Turk J. Pharm. Sci. 6 (3), 231-238, 2009

Short communication

EVALUATION OF ANTIBIOTIC RESISTANCE OF *Bacillus cereus* ISOLATES IN ICE-CREAM SAMPLES SOLD IN ANKARA

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Abstract

In this study a total of 34 Bacillus cereus isolated from ice-cream samples in different pastry shops in Ankara, Turkey were evaluated for antibiotic resistance, *which were collected from 34 samples of ice-cream. The isolates were identified by morphological, biochemical tests and confirmed by API 50 CHB. The susceptibility of 34 B. cereus to ampicillin (AMP), penicillin (P), tetracycline (TE), trimethoprim-sulphamethoxazole, erythromycin (E), ciprofloxacin (CIP), gentamicin (GM), and vancomycin (VA) were determined by both microdilution and disk diffusion methods. Among 34 B. cereus from isolated ice-cream samples no resistance were found to vancomycine,, gentamicin and ciprofloxacin, while they were found resistant to ampicillin (29.5%), penicillin (29.5%), and for trimethoprim-sulphamethoxazole (12%).. To tetracycline, another antibiotic investigated in this study, 6% resistance with microdilution method and 3% resistance with disc diffusion method was determined. To erythromycin, 3% resistance was determined with only disc diffusion method.*

Key words*: Bacillus, Identification, Resistance.*

Ankara Piyasasından Sağlanan Dondurma Örneklerinden İzole Edilen *Bacillus cereus* izolatlarında Antibiyotik Direncinin Değerlendirilmesi

Bu çalışmada Ankara ilinde satışa sunulan çeşitli pastanelerden toplanan 34 dondurma örneğinden izole edilen toplam 34 Bacillus cereus izolatlarının antibiyotik direnci araştırıldı. İzolatlar morfolojik, biyokimyasal testlerle tanımlandı ve API 50 CHB ile doğrulandı. Ampisilin (AMP), penisilin (P), tetrasiklin (TE), trimethoprim-sulphamethoxazole, eritromisin (E), siprofloksazin (CIP), gentamisin (GM), ve vankomisin (VA) karşı sıvı mikrodilüsyon ve disk difüzyon yöntemi ile 34 B. cereus izolatının duyarlılığı belirlendi. Dondurma örneklerinden izole edilen 34 B. cereus izolatı arasında vankomisine, gentamisine, ve siprofloksazine direnç bulunmazken ampisiline (% 29.5), penisiline (% 29.5), ve trimethoprim-sufametoksazole (% 12) dirençli bulundu. Çalışmada kullanılan diğer antibiyotiklerden tetrasiklin mikrodilüsyon yöntemi ile % 6, ve disk difüzyon yöntemi ile % 3. Eritromisine ise sadece disk difüzyon yönteminde % 3 oranında direnç bulundu.

Anahtar kelimeler*: Bacillus, İdentifikasyon, Direnç.*

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INTRODUCTION

The use of antimicrobials in both animals and humans can select for resistant bacterial populations. In food animals, antimicrobials are used for the control and treatment of bacterial associated infectious diseases as well as for growth promotion purposes (1), apart from the European Union (EU) ban of certain antibiotics that are used, or related to those used, in human medicine (European Commission; EC) (2). An undesired consequence of antimicrobial use in animals is the potential development of antimicrobial-resistant zootonic foodborne bacterial pathogens and subsequent transmission to human as food contaminants (EFSA) (3). In addition, spontaneous mutation in foodborne bacteria or the spread of resistant bacteria in the absence of selective pressure may also contribute to the antimicrobial resistance burden in food (EFSA) (4). In recent years, research has documented high level of antimicrobial resistance of *Bacillus* spp. isolated from food animals and in the environment (5). *Bacillus cereus* has a broad range of foods associated with infection including, cooked vegetables and meats boiled or fried rice, vanilla sauce, custards, soups, ice cream, herbs and spices, which are known that strains of *B. cereus* are able to grow at 5 or 7°C and could be of concern in refrigerated, pasteurized foods (6-10). *B. cereus* is important as it affects the shelf life of pasteurized milk and milk products. The organism is associated with defects as of flavours, sweet curdling and bitty cream by proteinase, lipase and phospholipase enzymes produced. They can grow over a wide range of temperature, pH and water activity. Its psychrotrophic properties enable *B. cereus* to grow and produce toxins in ice-cream at refrigeration temperatures (11). *B. cereus* has a high incidence in dairy products. As reported by Ahmet et al (7), 9% raw milk, 35% of pasteurized milk, 14% of cheese and 48% of ice-cream samples were contaminated with *B. cereus*; conversely, no fermented milk was found to be contaminated. Also, it is reported that ice-creams, soft ice-creams, milk powders, fermented milks, pasteurized milks were found to be contamined with *B. cereus*.

B. cereus is responsible for the majority of foodborne illnesses attributed to *Bacillus*. In addition to causing these effects in dairy products, *B. cereus* is recognized as causing diarrheal and emetic syndromes of food-poisoning outbreaks. *B. cereus* was linked to 14 outbreaks and caused 691 reported cases of foodborne illness in the United States. Both syndromes have occasionally been associated with dairy products. Although formerly considered an apathogenic species or a facultative pathogen, *B. cereus* has been ever more often isolated as an etiological agent in serious brain infections and in infections of patients with neutropenia and with carcinoma. The production of β-lactamases is one of potential virulence factors that make the producing strains resistant even to the 3rd generation of cephalosporin's (6, 8-10).

The purpose of this study is to determine antibiotic resistances agents' *B. cereus* isolated from ice-cream samples in different pastry shops of Ankara (Turkey) by microdilution and disc diffusion methods and evaluated the results with compare the two tests as percentage results for all tested antibiotics.

EXPERIMENTAL

Sampling

34 samples of ice-cream were collected under sterile conditions and purchased from retail outlet in different pastry shops of Ankara (Turkey) (12). The ice-cream samples were transported to the laboratory under cold chain and analyzed within 2h of sampling.

Ten ml of ice-cream were diluted with 90 ml of sterile saline in a conical flask with glass beads. 0.1 ml of diluted sample was surface–plated in duplicate on mannitol, egg yolk, and polymyxin (MYP agar). Plates were incubated at 30°C for 24h and examined for typical colonies. From each plate presumptive colonies (pink colonies surrounded by a zone precipitation) were selected onto blood agar, and identified using the API 50 CHB (13).

Microbiological analyses

Inoculum preparation, microdilution and disc diffusion method

Each isolate grown overnight on MYP agar at 37°C, was suspended in Mueller Hinton Broth (MHB; Merck) medium and vortexed thoroughly to achieve a homogen suspension. Turbidity was adjusted to the density of 0.5 McFarland macroscopically. This suspension (10^8 CFU/mL) was used for each method of susceptibility testing (13).

Microdilution method standard antibacterial powders of ampicillin (AMP; Wyeth), tetracycline (TE; Sigma), penicillin (P; Faco), trimethoprim-sulphamethoxazole (TMP-SMX; Roche), erythromycin (E; Faco), ciprofloxacin (CIP; Bayer), gentamicin (GM; Faco), vancomycin (VA; Lilly) were obtained from their respective manufacturers. The stock solutions of agents were dissolved in dimethylsulphoxide (DMSO; Merck) (tetracycline, vancomycin), 95% ethanol (erythromycin) and water (penicillin, trimethoprim-sulphamethoxazole, ciprofloxacin, gentamicin), pH: 8 phosphate buffer (ampicillin).

Microdilution technique was employed for the determination of MIC values with microplates 96-well Falcon^R (USA) microplates. Brinkman transferpette^R (Germany) was used for the two-fold dilution of the compound in the wells. The solutions of compounds were prepared at 128-0.063μg mL^{-1} concentrations in the wells of microplates by diluting with media. The microorganism suspensions used for inoculation were prepared at 10^5 CFU/mL by diluting fresh cultures at the density of McFarland 0.5 (10^8 CFU/mL). Suspensions of the microorganisms were inoculated to the two-fold diluted solution of the compound. Mueller-Hinton Broth (Oxoid) was used for diluting the microorganisms' suspension and for two-fold dilution of the compounds. The lowest concentration of the compounds that completely inhibits macroscopic growth was determined as Minimum Inhibitory Concentrations (MICs) were reported (8).

Cultures were analyzed for antimicrobial resistance using the disc diffusion assay recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) (14) on Mueller Hinton agar plates (Oxoid, CM 337). For testing *B. cereus*, Mueller Hinton agar plates contained 5% (v/v) sheep blood and were incubated under 37°C for 24h. Briefly, fresh bacterial colonies were inoculated in 0.8% NaCl suspension to a turbidity equivalent to a 0.5 McFarland standard. With a sterile cotton swab the culture was swabbed on the agar plate and standard discs (ampicillin 10μg, Oxoid), penicillin (10U, Oxoid), tetracycline (30μg, Oxoid), trimethoprim-sulphamethoxazole (23.75mcg SMX, 1.25mcg TMP, Oxoid), erythromycin (15μg, Oxoid), ciprofloxacin (5μg, Oxoid), gentamicin (10μg, Oxoid), and vancomycin (30μg, Oxoid) were applied using a disc dispenser. After incubation the diameter of the inhibition zone was determined according to the CLSI guidelines for aerobically grown bacteria.

Quality control (QC) was ensured by CLSI recommended strain *B. subtilis* ATCC 6633, and tested according to the guidelines of CLSI for microdilution/and disc diffusion method for AMP, TE, P, TMP-SMX, E, CIP, GM, and VA. Dimethylsulphoxide, pure microorganisms and pure media were used as control wells (15-17).

RESULTS AND DISCUSSION

Bacteria resistant to antimicrobial drugs, which spread into the human population from foods of animal origin, rank as the direct causative agents of foodborne diseases and represent a possible source of drug resistance of human pathogenic agents (16). Since the first description of foodborne illness from *Bacillus cereus* in the 1950s, this microorganism has received much attention. Nonetheless, outbreaks have been described in Denmark as well as in other countries. In the Netherlands and in England, *B. cereus* has been reported to be the causative organism of approximately 2% of the outbreaks of known origin. In France, the reported frequency of *B. cereus* outbreaks was 4–5%, and in the United States, 1–2% of the outbreaks have been attributed to *B. cereus* (19-22).

Table 1. Distribution of susceptibility of antimicrobial agents against isolated of 34 *Bacillus cereus* in disc diffusion and microdilution methods.

		MICs	Disc (mm)	*S
AMP	S	(B_fS ≤4) n=24 (70.5%)	(B_fS ≥15) n=24 (70.5%)	100%
	R	(B_fR≥32) n=10 (29.5%)	(B_fR ≤11) n=10 (29.5%)	
TE	S	(B_fS ≤4) n=32 (94%)	(B_fS ≤14) n= 33 (97%)	94%
	R	(B_fR ≥16) n=2 (6%)	(B_fR ≥19) n=1 (3%)	
P	S	(B_fS ≤0.1) n=24 (70.5%)	(B_fS ≤28) n=24 (70.5%)	100%
	R	(B_fR ≥32) n=10 (29.5%)	(B_fR ≥29) n=10 (29.5%)	
TMP-SMX	S	(B_fS ≤2) n=30 (88%)	(B_fS ≤ 11) n=30 (88%)	100%
	R	(B_fR ≥8) n=4 (12%)	(B_fR ≥15) n=4 (12%)	
E	S	(B_fS ≤0.5) n=34 (100%)	(B_fS ≤ 13) n=33 (97%)	97%
	R	(B_fR ≥8) n=0 (0%)	(B_fR ≥23) n=1 (3%)	
CIP	S	(B_fS ≤1) n=34 (100%)	(B_fS ≥21) n=34 (100%)	100%
	R	(B_fR >0.12) n=0 (0%)	(B_fR ≤15) n=0 (0%)	
GM	S	(B_fS ≤4) n=34 (100%)	(B_fS ≥12) n=34 (100%)	100%
	R	(B_fR >4) n=0 (0%)	(B_fR ≤15) n=0 (0%)	
VA	S	(B_fS ≤4) n=34 (100%)	(B_fS ≥15) n=34 (100%)	100%
	R	(B_fR >4) n=0 (0%)	(B_fR ≥17) n=0 (0%)	

S: Susceptibility rates of disk diffusion and microdilution methods of *Bacilli*, B_fR: Breakpoints for resistance, B_fS: Breakpoints for susceptibility, n: number of sample, R: Resistant, S: Sensitive, AMP: ampicillin, TE: tetracycline, P: penicillin, TMP-SMX: trimethoprim-sulphamethoxazole, E: erythromycin, CIP: ciprofloxacin, GM: gentamicin, VA: vancomycine

As it is shown in Table 1, all of the *B. cereus* isolates were found susceptible to vancomycin, gentamicin, and ciprofloxacin in both of broth microdilution and agar diffusion tests and similar results have been reported by other authors (12, 23). In our study, for ampicillin and penicillin, 29.5% (10/34) and for trimethoprim-sulphamethoxazole, 12% (4/34) of *B. cereus* isolates were observed resistant in both of disc diffusion and broth microdilution methods. Only in disc diffusion methods, one isolate was resistant to erythromycin (12%). In addition to that a 6 and a 3% of resistance to tetracycline have been found in *B. cereus* isolates from microdilution and from disc diffusion test methods. Control strains (*B. subtilis* ATCC 6633) was shown between the breakpoint according to the CLSI for AMP (MIC: 0.12 μg/ml; 30mm), TE (MIC: 0.12 μg/ml; 22mm), P (MIC; 0.12 μg/ml; 30mm), TMP-SMX (MIC: 0.12 μg/ml; 30mm), E (MIC: 0.25 μg/ml; 29mm), CIP (MIC: 0.25μg/ml; 25mm), GM (MIC: 0.5 μg/ml; 23mm), and VA (MIC: 0.12 μg/ml; 30mm) respectively.

Similarly, Rusul et al (24) indicated that *B. cereus* isolated from different food samples were susceptible to gentamicin and erythromycin which are used therapeutically in humans. In another study that was also conducted by Whong and Kwaga (25), strains of *B. cereus* isolated from some Nigerian foods were resistant to many antimicrobials. They found that all *B. cereus* were susceptible to ciprofloxacin (100%), chloramphenicol (100%), gentamicin (99%) but resistant to ampicillin (44 %) and to penicillin (80%). Jensen et al (24) and Guven et al (27) investigated the antimicrobial resistance among *B. cereus* group isolates from Danish agricultural soil and Turkish meat and meat products and obtained similar susceptibly to vancomycin, ciprofloxacin and gentamicin.

In this study, for ampicillin and penicillin, 29.5% (10/34) and for trimethoprim-sulphamethoxazole, 12% (4/34) of *B. cereus* isolates were resistant both disc diffusion and broth microdilution methods. Only in disc diffusion methods, of the *B. cereus* isolates, 3% (1/34) were resistant to erythromycin (Table 1). The presence of *B. cereus* strains resistant to β-lactam antimicrobial agents and sporadically to erythromycin and tetracycline suggests that ice-cream may be, under certain conditions, vectors of resistance to antimicrobial agents via *B. cereus.* It is previously reported that isolated *B. cereus* strains from milk were resistant to ampicillin with a relatively high rate of resistance to cotrimoxazole and sulphamethoxazole (28). In another study it was shown that strains of *B. cereus* were resistant to penicillins and cephalosporins, while they were susceptible to vancomycin and erythromycin in clinic isolates (29). Rosenquist et al (30) have reported penicillin resistance in *B. cereus* like organisms derived from ready-to-eat foods.

As a consequence of the continuous introduction of *B. cereus* spores into the dairy products, especially ice-cream, effective cleaning and disinfection procedures are important to avoid build up of high levels of *B. cereus*. The results of this study indicate that *B. cereus* could be a significant etiological agent of food poisoning.

In conclusion, public health risk of antimicrobial resistance in bacteria as a result of the use of antibiotics in animals for prophylactic and growth promoter agent. Antibiotic resistance associated with animal origin has been a global concern. This is the first report showing that *B. cereus* contamination and antibiotic resistance in ice-cream samples in Turkey. Therefore the Turkish regulation which is in compliance with the European Union (EU) regulation for the use of antibiotics in animals for prophylactic and growth promoter agent should apply in national area.

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Received: 26.02.2009
Accepted: 13.05.2009