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Research Article

Determination of Biofilm Formation, Antibacterial Resistance and Genotypes of *Bacillus cereus* Isolates from Raw Milk

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Abstract: *Bacillus cereus* is a foodborne pathogen that has a widespread presence in the environment and frequently found in foods especially in dairy products. Raw milk contaminated with *B. cereus* could be the cause of its widespreadness in the environment. In this study, it was aimed to determine the genotypes, biofilm formation, antimicrobial susceptibilities, and antibiotypes of *B. cereus* isolates from raw milk. For this aim, *B. cereus* isolated and identified from 10 of 250 raw milk samples were investigated. Biofilm forming abilities were determined *in vitro* by Congo Red Agar Method. Kirby Bauer Disc Diffusion Method was used for determining the antibiotic susceptibilities of the isolates. According to the antibiotic susceptibility results, quantitative antibiotyping was implemented. Genotyping of the isolates were performed by RAPD-PCR. Biofilm formation was determined in 40% of the isolates. The resistances against amoxicillin-clavulanic acid, gentamicin, erythromycin, vancomycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole were determined in 100%, 0%, 30%, 0%, 0%, 0%, and 50% of the isolates, respectively. In the quantitative antibiotyping, the isolates showed similarity between 0.75 to 1.00. The phylogenetic similarities were calculated between 29% to 82%. In conclusion, raw milks might threat the public health because of having potential of containing the antibiotic resistant *B. cereus*.

Keywords: *Antibacterial resistance, B. cereus, Biofilm, Genotyping, Raw milk*

Çiğ Süt Kökenli *Bacillus cereus* İzolatlarının Biyofilm Oluşturma, Antibakteriyel Direnç ve Genotiplerinin Belirlenmesi

Öz: *Bacillus cereus*, doğal ortamlarda yaygın olarak bulunan, gıdalarda, özellikle süt ürünlerinde sıklıkla bulunan, gıda kaynaklı patojendir. Çiğ süt, çevrede yaygın olarak bulunmasından dolayı *B. cereus* ile kolaylıkla kontamine olmaktadır. Bu çalışmada çiğ sütten izole edilen *B. cereus* izolatlarının genotip, biyofilm, antimikrobiyal duyarlılıkları ve antibiyotiplerinin belirlenmesi amaçlandı. Bu amaçla 250 adet çiğ süt örneğinden izole ve identifiye edilen 10 adet *B. cereus* izolatı incelendi. Biyofilm oluşturma yetenekleri Kongo Red Agar Metodu ile belirlendi. İzolatların antibiyotik duyarlılıklarının belirlenmesinde Kirby Bauer Disk Difüzyon testi kullanıldı. Antibiyotik duyarlılık sonuçlarına göre kantitatif antibiyotiplendirme gerçekleştirildi. İzolatların genotiplendirilmesi RAPD-PCR ile yapıldı. Suşların %40'ında biyofilm oluşumu saptandı. İzolatların amoksisilin-klavulanik asit, gentamisin, eritromisin, vankomisin, kloramfenikol, tetrasiklin, trimetoprim-sülfametoksazol dirençleri sırasıyla %100, %0, %30, %0, %0, %0 ve %50 olarak belirlendi. Kantitatif antibiyotiplendirme sonucunda izolatlar 0,75 ile 1,00 arasında benzerlik gösterdi. Filogenetik benzerlikler %29 ile %82 arasında hesaplandı. Sonuç olarak, çiğ sütler antibiyotiğe dirençli *B. cereus* içerme potansiyeline sahip olduğundan halk sağlığını tehdit edebilir bulunmuştur.

Anahtar sözcükler: *Antibakteriyel direnç, B. cereus, Biofilm, Çiğ süt, Genotiplendirme*

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Introduction

Composition of raw milk is a key point that influence the quality of milk and milk products. Microbiological ingredients play a major role in spoilage of raw milk. Some of the microorganisms, such as *Bacillus cereus,* affect on the safety and quality of raw milk [1]. *B. cereus* is a foodborne pathogen spread in environment and found in foods especially in dairy products [2]. The contamination of *B. cereus* has a higher rate than the other foodborne pathogens and its growth resulted to various dairy defects [3,4]. *B. cereus* remains a problem for dairy products in terms of shelf life and public health safety. Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) announced that *B. cereus* is the third agent reason of communal foodborne infections in Europe. In 2016, European Union announced that 5.5% of the outbreaks by foodborne pathogens are related to *Bacillus* [5]. The goal of dairy producers is to supply good quality products, but psychrotrophic bacteria in raw milk complicates this goal.

Microorganisms in milk have the ability like adhering and aggregating on stainless steel surfaces, resulted by biofilm formation in storage tanks and process surfaces. Adhesive biofilms formed by *B. cereus* can provide a source of contamination during production and processing. Biofilm formation increases the risk of cross-contamination by negatively affecting shelf life and reliability of dairy products. The structure of the biofilm also increases the resistance to immune system and antimicrobial agents as well as causes mechanical damage [6]. *B. cereus* can adhere to a wide variety of materials used in food processes to form biofilms. Biofilms often cannot be removed during Cleaning in Places (CIP) procedures. This makes *B. cereus* a foodborne pathogen causing deterioration of food quality and health hazards. *Bacillus* spp. can form resistant biofilms. This can result in continued contamination and is a significant risk for food quality and safety [7].

It is important not only to indicate the presence of *B. cereus* in foods, but also to detect disease-causing factors. Except beta-lactam antibiotics, most of the *B. cereus* isolates are susceptible to commonly used antimicrobial agents [8]. Antibiotic-resistant *B. cereus* strains are the cause of horizontal gene transfer [4]. *B. cereus* can also cause problems in the dairy industry due to its resistance to disinfectants. Establishing a *B. cereus* antibiotic resistance profile is important for public health [9,10]. Reports announced that *B. cereus* isolated from different foods are resistant to many antibiotics like ceftriaxone, tetracycline, streptomycin, trimethoprim, ampicillin, and penicillin. It is important to determine the resistance of foodborne *B. cereus* to antibiotics in order to better manage infectious diseases [11]. Mobile genetic elements lead to the spread of antibiotic resistance between *Bacillus* and the other pathogens through horizontal gene transfer [12]. Investigation of antibiotic resistance of *B. cereus* is important for food safety and public health.

Genome analyzes reveal that *B. cereus* was activated in protein metabolism, suggesting the adaptation of *B. cereus* to a symbiotic or parasitic life cycle. Evaluation of the relationships of microbial changes through chemical analyzes or analytical technologies should allow for the identification of the metabolic activity of the active degradation microbiota [13]. Methods for genotyping of *B. cereus* have been reported as multiple-locus variable-number tandem repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), repetitive element palindromic-PCR (rep-PCR), and randomly amplified polymorphic DNA PCR (RAPD-PCR) [9]. Molecular biology methods determine the specific genes of *B. cereus* and it is time-saving and highly specific, but have difficulties like equipment and personnel needs and in achieving constant temperature sensing. Methods based on PCR like RAPD-PCR are more common in use [14]. The VITEK2 BCL card method has made significant progress in the reliable identification of *Bacillus* spp. and related genera [15]. This method is an automatic microbial identification system that provides accurate and reproducible results, and is also a fast and reliable application for pathogen identification. The VITEK2 method is advantageous over PCR [16,17].

The aim of this study was to determine the biofilm formation, antimicrobial resistance, genotypes and antibiotypes of *B. cereus* strains isolated from raw milk.

Material and Methods

Isolation and Identification of *B. cereus*

Raw milk samples (n=250) from İzmir, Türkiye were brought to Bornova Veterinary Control Institute, Bacteriology Laboratory under cold chain conditions.

The raw milk samples were plated onto Columbia Agar (5% sheep blood, Liofilchem) and incubated at 37°C for 24-48 h. After the incubation period, colonies with strong β-hemolytic activity were applied Gram staining [18]. Gram-positive bacilli colonies were purified and then identified with the BCL ready card on the VITEK 2 (bioMérieux) instrument [16].

The identifications of the isolates were confirmed by PCR. The specific *motB* gene targeting PCR for the identification of *B. cereus* was carried out with the protocol as reported [19]. The presence of a 575 bp band after imaging was considered positive for *B. cereus*.

Determination of Biofilm Formation

Biofilm formation of isolates was detected *in vitro* by Congo Red Agar (CRA) method. CRA method was carried

out with the reported method [20]. By taking a single colony from the pure colonies in Trypticase Soy Agar (TSA), it was inoculated to CRA, and incubated at 37°C for 24-48 h under aerobic conditions. After incubation, color changes were observed. Isolates forming black-gray colony on CRA were determined as positive for the biofilm production and the pink-red colonies were determined as negative.

Determination of Antimicrobial Resistance and Antibiotyping

Antimicrobial resistances of *B. cereus* isolates were tested by Kirby-Bauer Disc Diffusion Method. A bacterial suspension was prepared from fresh cultures of the isolates in Physiological Buffer Solution (PBS) with a density of 0.5 McFarland. The prepared suspension (100 µL) was inoculated on Mueller Hinton Agar (MHA) surface. The 7 antibiotics including chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (30 µg), vancomycin (5 µg), trimethoprim-sulfamethoxazole (25 µg), amoxicillin-clavulanic acid (20 µg/10 µg) and tetracycline (30 µg) were selected. Antibiotic discs were placed on the medium and incubated at 37°C for 24 h. Zone diameters formed after incubation period were measured and evaluated according to Clinical and Laboratory Standards Institute (CLSI) 2020 (for chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole, tetracycline), CLSI 2012 (for amoxicillin-clavulanic acid) and to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023 (for erythromycin, vancomycin) guidelines. The interpretive categories and zone diameter breakpoints of the antibiotics (except erythromycin and vancomycin) against *S. aureus* ATCC 25923 were used for testing resistances of the isolates according to CLSI guidelines. For erythromycin and vancomycin breakpoints against *Bacillus spp*., EUCAST guideline was used [10,21-23].

The antibiotyping was performed according to the resistance profiles of the strains by means of the Unweighted Pair Group Method using arithmetic averages (UPGMA) cluster analysis, and the dendrogram was created for evaluation of relatedness between the strains [24,25]. For this method, the antibiotic resistance patterns were recorded to a table as susceptible (S), intermediate resistant (I) or resistant (R) for each antibiotics tested. This table was converted to a figure showing bands like a genotyping pattern. Then, this figure was analyzed in commercial band analyzes software (e.g. Quantitity One, BioRad). The phylophenotypic tree were drawn with using the software depending on the antibiotic resistance profiles.

Genotyping of *Bacillus cereus*

The ERIC-2 (Enterobacterial Repetitive Intergenic Consensus-2) primer (5'-AAG TAA GTG ACT GGG GTG AGC G-3') was used to evaluate RAPD-PCR patterns of *B. cereus* isolates. For PCR, a 25 µl RAPD master mix containing 1X PCR Buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 2.5 U *Taq* DNA polymerase, 25 pmol primer and 5 µL template DNA was prepared. This mixture was pre-denatured for 5 min at 94°C followed at 94°C by 1 min denaturation, at 40°C 1 min bonding, at 72°C 3 min elongation at 40 cyclus and for 7 min at 72°C were subjected to amplification at final elongation conditions. Amplification products were visualized by UV transilluminator with 1.5% agarose gel electrophoresis containing ethidium bromide (2 µg/mL) [26]. The UPGMA clustering method was used to generate dendrograms of RAPD patterns by using image analysis program. Genetic relationship between the isolates was also evaluated by considering the 70% similarity coefficient.

Results

Isolation and Identification of *B. cereus*

The Gram-positive bacilli colonies were purified after incubation period, and identified with BCL ready card on VITEK 2 (bioMérieux) instrument. The identifications of the isolates were confirmed by PCR, and all 10 isolates gave specific bands of 575 bp for B. cereus *(Fig. 1)*. *B. cereus* was isolated and identified from 10 of 250 raw milk samples at the rate of 4%.

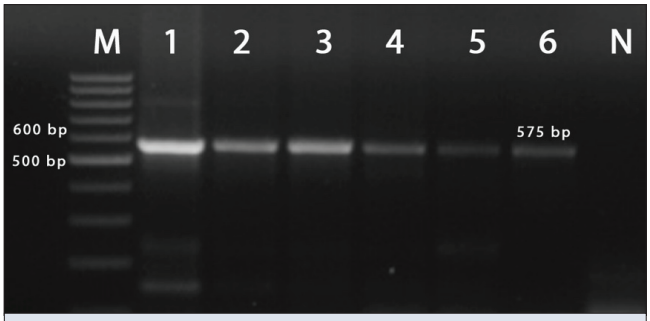

Fig 1. Confirmation of identifications by PCR. M: marker, 1-6: *B. cereus* isolates, N: negative control *(Escherichia coli)*

Determination of Biofilm Formation

Biofilm formations of *B. cereus* isolates (n=10) were evaluated with CRA method. It was determined that 4 of 10 *B. cereus* isolates at the percentage of 40% had biofilm activity.

Determination of Antimicrobial Resistance and Antibiotyping

B. cereus isolates were tested for antimicrobial resistance profiles against 7 selected antibiotics. All the isolates (n=10) were susceptible to chloramphenicol, gentamicin,

Table 1. *Antibiotic resistance/susceptibility of 10 Bacillus cereus isolates in this study*

Antibiotic	Susceptible*		Intermediate**		Resistant***	
	n	%	n	%	n	%
Amoxicillin-Clavulanic acid	0	0	0	0	10	100
Gentamicin	10	100	0	0	0	0
Erythromycin	7	70	0	0	3	30
Vancomycin	10	100	0	0	0	0
Chloramphenicol	10	100	0	0	0	0
Tetracycline	10	100	0	0	0	0
Trimethoprim-sulfamethoxazole	2	20	3	30	5	50

**Susceptible: indicates the diameter of inhibition zone (DIZ) against B. cereus strain was larger than the quality control strain; **Intermediate: indicates the DIZ against B. cereus strain was between SUS and RES; ***Resistant: indicates the DIZ against B. cereus strain was less than the quality control strain*

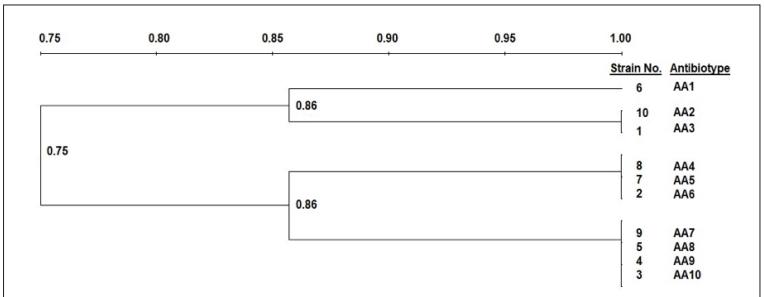

Fig 2. Dendrogram of 10 *Bacillus cereus* isolates according to their antibiotic susceptibility profiles

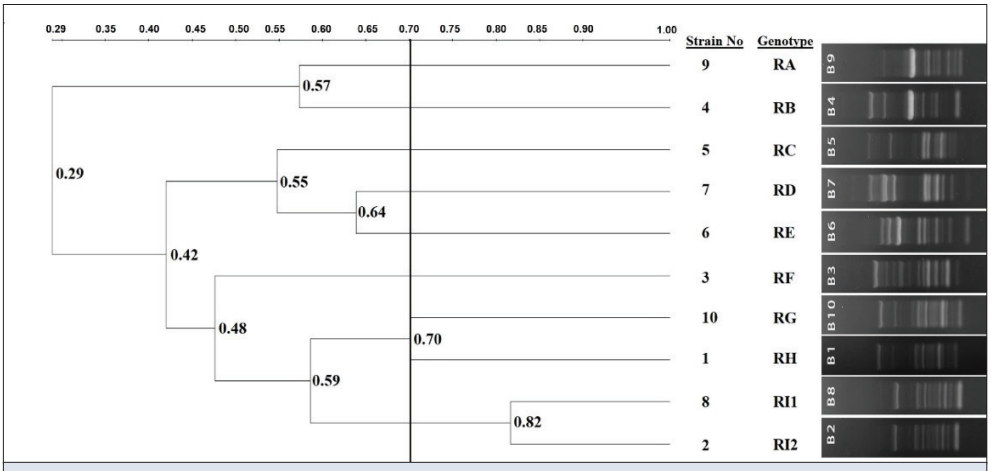

Fig 3. Dendrogram of 10 *Bacillus cereus* isolates after phylogenetic analysis for genotyping

tetracycline and vancomycin as well as resistant to amoxicillin-clavulanic acid *(Table 1)*. After determining antibiotic resistance/susceptibility status of the isolates, their phylophenotypicv similarities were determined by considering the resistance to the relevant antibiotics. The phylophenotypicv similarities of *B. cereus* isolates were calculated and they showed 75-100% similarity *(Fig. 2)*. The phylophenotypic relationship between isolates was evaluated by considering the 70% similarity coefficient. As the result of the evaluation, it was determined that isolates had 1 multiple antibiotype (AA).

Genotyping of *Bacillus cereus*

The phylogenetic analysis of *B. cereus* isolates by RAPD-PCR for genotyping was determined that the isolates showed similarity between 29-82% *(Fig. 3)*. Genetic relationships among isolates were evaluated by considering the 70% similarity coefficient. It was determined that

isolates had 8 single genotypes (RA-RH) and 1 multiple genotype (RI). It was observed that 2 isolates in the RI multiple genotypes showed 82% similarity.

Discussion

B. cereus has long been a health threat to human and animals, also have a significant impact on the food industry and agriculture [2,27]. *B. cereus* can cause hygiene problems and economic losses due to the deterioration of dairy products and sticking on process equipment [3]. *B. cereus* can weaken the bactericidal effect of disinfectants [10]. *B. cereus* has a higher contamination ability than other foodborne pathogens [4]. *B. cereus* isolates from pasteurized milk placed in same cluster, indicates that they came from a similar source, on the other hand raw milk isolates varied at a level showing different sources [28]. The studies can determine the distribution and genetic diversity of *B. cereus* strains found in raw milk and can provide a theoretical basis for controlling potential harms of this pathogen in dairy and dairy products [29]. In this study, *B. cereus* was detected in 10 of 250 raw milk samples at the rate of 4%. Compared to previous studies, it is the lowest contamination level of raw milk samples compared with 6.66% in a cheese plant at Alexandria [30], 9.8% in dairy farms in China [31], 23.3% in 60 raw milk samples [1], 26% in China [4], 29.5% in milk samples from the dairy animals [32], 33.3% in Guangxi, Yunnan, and Guizhou, the provinces of China [10], 37.5% in household milk from dairy environments [33], 40% in Pakistan [34], 40% in Zagazig city [35], 47% in Ghana [36], and 85% in Egypt [37].

In our research, it was determined that 4 of the 10 *B. cereus* isolates at the percentage of 40% had biofilm activity. Compared to previous studies, it is lower compared with the isolates in Victoria, Australia those had 53.7% biofilm forming ability [28]. In a previous study, they measured the ability to form biofilms of 5 groups of *B. cereus* with 41 ST (sequence type) on stainless-steel tubes [10]. In another study, all the isolates of *B. cereus* bacteria were selected to verify biofilm formation ability in microtiter plates and results showed that all isolates (100%) could form biofilm. Data highlights that dairy industry needs to reinforce control in the initial quality of raw material and in CIP cleaning applications [38].

In this study, it was determined that all isolates were susceptible to chloramphenicol, gentamicin, tetracycline and vancomycin as well as resistant to amoxicillin-clavulanic acid. Compared to a previous study, *B. cereus* isolates had resistance to amoxicillin at the rate of 80% whereas our rate was 100%. The isolates were susceptible to erythromycin, vancomycin at the rates of 100%, 93.33% [35] respectively, whereas our rates were respectively 70% and 100%. In another study, *B. cereus* isolates showed resistance to amoxicillin and tetracycline with 68.9% and 51.1% [33] whereas our rates were 100% and 0%, respectively. The study in Ghana presented that, *B. cereus* isolates were resistant to amoxicillin at the rate of 100% that was same in our study. The isolates susceptible to gentamicin and chloramphenicol at the rates 100% and 99% [36] whereas similar to our study 100% and 100%, respectively. The study in Southwestern China introduced that all isolates were susceptible to gentamicin and chloramphenicol [10] same as in our study. The antibiotic susceptibility of 54 *B. cereus* isolates to 17 antibiotics was tested and all isolates were determined susceptible to chloramphenicol and gentamicin [4] same as our study. Isolates were susceptible to tetracycline, trimethoprim-sulfamethoxazole, erythromycin at the rates of 98.15%, 85.18% and 83.33% [4] whereas 100%, 20% and 70% in our study, respectively. Accept trimethoprim-sulfamethoxazole, the other rates are quite similar with our study. In another study, *B. cereus* isolates were susceptible to gentamicin at the rate of 100% as same in our study [39].

The phylogenetic analysis of *B. cereus* isolates determined that the isolates showed similarity between 29-82%. It was determined that the isolates had 8 single genotypes (RA-RH) and 1 multiple genotype (RI). It was observed that 2 isolates in the RI multiple genotype showed 82% similarity. A previous study determined that the 96 *B. cereus* strains containing 41 ST (sequence type) were divided into 5 clusters using 90% similarity for the critical threshold [10]. Another study presented that 56 *B. cereus* strains were detected from 300 environmental samples and 50 raw milk samples divided into 18 sequence types (STs) using multilocus sequence typing method. The results could reveal the distribution and genetic diversity of *B. cereus* strains in raw milk and cattle farm environments, and provide a theoretical basis for controlling the potential harm of these pathogenic bacteria in dairy products [29]. It was presented that 54 strains of *B. cereus* isolates were divided into 24 ST in raw milk samples. An elaborated phylogenetic relationship of the 54 *B. cereus* strains was clustered into 3 groups. The results showed no obvious association between *B. cereus* genotype and collection regions [4]. A previous study introduced that 14 sequence types of *B. cereus* were found in raw bovine milk samples and all isolates in the research and 13 selected reference strains showed phylogenetic relationship [40].

As the biofilm formation ability positive strains (no. 3,6,7,9) were evaluated, it was determined that the phylophenotypic similarities of antibiotic resistance/susceptibility of the strains 3 and 9 showed 100% similarity while their genetic relationship showed 29% similarity. The strains 6 and 7 showed 64% genetic similarity while their phylophenotypic similarity of antibiotic resistance/susceptibility was determined as 75% similarity. The strains 2 and 8 which were biofilm formation ability

negative, showed the highest genetic similarity rate as 82% while their phylophenotypic similarity of antibiotic resistance/susceptibility was 100%.

In conclusion, the widespread presence in the natural environment, high biofilm formation ability, resistance to heat treatment and antibiotics make *B. cereus* an important bacterium to follow in terms of public health. *B. cereus* shows a wide diversity from raw milk to the final product. In order to reduce the risks of this foodborne pathogen in terms of raw milk, dairy products and public health, it is very important to implement sanitation rules at all stages from raw milk to the final product. Determination of critical control points such as transport tanks, storage tanks, production equipment, in places and personnel from the raw milk stage to the final product stage and the implementation of sanitation practices at these points is highly important against *B. cereus* contamination.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (S. Savaşan) on reasonable request.

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Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee Funding Support.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SS, ÇN, VEE and SS planned, designed and performed the analysis. The manuscript was written by SS and SS. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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