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ANTIBIOTIC TOLERANCE FOR CEFTRIAXONE, CEFOTAXIME, CEFOPERAZONE, CEFAZOLIN AND VANCOMYCIN IN METHICILLIN - RESISTANT STAPHYLOCOCCUS AUREUS STRAINS*

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ÖZET

Metisiline dirençli Staphylococcus aureus suşlarında seftriakson, sefotaksim, sefoperazon, sefazolin ve vankomisine antibiyotik toleransı.

In-vitro deneylerde seftriakson, sefotaksim, sefoperazon, sefazolin ve vankomisine duyarlı bulunan MRSA suşlarında duyarlı bulundukları antibiyotikler için antibiyotik toleransı aranmıştır. MBK/MİK oranı 32 veya daha yüksek olan suşlar "olası toleran" olarak kabul edilmiş ve olası toleran suşlar ölüm-zaman grafikleri çizilerek incelenmiştir. Antibiyotiğin 8x MİK konsantrasyonunda 24 saatte canlı bakteri sayısında 3 log'dan az düşme olan suşlar "yüksek derecede toleran", 3-5 log arasında düşme olan suşlar "düşük derecede toleran" olarak değerlendirilmiştir. 50 MRSA suşundan diğer antibiyotiklere in-vitro duyarlı, olası toleran, yüksek derecede toleran ve düşük derecede toleran bulunan suş sayıları, sırasıyla, seftriakson için 28, 7, 6, ve 1; sefotaksim için 28, 15, 7 ve 3; sefoperazon için 26, 9, 5 ve 1; sefazolin için 31, 6, 4, ve 1; vankomisin için 50, 19, 4 ve 4 olarak bulunmuştur.

SUMMARY

Antibiotic tolerance for ceftriaxone, cefotaxime, cefoperazone, cefazolin and vancomycin was searched in MRSA strains which were found susceptible to these antibotics in in-vitro tests. A MBC/MIC ratio equal to or greater than 32 for susceptible strains was regarded presumptive for tolerance and time-kill curves were drawn for presumptively tolerant strains. Strains with a decrease less than 3 logs in live-counts (in 8xMIC antibiotic, 24 hours) were labelled as highly tolerant, strains with a decrease of 3-5 logs as moderately tolerant. Out of 50 MRSA, the numbers of in-vitro susceptible, presumptively, highly and moderately tolerant strains were 28, 7, 6 and 1 for ceftriaxone; 28, 15, 7 and 3 for cefotaxime; 26, 9, 5 and 1 for cefoperazone; 31, 6, 4 and 1 for cefazolin; 50, 19, 4 and 4 for vancomycin, respectively.

INTRODUCTION

When a bactericidal antibiotic exerts only a bacteriostatic effect on a bacterial strain, the phenomenon is called antibiotic tolerance (5,9).

Although there were many older observations, antibiotic tolerance was thoroughly described by Tomasz et al (13) in 1970 for the first time in a mutant *S. pneumoniae* strain. In 1974, Best el al (1) observed antibiotic tolerance in a *Staphylococcus* strain. This was the first *Staphylococcus* strain tolerant to penicillin.

In 1977, Sabath et al (12) reported that tolerant staphylococci were encountered more

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frequently among strains isolated from deep infections. Following this report, the number of papers about tolerance increased sharply. Until now, tolerance was observed in more than 20 Gram positive bacterial species. The only Gram negative tolerant strains reported until now are some laboratory mutants of *E. coli* (5).

The bactericidal activity of beta-lactam antibiotics are considered to be important in the treatment of infections such as endocarditis, meningitis, osteomyelitis and infections in immunocompromised patients. Thus, antibiotic tolerance may have adverse effects on these occasions. On the other hand, beta-lactam antibiotics are known to be ineffective in the treatment of infections caused by methicillin resistant *S. aureus* (MRSA) strains although MIC values indicative for susceptibility were obtained for some of these strains in in-vitro tests.

MATERIALS AND METHODS

Fifty MRSA strains isolated from various clinical specimens were used. The susceptibility tests were performed in Mueller-Hinton broth. For methicillin susceptibility, the medium was supplemented with NaCl, $CaCl_2$ and $MgCl_2$.

Minimal inhibitory concentrations (MIC) were determined by the tube dilution method. To ensure that inoculum consists of logarithmically growing bacteria, broth was lightly inoculated from 18-hour old culture and incubated at 37 °C until the turbidity of McFarland 0.5 tube was achieved. So, an inoculum containing $5x10^5$ - $1x10^6$ bacteria per ml was obtained.

Minimal bactericidal concentrations (MBC) were determined by inoculating 0.01 ml on agar plates from tubes without any visible growth in 18 hours in MIC determinations. The lowest concentration yielding less than 5 colonies (at least 99.9 % kill) was accepted as MBC.

When the ratio of MBC to MIC of an antibiotic is equal to or greater than 32, the strains was regarded "presumptively tolerant" (12). Time-kill curves in 8xMIC antibiotic concentrations were drawn for such strains. In these experiments, antibiotic was added to a broth culture containing app. 10^6 logarithmically growing bacteria per ml at time zero, the culture was incubated at 37 °C and live counts were determined at 2, 4, 6 and 24 hours. Since a quantitative standard is not established for antibiotic tolerance, less than 3 logs decrease in live count (more than 0.1 % of bacteria in inoculum alive) in 24 hours was arbitrarily accepted indicative for high degree tolerance, and 3 to 5 logs decrease for moderate tolerance. If the live count decreased more than 5 logs, the strain was regarded nontolerant.

In all experiments, *S. aureus* ATCC 25923 which was susceptible and nontolerant to all antibiotics used in this investigation, was used as a control strain. All counts given below are the means of two determinations.

RESULTS

Fifty-two (34%) of 152 *S. aureus* strains isolated from clinical specimens at the beginning of this investigation were found to be methicillin resistant (MIC≥16 mg/l) (Table 1). Fifty of them were used in the experiments.

Table 1. MIC values of 52 MRSA strains.

MIC (mg/l):	16	32	64	128	256	>256
No. of strains:	13	7	4	4	4	20

MIC values indicated in-vitro susceptibility to ceftriaxone (≤32 mg/l) for 28 strains, to cefotaxime (≤32 mg/l) for 28 strains, to cefoperazone (≤32 mg/l) for 26 strains, to cefazolin (≤16 mg/l) for 31 strains and to vancomycin (≤2 mg/l) for all strains. MBC/MIC ratios of these strains were given in table 2.

As it is seen in table 2, MBC/MIC ratios equal to or greater than 32 which may be considered presumptive for tolerance were obtained in 7 strains for ceftriaxone, in 15 strains for cefotaxime, in 9 strains for cefoperazone, in 6 strains for cefazolin and in 19 strains for vancomycin.

Table 2. MBC/MIC ratios for MRSA strains which were found in-vitro susceptible to other antibiotics.

Antibiotic	No. of strains	MBC / MIC ratio									
		1	2	4	8	16	32	64	128	>128	(≥32)
Ceftriaxone	28	1	10	4	4	2	5	2			(7)
Cefotaxime	28			9	3	1	7	2	5	1	(15)
Cefoperazone	26			6	7	4	7	2			(9)
Cefazolin	31	4	1	2	12	6	3	1		2	(6)
Vancomycin	50	1	2	13	7	8	11	8			(19)

When the time-kill experiments were performed with these strains (MBC/MIC≥32), the numbers of highly tolerant, moderately tolerant and nontolerant strains were found to be 6, 1 and 0 for ceftriaxone; 7, 3 and 5 for cefotaxime; 5, 1 and 3 for cefoperazone; 4, 1 and 1 for cefazolin; 4, 4 and 11 for vancomycin. The results were summarised in table 3.

When a MBC/MIC ratio equal to or greater than 32 is considered presumptive for tolerance, 24 strains were found to be tolerant to 1 of the 5 antibiotics used, 6 strains to 2 antibiotics, 4 strains to 3 antibiotics, 2 strains to 4 antibiotics. Fourteen strains were found to be nontolerant to all antibiotics. The strains showing cross-tolerance to two or more antibiotics according to this criterion were shown in table 4.

Table 3. Summary of the results obtained with 50 MRSA strains.

Antibiotics	In-vitro susceptible	Presumptively tolerant (MBC/MIC≥32)	Time-kill studies		
			Highly tolerant	Moderately tolerant	Nontolerant
Ceftriaxone	28	7	6	1	0
Cefotaxime	28	15	7	3	5
Cefoperazone	26	9	5	1	3
Cefazolin	31	6	4	1	1
Vancomycin	50	19	4	4	11

Table 4. Cross-tolerance determined by MBC/MIC ratios presumptive for tolerance.

Strains	Number of strains	Ceftriaxone	Cefotaxime	Cefoperazone	Cefazolin	Vancomycin
K23,K3	2	+	+	+	+	
24	1		+	+	+	
31,42	2		+	+		+
5691	1	+	+			+
K9	1		+		+	
K14	1			+		+
C22	1		+	+		
26,A23,5796	3	+	+			

When the results of time-kill experiments are considered, cross-tolerance was recorded in only 8 strains. Five of them showed cross-tolerance for 2 antibiotics, 2 for 3 antibiotics and 1 for 4 antibiotics. These results were shown in table 5.

Table 5. Cross-tolerance determined by time-kill studies.

Strains	Number of strains	Ceftriaxone	Cefotaxime	Cefoperazone	Cefazolin	Vancomycin
K23	1	+	+	+	+	
K3	1	+		+	+	
5691	1	+	+			+
K9	1		+		+	
24	1			+	+	
C22	1		+	+		
A23,5796	2	+	+			

DISCUSSION

Antibiotic tolerance is accepted by some authors as a special mechanism of bacterial resistance since the tolerant bacteria resist to the killing and many times to the lysis effect of an antibiotic in concentrations higher than MIC (5,6,14). On the other hand tolerance is an entirely different phenomenon from resistance which simply means an increased MIC. A bacterial strain may be resistant and tolerant, resistant and nontolerant, sensitive and tolerant, or sensitive and nontolerant to an antibiotic (5). The main future in tolerance is the difference between MBC and MIC whether the MIC value is high or low. None of known resistance mechanisms plays a role in tolerance (4). On the other hand, a decrease in autolytic activity was observed in tolerant strains. This decrease in autolytic activity depends on the increased production of inhibitors of major autolytic enzyme, amidase, but other mechanisms were also proposed (5,10).

Tolerance is a phenomenon between Gram positive bacteria and cell-wall inhibitor antibiotics such as beta-lactams and vancomycin. The term tolerance is used to mean genetic tolerance. This is entirely different from phenotypic tolerance which is observed in some physiological growth conditions. For example, beta-lactam antibiotics are effective on growing bacteria and bacteria in stationary phase resist to the killing effect of these antibiotics. This is a phenotypic tolerance. Cultures growing in low pH may also exert a phenotypic tolerance. Phenotypic tolerance is observed in all strains of a species. Genetic tolerance, on the other hand, is the lowered killing rate of a bacterial strain in conditions in which many other strains are killed rapidly.

Many methods have been reported to test tolerance, but probably the best methods to distinguish tolerant and nontolerant strains are the kinetic tests in which lowered killing rates are shown in tolerant strains. The results obtained by this method vary greatly according to the conditions of cultures (e.g. media, pH, temperature) and the method is not practical for a routine laboratory. For this reason, Sabath et al (12) proposed that a MBC/MIC ratio equal to or greater than 32 may be accepted as indicative for tolerance in a routine laboratory where the MBC means the lowest antibiotic concentration causing 99.9 % kill in 24 hours (2). To determine this ratio is easy in laboratories where the MIC determinations were done with an adjusted inoculum. In such laboratories a live count may be performed from the tube containing 32xMIC (the fifth tube after the first no growth tube in a two-fold dilution series) at the time MIC is read. If the live count is higher than 0.1 % of that in the inoculum, the strains are labelled as tolerant.

Another method to test tolerance is to determine the kill ratio in 24 hours in the presence of high concentration of antibiotic. In nontolerant strains the live counts decrease more than 5 logs in 24 hours while in tolerant strains this decrease is usually 1 to 2 logs (7).

In tolerance determinations, experiments should be done in conditions which will not result in phenotypic tolerance (e.g., not stationary phase but logarithmically growing bacteria should be used). The big differences in the incidences of tolerant strains in a bacterial species in the literature are probably due to the different test conditions (5).

The opinions about clinical significance of antibiotic tolerance are controversy. Since bactericidal antibiotics are preferable in immunocompromised patients and in infections such as endocarditis, meningitis and osteomyelitis, it may be expected that the treatment of infections caused by tolerant strains would be more difficult. In-vivo experiments on rabbit endocarditis model support this view (5). There are also some reports about the difficulty in the treatment of human infections caused by tolerant strains (5,8,11,12). But the clinical significance of tolerance has not been clarified yet.

The first example of antibiotic tolerance was observed in *S. pneumoniae* for penicillin. Then, it was observed in *S. viridans, S. mitior, S. mutans, S. milleri, S. salivarius, S. bovis,* streptococci from serogroups A, B, G and D, *E. faecalis, E. faecium, Lactobacillus, L. monocytogenes, S. aureus* and *C. perfringens* strains. The most frequently studied bacterial genus for tolerance is *Streptococcus* which is followed by *Staphylococcus* (5).

Cross-tolerance between beta-lactam antibiotics is a common finding in *S. aureus* strains. Although less frequently, some examples of cross-resistance are also observed between beta-lactam antibiotics and vancomycin. Sabath et al (12) isolated 7 penicillin-tolerant *S. aureus* strains from patients with poor reply to penicillin therapy for infections such as endocarditis, osteomyelitis and pneumonia. Most of these strains showed cross-tolerance to cephalosporins and vancomycin. When the tolerant strains were investigated, only 7% or less of the bacterial cells in the culture were found to be tolerant. The autolytic activity exerted by these cells decreased the lysis of other cells without this activity as well. If the tolerant cells consist of about 7% of the bacterial population in the culture, then the probability that tolerance could be observed in a pure culture prepared by picking up one colony would be lower than the reality. That may also be the reason for the contraversial results in reported experiments. Preparing an inoculum by picking up many colonies from original plates may be a solution for this problem in tolerance experiments. Bradley et al (3) used all bacterial population grown by culturing 0.1 ml blood samples, and found that all of 34 *S. aureus* strains were tolerant.

In this paper, we tested 50 MRSA for in-vitro susceptibility to ceftriaxone, cefotaxime, cefoperazone, cefazolin and vancomycin by the tube dilution method and looked for tolerance to these antibiotics in strains found susceptible in-vitro. In the first hand MBC/MIC ratio was determined. If this ratio indicated tolerance, as a more precise method, the time-kill curves were drawn for these strains. Results showed that many of the strains having presumptive MBC/MIC ratios are not really tolerant and are killed rapidly in time-kill experiments. They showed that methicillin resistance and tolerance to cephalosporins and vancomycin are unrelated phenomenons since only some of MRSA strains are found tolerant. Actually different mechanisms are of value in these phenomenons (12). A *S. aureus* strain tolerant to one cephalosporin may be nontolerant to another even if they are from the same generation although examples of cross-resistance among cephalosporins and/or different antibiotics such as vancomycin are encountered.

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