DETERMINATION OF β-LACTAMASE ACTIVITIES AND ANTIBIOTIC SUSCEPTIBILITY OF SOME *BACILLUS* STRAINS CAUSING FOOD POISONING

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SUMMARY

Some *Bacillus* species are important food pathogens. For example, *B. cereus* is an opportunistic pathogen found in raw milk that is a common cause of food poisoning. It is of interest to investigate the virulant profiles of *Bacillus* strains isolated from foods and samples associated with food-poisoning outbreaks. Nineteen *Bacillus* strains were isolated from various milk samples. β-Lactamase enzyme activities of these *Bacillus* strains were evaluated with iodometric and chromogenic cephalosporin (nitrocefin) test methods. Five of 19 *Bacillus* strains isolated were positive for β-lactamase activity. Clavulanate-amoxycillin and cephazolin were chosen to test the antibiotic susceptibilities of the β-lactamase positive and negative *Bacillus* strains. Of the five β-lactamase positive *Bacillus* strains, three were susceptible, and two strains intermediate to clavulanate-amoxycillin; one was susceptible, and four strains were intermediate to cephazolin. None of the β-lactamase positive *Bacillus* strains was resistant to both antibiotics. Of the 14 β-lactamase negative strains, five were susceptible to clavulanate-amoxycillin, four strains were intermediate, and five strains were resistant; three were susceptible,

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one intermediate, and ten β-lactamase negative strains were resistant to cephazolin.

KEY WORDS

Bacillus, inhibitory effect, β-lactamase, antibiotic susceptibility test

INTRODUCTION

Bacillus may contaminate raw milk in different ways. Because these spore-forming bacilli are resistant to heat, they may persist in milk and dairy products even after pasteurization. Contaminated milk and dairy products may lead to food poisoning. There are reports indicating that some of the enzymes secreted by *Bacillus* persist even after pasteurization /1/.

β-Lactamase is one of the enzymes synthesized by bacilli. Bacteria secreting β-lactamase are resistant to penicillin and its derivatives. Thus, β-lactamase enzymes are important in human health. There are pathogenic species amongst the bacilli. *B. cereus* and *B. subtilis* are especially important in terms of public health, because of food poisoning /2/.

β-Lactamases can be classified into three groups, according to whether they are chromosomal enzymes, synthesized by a plasmid mediator, or hydrolyze cephalosporins. The chromosomal enzymes are specific to the bacterial species and include penicillinase. β-Lactamase synthezised in plasmids is peculiar to the plasmid but not to the species, because plasmids can enter easily from one bacterium to another. This group includes carbenicillines and cloxacillines. The third group includes wide spectrum β-lactamases that may hydrolyze cephalosporin and aztreonam /2/.

β-Lactamases are protoplast membrane-bound enzymes in Gram positive bacteria. Membrane-bound β-lactamases were first called γ-penicillinases.

Chand *et al.* /3/ isolated penicillinase enzymes of *Bacillus* species from milk. In this study, it was emphasized that penicillinase production of *Bacillus* strain at pH 6.5-7.0 was very easy.

Nielsen and Lampen identified a third β-lactamase enzyme from *B. cereus* 567 and researched its characteristics /4/. It was determined

that this enzyme was similar to γ-penicillinase, and this enzyme was called β-lactamase III. They reported that this enzyme occurred in a membrane-bound form. β-Lactamases in other Gram positive bacteria were have been found bound to glyceride-cysteine with lipoprotein.

Connolly and Waley studied the characteristic properties of β-lactamase produced by *B. cereus* 569/H9 /5/. They determined that membrane-bound β-lactamase was immunochemically different from extracellular enzymes.

The purpose of this study was to identify *Bacillus* strains causing food poisoning and to determine β-lactamase enzyme activity and antibiotic susceptibility.

MATERIALS AND METHODS

In the present study, 19 *Bacillus* strains were isolated from 111 raw milk samples, and identified by biochemical tests. β-Lactamase enzyme activities of these identified bacilli were studied by the iodometric test and the chromogenic cephalosporin (nitrocefin) test.

Iodometric test

Bacillus strains were grown on nutrient agar with 0.2% starch. The test mixture was prepared by adding 1 ml iodine to penicillin solution. Prepared penicillin-iodine solution was dropped onto the growing *Bacillus* colonies on the agar plates. The reaction around the colonies was observed by adding 1 drop of starch solution. After applying this solution, a blue color developed around the colonies because of iodine bound to starch. In β-lactamase positive colonies, a ring-shaped colorless region appears, because the β-lactamase dissolves the penicillin in the applied solution /6/.

Chromogenic cephalosporin test

β-Lactamase enzyme activity of *Bacillus* strains was tested by using β-Lactamase Identification Sticks Oxoid BR 66. The yellow part of the sticks is applied to *Bacillus* colonies growth on glucose agar. β-Lactamase positive bacilli cause a color change from yellow to pink on the end of the stick /6,7/. Nitrocefin was an effective

substance for the sticks used in our study. Nitrocefin sticks need to be wetted before use.

In addition, antibiotic susceptibilities of *Bacillus* strains were tested by clavulanate-amoxycillin and cephazolin by using the Kirby Bauer disk diffusion method /8-11/. Antibiotic susceptibilities were evaluated by measuring zone diameters formed around antibiotic disks. Table 1 shows the antibiotics used in this study and the antibiotic dose in each disk.

TABLE 1

Antibiotics used in this study and doses used in each disk

Antibiotic	Dose in each disk	Zone diameter		
		Resistant	Intermediate	Susceptible
Clavunate-amoxycillin	10+20 μg	< 13 mm	14-17 mm	≥ 18 mm
Cephazolin	30 μg	< 14 mm	15-17 mm	> 18 mm

RESULTS

Nineteen *Bacillus* strains were isolated from 111 raw milk samples. The iodometric test and the chromogenic cephalosporin (nitrocefin) test used to detect β-lactamase enzyme activity both gave the same results. The types of strains, numbers isolated and β-lactamase activities are shown in Table 2.

In the study, of β-lactamase positive *Bacillus* strains, *B. mycoides* II and *B. coagulans* were susceptible to clavulanate-amoxycillin, and *B. subtilis, B. brevis* I and *B. brevis* II strains were intermediate to clavulanate-amoxycillin. Only one strain (*B. mycoides* II) was susceptible to cephazolin. Four strains (*B. subtilis, B. coagulans, B. brevis* I and *B. brevis* II) were intermediate to cephazolin. None of the β-lactamase positive strains isolated in this study was resistant to clavulanate-amoxycillin or cephazolin.

Of the β-lactamase negative bacilli, *B. cereus, B. firmus, B. mycoides* I, *B. polymyxa* I and *B. polymyxa* II were resistant to clavulanate-amoxycillin. *B. macerans, B. cereus, B. firmus, B. megaterium, B. mycoides, B. polymyxa* I and II, and *B. stearothermophilus* I, II, III were resistant to cephazolin (see Table 3).

TABLE 2

Bacillus strains isolated from various raw milk samples analyzed according to positive and negative β-lactamase enzyme activity

	No. of strains	β-Lactamase activity	Total
B. subtilis	1	Positive	5
***B. mycoides* II**	1	Positive	
B. coagulans	1	Positive	
B. brevis	2	Positive	
B. cereus	1	Negative	14
B. sphaericus	1	Negative	
B. licheniformis	1	Negative	
B. macerans	1	Negative	
B. firmus	1	Negative	
B. megaterium	1	Negative	
B. lentus	1	Negative	
B. mycoides I	1	Negative	
B. circulans	1	Negative	
B. polymyxa	2	Negative	
B. stearothermophilus	3	Negative	

TABLE 3

The comparison of antibiotic susceptibilities of 14 β-lactamase negative and five β-lactamase positive *Bacillus* strains

	Clavulanate-amoxicillin			**Cefazolin**		
	S	**I**	**R**	**S**	**I**	**R**
β-Lactamase negative						
B. cereus			R			R
B. sphaericus	S			S		
B. licheniformis		I			I	
***B. macerans* I**	S					R
B. firmus			R			R
B. megaterium	S					R
B. lentus	S			S		
***B. mycoides* I**			R			R
B. circulans	S			S		
***B. polymyxa* I**			R			R
***B. polymyxa* II**			R			R
***B. stearothermophilus* I**		I				R
***B. stearothermophilus* II**		I				R
***B. stearothermophilus* III**		I				R
β-Lactamase positive						
B. subtilis		I			I	
***B. mycoides* II**	S			S		
B. coagulans	S				I	
***B. brevis* I**		I			I	
***B. brevis* II**		I			I	

S = susceptible; I = intermediate; R = resistant.

DISCUSSION

Not only do *Bacillus* strains cause food poisoning, but they are also found in infected wounds and abscesses, and they can cause septicemia, endocarditis, meningitis, and lung and urinary system infections. The number of *Bacillus* organisms present in a sample must be $\geq 10^5$ to be accepted as the etiological agent, and also toxins must be detected. However, as it is not easy to define toxin properties, antibiotic treatment is administered to patients if *Bacillus* is thought to be the disease agent.

The antibiotics used until now in *Bacillus* treatment have been penicillin derivatives with broad-spectrum activity. However, there are *Bacillus* species which secrete β-lactamase. β-Lactamases are enzymes that hydrolyze β-lactam antibiotics, such as penicillin, and cause the development of resistance to these antibiotics. β-Lactam antibiotics have been tried in the treatment of infections caused by β-lactamase-producing bacteria. When used together with amoxycillin, clavulanic acid, a β-lactamase inhibitor, extends the antibacterial spectrum of β-lactam antibiotics by irreversibly binding and thus inhibiting many bacterial β-lactamase enzymes. Some cephalosporin antibiotics are potent against β-lactamase-producing bacteria. Cephazolin, a first-generation cephalosporin, effective against Gram positive bacteria, was chosen for this study, as well as clavulanate-amoxycillin.

The susceptibilities of *Bacillus* strains, tested for β-lactamase enzyme activity, to clavulanate-amoxycillin and cephazolin were investigated in this study. Of 19 *Bacillus* strains isolated and identified from raw milk, five strains were β-lactamase positive, and the other 14 strains were β-lactamase negative. Two of the β-lactamase positive strains were susceptible to clavulanate-amoxycillin, while three strains were intermediate. Only one of the β-lactamase positive strains was susceptible to cephazolin, and four strains were intermediate. Five of the β-lactamase negative strains were susceptible to clavulanate-amoxycillin, four strains were intermediate and five strains were resistant. To cephazolin, three strains were susceptible, one intermediate, and ten strains were resistant.

Doganay and Aydın tested the antibacterial susceptibility of *B. anthracis* /12/. These investigators tested the susceptibilities of 22 *B. anthracis* strains to 27 antibacterial agents by using an agar diffusion

technique. They observed that all the isolates were susceptible to cephazolin. In their study, ofloxacin, a new antimicrobial agent, also showed very good activity.

Gedek determined β-lactamase activity in *B. cereus* strains and also investigated cephalosporins in raw milk /13/. In his study, penicillinase antibiotics, such as cephalonium, cephapirin and cephacetril, were exposed to β-lactamases concentrated from *B. cereus* by an agar diffusion technique. Finally, they observed that microbiological activity in raw milk was lost partially or completely.

The most widely used methods to detect β-lactamase enzyme activity are the iodometric test and the chromogenic cephalosporin (nitrocefin) test. Tal *et al.* studied β-lactamase enzyme activities of *Bacillus* strains /14/, using the iodometric test, as we did. The chromogenic cephalosporin (nitrocefin) test method has been frequently used in recent years. In the present study, *Bacillus* strains were tested for β-lactamase enzyme activity by two tests, the iodometric method and the chromogenic cephalosporin test, using the antibiotic nitrocefin as substrate. There are many different test methods in the literature to detect β-lactamase enzyme activity /7,14-18/.

Baker used the chromogenic cephalosporin test method in his investigation /7/. In this study, nitrocefin was used as substrate, as in the present study. He determined that *B. subtilis* and *B. cereus* synthesized extracellular β-lactamase enzymes. In our study, the β-lactamase activity of the *B. cereus* strain isolated was negative.

Clavulanate-amoxycillin is more effective against β-lactamase positive bacterial strains, as the clavulanate binds to the β-lactamase and destroys its activity by irreversibly forming an acyl enzyme complex. In this study, of the β-lactamase positive strains isolated, *B. mycoides* II was susceptible to both clavulanate-amoxycillin and cephazolin, and *B. coagulans* was susceptible to clavulanate-amoxycillin.

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