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Effect of Beta-Lactamase Inhibitors on Minimum Inhibitory Concentration of Ampicillin and Amoxicillin for *Staphylococcus aureus* Strains

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ABSTRACT. Seventy strains of *Staphylococcus aureus* isolated from the nasopharinx (80%), urinary tract (16%), skin (1 strain) and eyes (2 strains) of patients at the clinical laboratory "El eritrocito" were analyzed. Susceptibility to 12 antibiotics was tested by the method of Kirby-Bauer. Minimal inhibitory concentration (MIC) of ampicillin, ampicillin + sulbactam, amoxicillin and amoxicillin + clavulanic acid were determined by plate dilution. Percentages of resistance were: Penicillin and ampicillin (100%), ceftazidim (81.4%), erythromycin (68.6%), tetracyclin (31.4%) trimetoprim-sulphametoxasol (25.7%), dicloxacillin and pefloxacin (12.8%), cefuroxime and cefotaxime (4.3%), gentamicin (2.8%), cephalothin (0%). All strains were resistant to three or more antibiotics, with higher percentages of resistance to four (31.4%), three (27.1%), five (21.4%) and six (12.9%) drugs. One strain was resistant to nine antibiotics and 5.9% were resistant to seven. 97.5% of the strains were β-lactamase-positive. The MIC_{50} of ampicillin and amoxicillin was 500 μg/ml and the MIC_{90} were 1727 μg/ml and 2000 μg/ml, respectively. β-lactamase inhibitors sulbactam and clavulanic acid reduced these values eightfold, except for the MIC_{50} of ampicillin + sulbactam whose reduction was sixteen fold. These results show that the combination of β-lactamic + β-lactamase inhibitor was more efficient than cephalosporins for killing these β-lactamase-positive strains.

RESUMEN. Se analizaron 70 cepas de *Staphylococcus aureus* aisladas de la nasofaringe (80%), de las vías urinarias (16%), de infecciones cutánea (1 cepa) y oculares (2 cepas) de pacientes que acudieron al Laboratorio de Análisis Clínicos "El eritrocito". Las cepas se identificaron por morfología colonial y microscópica y mediante pruebas bioquímicas. La susceptibilidad a 12 antibióticos se determinó por el método de Kirby-Bauer. Las concentraciones mínimas inhibitorias (CMI) de ampicilina, ampicilina + sulbactam, amoxicilina y amoxicilina + ácido clavulánico se determinaron por dilución en placa. Los porcentajes de resistencia fueron: penicilina y ampicilina (100%); ceftazidima (81.4%); eritromicina (68.6%); tetraciclina (31.4%); trimetoprim-sulfametoxazol (25.7%); dicloxacilina y pefloxacina (12.8%); cefotaxima y cefuroxima (4.3%); gentamicina (2.8%); cefalotina (0%). Todas las cepas fueron resistentes a 3 o más antibióticos; los porcentajes mayores de cepas multirresistentes correspondieron a tetra-resistentes (31.4%), tri-resistentes (27.1%), penta-resistentes (21.4%), sexta-resistentes (12.9%) y hepta-resistentes (5.9%); sólo 1 cepa fue resistente a 9 antimicrobianos. La mayoría de las cepas (97.5%) produjo β-lactamasa. La CMI_{50} de ampicilina y amoxicilina fue 500 μg/ml y la CMI_{90} de 1727 μg/ml y 2000 μg/ml, respectivamente; los inhibidores de β-lactamasas, ácido clavulánico y sulbactam, disminuyeron 8 veces estos valores, excepto para la CMI_{50} de ampicilina + sulbactam, cuya disminución fue de 16 veces. Estos resultados muestran que las cefalosporinas fueron menos eficaces que la combinación β-lactámico + inhibidor de β-lactamasa contra estas cepas productoras de β-lactamasas.

INTRODUCTION

β-lactam antibiotics inhibit enzymes that participate in bacterial cell wall biosynthesis during cellular division. They bind to cytoplasmic membrane proteins (PBPs, by penicillin binding proteins). Gram-negative bacteria are resistant to several β-lactam antibiotics due to the incapacity of these drugs to diffuse across the outer membrane pores,[5] and also to the presence of low levels of β-lactamase chromosomically encoded;[4,18] an additional resistance mechanism to β-lactamase-non-labile cephalosporins is based on the capacity of the periplasmic enzyme to bound the antibiotic avoiding contact with its target.[26] Acquired resistance to β-lactam antibiotics by clinical isolates may be due to plasmid-encoded β-lactamases or to chromosomal mutations that alter the amount of PBPs or their affinity for the drugs. In Gram-negative bacteria resistance to β-lactam can be achieved by mutations increasing outer membrane impermeability.[9,10,14] There are mutations in *Staphylococcus aureus* that alter PBPs, conferring cefradine resistance.[8]

Penicillin-resistance in *S. aureus* due to production of β-lactamases appears firsts at the beginning of the 1950s, quickly followed by resistance to other antibiotics (macrolides, aminoglycosides and tetracycline). The genes responsible of these phenotypes were probably present in staphylococci populations and other bacteria before the ample use of antibiotics, which could explain the swiftness in prevalence of resistant microorganisms. For example, 5-10 % of the *S. aureus* strains stored in the pre-antibiotic era are resistant to penicillin due to the fact that they produce β-lactamase.[16] Introduction of penicillin-derived compounds non-labile to β-lactamase, as methicilin, in 1960 was quickly followed by the emergence of resistant strains, principally in hospitals. Methicillin resistance is probably due to the PBP2, a protein whose affinity for β-lactam compounds is extremely low.[6,11]

Most penicillin-resistant *S. aureus* strains produce β-lactamases, usually plasmid-encoded. There are four penicillinase variants describe in *S. aureus* (A to D).[20] These enzymes quickly hydrolyze ampicillin and bencilpenicillin, but little to methicillin oxacillin and cloraxil. Except for the type D enzyme, whose expression is constitutive,[22] all of them are inducible and most of their activity is extracellular.[22]

A transposon-encoded β-lactamase has been described in *S. aureus*. This mobile genetic element, designated Tn552 (6.7 kb) has been found in the chromosome as in plasmids.[23]

β-lactamase hydrolysis of β-lactam antibiotics is accomplished by an intermediate acyl-enzyme with subsequent penicillinoic acid liberation. Two strategies have been employed for killing β-lactamase-producing bacterial strains: i) Combination of β-lactam antibiotics with β-lactamase inhibitors, particularly ampicillin + sulbactam, or amoxicillin + clavulanic acid. ii) Synthesis of β-lactam antibiotics non-labile to β-lactamases (cephalosporins).

Actually there are antibiotics non-labile to the β-lactamases produced by *S. aureus*, one group includes the semisynthetic penicillins methicillin, oxacillin, nafcillin and others.[19] It has also been developed the cephalosporins, whose resistance to β-lactamases varies; they include the second generation (cephoxitin, cephamandole, cephuroxime) and third generation cephalosporins (cephotaxime, moxolactam, cephoperazone and others).

The utilization of β-lactam antibiotics non-labile to β-lactamases, and combinations of β-lactam + β-lactamase

Table 1. Antibiotic Employed Against *S. aureus* Strains

ANTIBIOTIC	ABREVIATION	FAMILY	INHIBITION ZONE (mm)[a]		
			R	I	S
Ampicillin	AMP	Aminopenicillin	≤ 28		≥ 29
Cefalotin	CF	First generation Cephalosporin	≤ 14	15-17	≥ 18
Cefotaxime	CTX	Third generation cephalosporin	≤ 14	15-22	≥ 23
Ceftazidime	CAZ	Third generation cephalosporin	≤ 14	15-17	≥ 18
Cefuroxime	CXM	Second generation cephalosporin	≤ 14	15-17	≥ 18
Dicloxacillin	CLOX	Semisynthetic Penicillin	≤ 10	11-12	≥ 13
Eritromicin	ERI	Macrolide	≤ 13	14-17	≥ 18
Gentamicin	GEN	Aminoglycoside	≤ 12	13-14	≥ 15
Pefloxacine	PEF	Quinolone	≤ 14	15-22	≥ 23
Penicillin	PEN	Penicillin	≤ 28		≥ 29
Tetracycline	TET	Tetracycline	≤ 14	15-18	≥ 19
Trimetoprim-sulfametoxazol	SXT	Diaminopyrimidine and sulfonamide combination	≤ 10	11-15	≥ 16

[a] R= Resistant I= Intermediate S= Susceptible

Revista Latinoamericana de Microbiología (1998) 40:128-134
Asociación Latinoamericana de Microbiología

inhibitor constitutes a selection factor in pro of new β-lactamase-producing bacterial strains, with higher enzyme expression levels, plasmid-encoded and with altered catalytic properties as to possess greater affinity for substrates non-labile today or with reduced affinity for β-lactamase inhibitors.[12] Indeed, it has been reported that β-lactamases, commonly plasmid-encoded and restricted initially to enterobacteria, are found now in diverse bacterial species, including *Pseudomonas aeruginosa, Haemophilus influenzae* and *Neisseria gonorrhoeae*. These enzymes can hydrolyze second and third-generation cephalosporins and, of course, the older β-lactam antibiotics; in this way, these strains are practically resistant to all β-lactam antibiotics, except to carbapenems.[7] However, it seems that an increase in production of inhibitor-resistant β-lactamases have not occurred yet, as a report of the review of 1500 published papers about this topic between 1978 and 1993 shows that in spite the increase of β-lactamase-producing strains resistant to ampicillin and amoxicillin, there are no evidence for the increased resistance to amoxicillin + clavulanic acid.[21]

β-lactamase detection in enterobacteria has very little predictive value about resistance or sensibility to distinct β-lactam antibiotics because one single strain can produce several β-lactamases with different substrate specificities.[25] However, in other penicillin-resistant bacteria as *Neisseria gonorrhoeae*,[2] *S. aureus*,[1] *Moraxella (Branhamella) catarrhalis*,[15] and *Haemophilus influenzae*,[24] the strains produce a single β-lactamase, and determination of the enzymatic activity allow the prediction about resistance or susceptibility to β-lactam antibiotics, at least 24 h before the results of the antibiogram can be obtained.

In this work susceptibility to twelve antibiotics and the effect of β-lactamase inhibitors sulbactam and clavulanic acid on the MIC of amoxicillin and ampicillin, respectively, were evaluated in 70 clinical strains of *S. aureus*.

MATERIAL AND METHODS

Source of the strains. Seventy *S. aureus* strains isolated from patients that went to the clinical laboratory "El eritrocito" were analyzed. Strains were identified by colony and microscopic morphology and by coagulase positive test. Eighty percent of the strains were isolated from nasopharynx, 16 % from urinary tract and the other from a cutaneous infection (one strain) and ocular infection (two strains). Most patients were young people and children, almost 70 % were 30 years old or less. The most abundant patient group was 0-10 years old (28 %), followed by 11-20 years old (20 %). Lowers patient groups were 61-70 years old and 71-80 years old (1.4 % in each one).

Antibiotic Resistance. Susceptibility testing to the following 12 antibiotics was determined three times by the de Kirby-Bauer method,[3] diameter of inhibition zone was recorded and the strains were classified as susceptible or resistant according with the indicated break points (Table 1).

MIC Determination. Minimum inhibitory concentration of ampicillin, ampicillin + sulbactam, amoxicillin, and amoxicillin + clavulanic acid were determined three times by plate dilution in Mueller-Hinton agar with a multiple inoculator as been described previously.[27] MIC is the minimum antibiotic concentration that inhibits visible bacterial growth after incubating at 37°C for 24 h.

Combinations of β-lactam antibiotic + β-lactamase inhibitor used were: clavulin (SmithKline Beecham Farmacéutica, SA de CV; which contains amoxicillin + 25 % potassium clavulanate) and Unasyna (Pfizer, S.A. de C.V.; which contains ampicillin plus 66.8 % sulbactam).

β-lactamase detection. Paper disks impregnated with the chromogenic cephalosporin nitrocephin (BBL) were used for detection of β-lactamases. This substrate change from yellow to red after the amide bond of β-lactam ring is hydrolyzed by β-lactamase.

A nitrocephin-impregnated disk was moistened with a water drop and several colonies of the *S. aureus* strain were poured on it with an inoculating loop. The change in color was observed in 1-2 min.

RESULTS

Antibiotic resistance. All strains were resistant to the β-lactam antibiotics ampicillin and penicillin (Fig. 1), about 80 % were resistant to the third generation cephalosporin ceftazidime and almost 70 % to the macrolide erythromycin. Approximately one third of the strains showed resistance to tetracyclin, and one fourth to trimetoprim + sulphametoxazol. Near 13 % of the strains were resistant to the semisynthetic penicillin dicloxacillin or to the quinolone pefloxacin. Only 4.3 % of the strains were resistant to the cephalosporins cefuroxime (second generation) or cefotaxime (third generation). The frequency of gentamicin-resistant strains was very low (2.8 %) and all of them were susceptible to cephalothin. Greater intermediate susceptibility were observed for pefloxacin and cefotaxime (71.4 % for each one).

Multirresistance to antibiotics. All the strains tested were resistant to three or more drugs (Fig. 2), one strain (1.4 %) showed resistance to nine antibiotics. Greater proportions of multirresistant strains were to four (31.4 %), three (27.1 %), five (21.4 %) antibiotics; whereas lower proportions were observed for resistance to six (12.9 %) and seven (5.9 %) drugs. Strains resistant to eighth antibacterial drugs were not observed (Fig. 2).

β-lactamase production. Production of β-lactamases by the *S. aureus* strains was qualitatively determined by its capacity to hydrolyze the chromogenic cephalosporin nitrocephin. Nearly all strains were β-lactamase producers

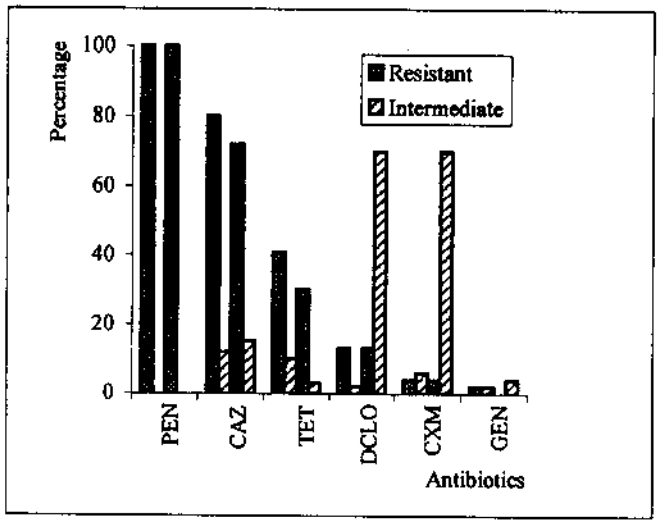

Fig. 1. Antibiotic resistance by the *S. aureus strains.* Susceptibility to antibiotics was determined by the Kirby-Bauer method. The strains were classified as resistant, intermediate or susceptible according to the zone inhibition diameter, as stated in Table 1

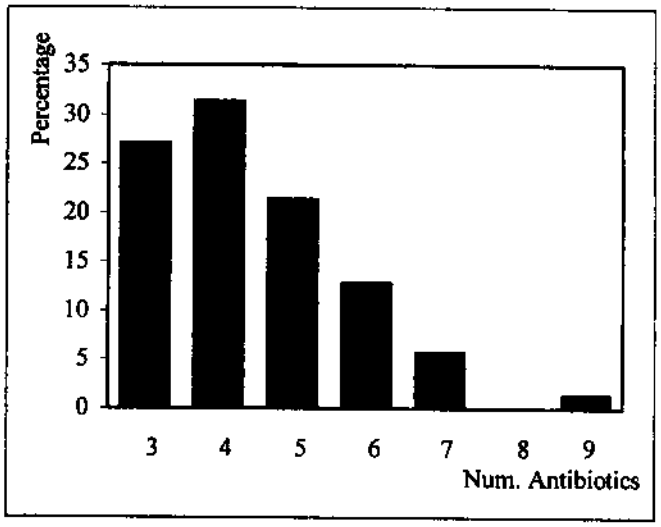

Fig. 2. Multirresistance to antibiotics by the *S. aureus* strains. Percent of strains resistant to three or more antibiotics.

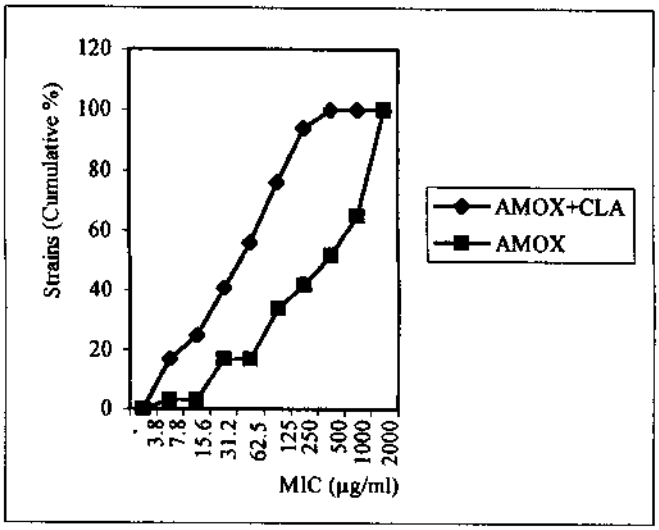

Fig. 3. Minimum inhibitory concentration of beta-lactam antibiotic with and without beta-lactamase inhibitor. MICs were measured by plate dilution. MIC_{50} and MIC_{90} of amoxicillin were 500 µg/ml and 2000µg/ml respectively; whereas those of amoxicillin + clavulanic acid were 62.5 µg/ml and 250 µg/ml respectively.

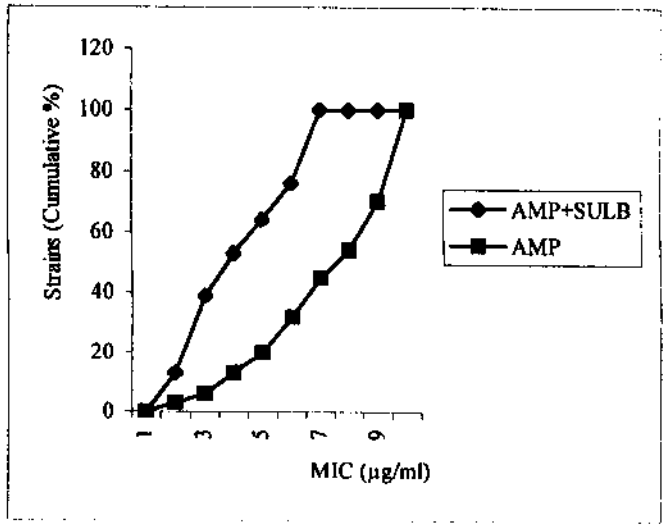

Fig. 4. Minimum inhibitory concentration of beta-lactam antibiotic with and without beta-lactamase inhibitor. MICs were measured by plate dilution. MIC_{50} and MIC_{90} of ampicillin were 500 µg/ml and 1727 µg/ml respectively; whereas those of ampicillin + sulbactam acid were 31.2 µg/ml and 216 µg/ml respectively.

(95.7 %). Only three strains (4.3 %) did not produce β-lactamase.

Minimum inhibitory concentrations of β-lactam antibiotics with and without β-lactamase inhibitors. In order to quantitate the effect of the β-lactamases inhibitors sulbactam and clavulanic acid on ampicillin and amoxicillin MICs respectively, these concentrations were measured for each antibiotic alone and combined with the inhibitor. As can be seen on Fig. 3, MIC_{50} of amoxicillin (concentration that inhibits the growth of 50 % of the strains) was 500 µg/ml and MIC_{90} was 2000 µg/ml. Addition of clavulanic acid diminished eightfold these values (62.5 µg/ml and 250 µg/ml, respectively) (Fig. 3).

MIC_{50} and MIC_{90} of ampicillin were 500 µg/ml and 1727 µg/ml, respectively (Fig. 4). Sulbactam addition to ampicillin reduced sixteenfold the MIC_{50} (31.2 µg/ml) and eightfold the CMI_{90} (216 µg/ml) (Fig. 4).

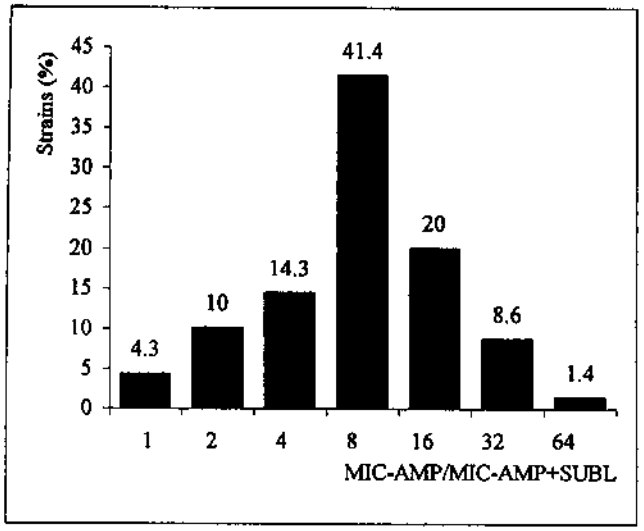

Fig. 5. Effect of sulbactam on minimum inhibitory concentration of ampicillin. Ratio of ampicillin MIC value divided by the MIC value of ampicillin + sulbactam.

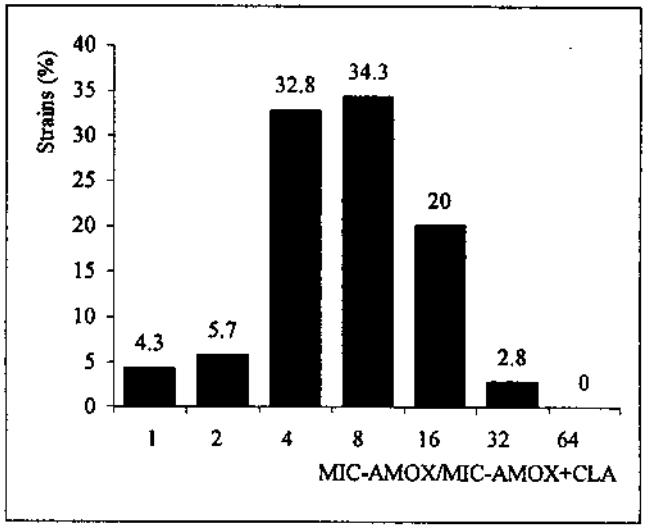

Fig. 6. Effect of clavulanic acid on minimum inhibitory concentration of amoxicillin. Ratio of amoxicillin MIC value divided by the MIC value of amoxicillin + sulbactam.

Variety of β-lactamases produced by the *S. aureus* strain population. In spite of the observed reduction of ampicillin and amoxicillin MIC_{50} and MIC_{90} caused by sulbactam or clavulanic acid, respectively, when the ratio MIC-β-lactam/MIC β-lactam + β-lactamase inhibitor was analyzed, it was observed that β-lactamases produced by the strains differ in their sensibility to the inhibitor. The sulbactam-mediated reduction in MIC of ampicillin showed a distribution in the range twofold to sixtyfourfold, with a mean value of eleven (Fig. 5). Only three strains (4.3 %) did not show reduction in ampicillin MIC by sulbactam. A similar distribution was obtained for the ratio MIC-amoxicillin/MIC-amoxicillin + clavulanic acid, whit 8.3 as the mean value in a range between 2 and 32. Also, the same three strains did not show reduction in amoxicillin reduction by the addition of clavulanic acid (Figure 6).

DISCUSSION

Source of the strains. Eighteen percent of the *S. aureus* strains studied were isolated from nasopharynx which reflects the high incidence of this pathogen as etiologic agent of respiratory tract infections. This high frequency is consistent with that reported in a previous study, in which 1454 bacterial strains isolated from ambulatory patients at a University Clinic during a period of seven years; 651 of these strains were *S. aureus* and 603 (92.6 %) of them were isolated from nasopharing.[13]

It is known that children and aged people are very susceptible to respiratory tract infections. Most of the *S. aureus* strains analyzed here were isolated from children of ten years or less (28.8 %) and from young people (38.6 % from patients between 11-30 years old). The lowest number of strains corresponded to those isolated from aged patients (2.8 % for people between 61-80 years old) owing to the fact that this age group was quite low in patients that went to "El eritrocito".

Antibiotic resistance. All *S. aureus* strains reported here were resistant to the β-lactam antibiotics penicillin and ampicillin (Fig. 1). This proportion is greater than that reported in other countries[17] and could be due to a higher use of these antibiotics by the people from this community, so the bacterial population probably was selected as resistant principally by its β-lactamases production capacity.

Resistance to some β-lactamase-non labile antibiotics was also high: a few more than 80 % of the strains were resistant to the third-generation cephalosporin ceftazidime and about 13 % were resistant to dicloxacillin. On the other hand, almost all strains (95.4 %) were susceptible to the cephalosporins cefuroxime (2nd-generation) or cefotaxime (3rd generation) and all the strains were susceptible to cephalothin (first generation). These remarkably differences between resistance to new cephalosporins and sensitivity to older ones clearly shows that introduction date of an antibiotic to the market does not guarantee its effectiveness, since selection of resistant strains is greatly influenced by the bad use and abuse of antimicrobial drugs. The data reported here demonstrate that it is impossible to consider any antibiotic as of first election.

The lowest frequency of resistant strains were for gentamicin (2.8 %) and cephalothin (0 %) (Fig. 1). All the strain tested were resistant to three or more antibiotics with the higher frequency for strains resistant to four drugs (31.4 %) (Fig. 2) which probably reflects that they possess plasmids.

Effect of β-lactamases inhibitors on the MIC of β-lactam antibiotics. We reported here that 95.7 % *S. aureus* strains were β-lactamase-producers, and that the β-lactamases inhibitors clavulanic acid and sulbactam reduced eightfold and sixteenfold the MIC_{50} and MIC_{90} of amoxicillin and ampicillin for the *S. aureus* population studied (Fig. 3 and 4). However, a more detailed analysis permits to distinguish six (Fig. 5) or five (Fig. 6) subpopulations which probably correspond to strains that produce β-lactamase in different concentrations or with distinct sensitivity to the inhibitor. The three strains (4.3 %) that did not produce β-lactamase had identical MICs of β-lactam antibiotic with and without β-lactamase inhibitor (Fig. 5 and 6). In spite that these strains were not β-lactamase-producers, they were resistant to penicillin and ampicillin. These data strongly suggest that resistance of these strains to both β-lactam antibiotics is due to an alteration of the PBPs.

CONCLUSIONS

The data reported here shows that *S. aureus* is a common cause of respiratory tract infections and that most strains are resistant to the antibiotics traditionally considered as of first election, especially to penicillin and ampicillin. The data also shows that all strains studied were multirresistant. Finally, of the two strategies employed to combat β-lactamase-producer strains, the introduction of cephalosporins was less efficient than the combination of β-lactam antibiotic + β-lactamase inhibitor, with the exception of cephalothin, to which there were no resistant strains.

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