SHV* and *CTX-M* specific primers for each colony of each separate milk sample listed in Table 1. To amplify the products following conditions were maintained;denaturation at 94 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 60 °C for 30 seconds followed by extension at 72 °C for 1 minute and a final extension at 72 °C for 3 minutes (16).

Result

During a 2-month period, *Bacillus cereus* was isolated from 200 milk samples. It was observed

that 38% of raw milk samples and 22% of pasteurized milk samples contained beta-lactamases producing *Bacillus cereus* which is responsible for the antibiotic resistance profile observed in these isolates It was observed that 38% of raw milk samples and 22% of pasteurized milk samples 3 were found to contain beta-lactamases producing *Bacillus cereus* strains as confirmed by ceftazidime/clavulanate and cefotaxime/clavulanate disc diffusion tests.

Further, the DNA of all the ESBL positive species were isolated and used for molecular characterization of the *Bacillus cereus* isolates using PCR. Out of 200 samples analyzed, 38 *Bacillus* strains isolated from raw milk and 22 from pasteurized milk were screened for expression of beta-lactamases synthesizing genes such as *blaSHV*, *blaTEM*, and *blaCTX-M*. Antibiotic susceptibility profile showed results as shown in figure 1.

The gene expression analysis of *blaSHV*, *blaTEM*, and *blaCTX-M* genes were carried out using gene specific primers (Table 2) and the PCR products were analyzed using Agarose gel electrophoresis with the help of a 100 base pair DNA ladder as shown in figure 2. It has been observed that ESBL gene expression profile of *Bacillus cereus* strains varied depending on their isolation from raw or pasteurized milk. In isolates obtained from raw milk, *TEM* gene was expressed in highest number of isolates (50%) while in pasteurized milk *CTX-M* was expressed in highest number of isolates (45%).

Discussion

B. cereus is one such bacteria which is frequently causes food-borne disorders in humans. It has been observed that due to its high resistance for elimination during pasteurization process, the spores of *B. cereus* do not eliminate completely and cause a very serious threat to human health (4, 17). Food poisoning by B. cereus occurs either due to an infection or an intoxication, which leads to the sever disease. It has been observed that meat products and dairy products are often associated with food poisoning caused by food borne pathogens of those important *B. cereus* (18). β-lactamases are hydrolytic enzymes which acts on the β-lactam ring of common antibiotics such as penicillins and cephalosporins and render them ineffective thus confer the bacterium *Bacillus cereus* resistance towards lactam antibiotics 919). Extended-spectrum beta-lactamases (ESBLs) render beta-lactam antibiotics such as penicillins, cephalosporins, and monobactams ineffective. An alteration in the amino acid sequence around the active site of the enzyme due to mutation in the narrow-spectrum beta-lactamases (TEM-1, TEM-2, or SHV-1) gives rise to ESBLs (19,20). Fenselau et al. identified the β-Lactamase in penicillin resistant *Bacillus cereus* spores (21). As the beta lactams are the most commonly used antibiotics, the emergence of beta-lactam resistant bacterial species represents a serious threat to continuous and effective use of beta-lactams (22).

Table 1. Representing list of primers used and their sequence.

Primers	Nucleotide Sequences (5′-3′)
SHV	CGCCTGTGTATTATCTCCCT
SHV	CGAGTAGTCCACCAGATCCT
TEM	TTTCGTGTCGCCCTTATTCC
TEM	ATCGTTGTCAGAAGTAAGTTG G
CTX-M	CGCTGTTGTTAGGAAGTGTG
CTX-M	GGCTGGGTGAAGTAAGTGAC

Table 2. Total number of genes that were amplified during PCR.

Genes	Raw milk (38 isolates)	Pasteurized milk (22 isolates)
SHV	7	4
TEM	19	8
CTX-M	12	10

The present study shows that 38% of raw milk samples and 28% of pasteurized milk sample were found to be contaminated with *Bacillus cereus*. Moreover, the antibiotic sensitivity profile of the isolates reveal that most of the isolates (80%) were resistant to nitrofurantoin and amoxicillin (80%) while 98% isolates were sensitive to ceftizoxime. For other antibiotics, the level of resistance varied between 20-45%. In a similar study, Fossi et al. have observed that occurrence of *B. cereus* in raw milk was 8.22% and in processed milk powder it was 13.33%. Our study found a very high rate of contamination of raw milk compared with the study by Fossi et al (38% v/s 8.22%). Moreover, Fossi et al. have observed that raw milk has lesser contamination rate compared with processed milk powder (8.33% v/s 13.33%). This is in contrast with our study where raw milk had higher contamination rate than processed milk (38% v/s 22%). The same study reported that isolates from raw milk and processed milk showed sensitivity to tetracycline, gentamicin, chloramphenicol and nalidixic acid, but resistance to penicillin, ampicillin and trimethoprim/sulphamethoxazole (23). However, an Egyptian study by Shawish et al. reported that strains of *B. cereus* were completely resistant to penicillin G and completely sensitive to oxacillin, clindamycin, vancomycin, erythromycin, gentamicin, ciprofloxacin, and ceftriaxone (24). In our study, we have reported intermediate sensitivity (20-45%) towards most of the antibiotics such as amikacin, ceftazidime, cefotaxime, cefotaxime, gentamicin and ciprofloxacin. This data shows that antibiotic sensitivity considerably varies between the regions and *Bacillus cereus* from different regions/countries possess different levels of sensitivity towards commonly used antibiotics possibly due to environmental factors and/or different levels of gene expression.

An efficient treatment of infections requires detailed information about antibiotic sensitivity profile of the pathogen(s) and potential antibiotic resistance mechanism involved. The polymerase chain reaction is one such method which helps in understanding the resistance mechanism involved at the genetic level. It is reported that ESBL resistance is often attributed to expression of (*bla*)*SHV*, (*bla*)*TEM* and (*bla*)*CTX-M* genes in Enterobacteriaceae and other Gram-negative bacteria (25). In the present study, PCR amplification products of the *Bacillus* isolates from raw milk show that 18.5% isolates (7/34) carried the *blaSHV* gene, 50% (19/38) carried *blaTEM* gene and 35% (12/38) isolates possessed *blaCTX-M* gene. In *Bacillus* isolates from pasteurized milk samples, the expression profile of *blaSHV*, *blaTEM*, and *blaCTX-M* genes were found to be 18% (4/22), 37% (8/22) and 45% (10/22) respectively. The outcome of present study reflects a growing trend of resistance towards beta-lactams due to wide spread expression of ESBL genes in *B. cereus* species.

Conclusion

Findings of the present study demonstrate that pasteurization does not necessarily remove contamination of *B. cereus* from the raw milk and better sterilization techniques are required to completely eliminate the chances of *Bacillus* contamination. This will insure that public health hazards associated with *Bacillus* contamination can be avoided completely. Moreover, the findings of the present study reveal that antibiotic resistance is rising in *B. cereus* isolates and ESBL genes are one key factor in conferring antibiotic resistance to the *Bacillus* species. The World Health Organization (WHO) has declared antibiotic resistance as the biggest threat to global health, food security and development due to rising incidences of antimicrobial resistance in common pathogens and its huge ramifications on global health (26). The present study confirms the rising trend of antibiotic resistance in *B. cereus* and advocates a prudent use of antibiotics for treatment of *B. cereus* associated disorder to

avoid/stop further emergence of resistance toward commonly used antibiotics.

Conflict of interest

No conflicts of interest were disclosed.

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