Return to contents page

PREVALENCE AND PATTERN OF ANTIBIOTIC SENSITIVITY OF METHICILLIN-SENSITIVE AND METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS IN SHIRAZ-IRAN

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- **ABSTRACT**

Background: There is a growing concern about the rapid rise in resistance of *Staphylococcus aureus* to antimicrobial agents.
Objective: To determine the prevalence and pattern of antibiotic sensitivity among methicillin-resistant and -sensitive *S.aureus* in Shiraz, south of Iran.
Methods: One hundred and six *S. aureus* isolates from patients with staphylococcal infections were collected. The isolates were screened for methicillin-resistance by minimum inhibitory concentration (MIC) determination and agar screen plate. Detection of acquired resistance was accomplished by addition of a β -lactamase inhibitor to the test media. Tolerance was detected by determination of minimum bactricidal concentration (MBC)/MIC ratio.
Results: The frequency of intrinsic-resistant *S. aureus* was 33.0% (90% in isolates from burn unit patients). Acquired-resistant and tolerant isolates comprised 4.7% and 0.9% of all isolates respectively. None of the isolates showed MOD-type resistance [for "modified" penicillin binding proteins (PBPs)]. The pattern of resistance to 13 antibiotics were determined by disk diffusion method.None of the intrinsic -resistant isolates were resistant to vancomycin or rifampin but the frequency of resistance to other compounds was high. The methicillin-sensitive and acquired-resistant isolates were highly sensitive to all tested antibiotics except penicillin.
Conclusion: In this study, methicillin-resistance proved to be a useful marker in selecting appropriate antimicrobial agents for treatment of infections caused by *S. aureus*. Changing pattern of resistance of *S.aureus* makes its periodic surveillance (each 3 to 4 years once) mandatory.

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Key Words • Prevalence • methicillin resistance • Staphylococcus aureus • microbial sensitivity tests

Introduction

"Methicillin-resistant *Staphylococcus aureus"* (MRSA) are isolates with resistance to the penicillinase-resistant penicillin (PRP) class of antibiotics. At least four mechanisms have been proposed to explain the resistance in MRSA: Intrinsic resistance, that refers to all mechanisms of resistance not mediated by antibiotic inactivation.[1] It is a chromosomally mediated phenomenon, and in MRSA, involves production of a low affinity penicillin-binding protein (PBP-2a) which can perform the function of other high affinity PBPs at antibiotic concentrations that inactivate the other PBPs and would otherwise be lethal.[2,3] Most isolates of methicillin-resistant *Staphylococci* isolated from nature and usual laboratory culture are heterogeneous in their phenotypic expression of the resistance and only a small number of cells (one in 10^4 to 10^8) are able to express their methicillin-resistant trait.[4,5] The *in vitro* detection of a minority population of resistant cells requires manipulation of the standard test conditions. Conditions that favor the detection of these isolates include (a) lower incubation temperature, (b) prolonged incubation, and (c) addition of sodium chloride to the medium.[6-7]

Acquired resistance, which is characterized *in vitro* by a borderline MIC, lowered to a susceptible range by the addition of a β -lactamase inhibitor such as clavulanic acid.[6]

MOD-type resistance (for "modified" PBPs), that confers a borderline resistance to the strain by some modification of normal PBPs resulting in an altered affinity for the antibiotic.[8]

Although the clinical significance of methicillin-resistance has been questioned in the past, there is now a widespread acknowledgment of the pathogenicity of MRSA. It has emerged as a significant cause of both nosocomial and community-acquired infections. Furthermore, during the past decade there has been a steady increase in the incidence of infections caused by this bacterium.

This study was undertaken to document: (1) the prevalence of methicillin-resistance among *S. aureus* isolates at three tertiary care teaching hospitals and (2) the pattern of antibiotic susceptibility of methicillin-sensitive and resistant isolates to the commonly used antimicrobial agents.

Materials and Methods

One hundred and six isolates of *S. aureus* obtained from three large university affiliated hospitals during one year made up our study population. Isolates were identified as *S. aureus* by gross and microscopic morphology and by positive coagulase, catalase and DNase tests. All isolates were isolated from patients in whom *S. aureus* was the sole causative infectious agent. The staphylococcal infection was confirmed by clinical and paraclinical correlation. Mixed specimens and samples obtained from nose, throat, sputum were excluded. The sources of specimens are shown in Table 1.

Antibiotic susceptibility test

The pattern of antibiotic sensitivity of *S. aureus* to 13 antibiotics (penicillin, cephalothin, cephalexin, gentamicin, amikacin, trimethoprime-sulfamethoxazole (TMP-SMZ), erythromycin, clindamycin, lincomycin, rifampin, chloramphenicol, ciprofloxacin, and vancomycin) was determined by disk diffusion (Kirby-Bauer) susceptibility test. All tests were performed on Muller Hinton agar (Oxoid Co., Hampshire, UK) and were interpreted after incubation for 24 hours at 35^0 C. No supplemental NaCl was added to the agar medium. The zone diameters measured around each disk were interpreted on the basis of guidelines published by the NCCLS.[10]

Oxacillin (Sigma Co. St. Louis, USA) MICs were determined by broth macrodilution method as recommended by NCCLS.[10] We first determined MICs in all isolates with and without the addition of 2% NaCl to the test media. All isolates with oxacillin MIC> 8 μ g /ml in the presence of NaCl were defined as "intrinsic resistant".[11,12] For all other isolates, the true MICs were considered MIC values without the addition of NaCl. Isolates with oxacillin MIC<2 μ g/ml were considered as "sensitive", and isolates with MICs between 2 to 8 μ g/ml were considered as "borderline-resistant".[11,12] A direct inoculum from an overnight culture on tryptone soya broth (TSB) (Oxoid Co. Hampshire, UK) and a mid-log phase inoculum at a final concentration of 5? 10^5 were used. The antibiotics were diluted in Muller-Hinton broth (Difco Co, Detroit, USA.) with 2% NaCl. The trays were incubated at 35^0 C and readings were were done at 24 and 48h.[10,13]

β -lactamase production was assayed by the iodometry method. To define the role of β -lactamase in methicillin resistance, we also determined the MICs of "borderline-resistance" isolates after addition of the β -lactamase inhibitor, clavulanic acid (Gist-brocades Co.), to the test tubes at a concentration of 4 μ g/ml. Reduction in MIC of 2 or more dilutions was considered to be indicative of "acquired resistance".[13] Isolates with borderline resistance that either did or did not produce β -lactamase, and the inhibition of β -lactamase caused minimal or no reduction in MIC, were considered resistant by "MOD-type" or some other unknown mechanisms. MBCs were determined by transferring 100 μ l of broth from the MIC tubes with no visible growth to triptose-soy agar and counting the colonies after 24h of incubation. The ratio of MBC to MIC equal to or greater than 32 was assumed as "tolerance".[9] Muller-

Hinton agar supplemented with 4% NaCl and oxacillin 6µ g/ml was used for the agar screen plate, as recommended by the NCCLS.[10] In the agar screening test, the inoculum suspension was prepared by the direct inoculum preparation method by adjusting the turbidity of overnight growth medium to match that of a 0.5 McFarland standard (approximately 10^8 CFU/ml). This suspension was used to inoculate the oxacillin agar screen plate. Ten microliters of a 1:100 dilution of the test suspension was deposited on the agar screen surface, resulting in 10^4 CFU per spot. Test plates were incubated at 35^0C in ambient air and examined for any evidence of growth after 24h. If no growth was observed at 24h, the plates were incubated for an additional24h. Isolates that grew on the agar were considered "intrinsic resistant".

Results

MIC values of one hundred and six staphylococcal isolates to oxacillin are shown in Figure 1. Altogether, 40 (37.7%) isolates were methicillin-resistant. Thirty-five (33.0%) isolates had oxacillin MIC>8 µ g/ml and were designated as "intrinsic resistant". All 35 isolates grew on agar screen plate. Among the 10 isolates isolated from burn unit patients, 9 (90%) were intrinsic resistant; all with oxacillin MIC>128 µ g/ml. If burn unit isolates are excluded, the frequency of intrinsic resistant decreases to 27.0% (Table 2). Five (4.7%) isolates showed a "borderline oxacillin resistance" i.e., oxacillin MIC between 2 to 8 µ g/ml. After addition of clavulanic acid (4 µ g/ml), all these isolates showed marked decrease (greater than two-fold dilutions in their MICs), hence, all of them belonged to the category of "acquired resistant". Sixty-six (62.2%) isolates had oxacilin MIC<2 µ g/ml and were classified as methicillin-sensitive". None of the sensitive or borderline resistant isolates grew on the agar screen plate. Among isolates examined in this study, only one strain (with borderline resistance) showed the property of "tolerance" (MIC=8µ g/ml, MBC=256 µ g/ml, MBC/MIC=32). It was isolated from a neonate with an infected surgical wound admitted in NICU. MBC to MIC ratio for all other isolates were no more than 4 and for most isolates MBC was exactly equal to MIC.

The prevalence of intrinsic-resistance varies according to the site of infection (Table 3) the highest prevalence occurring in isolates isolated from ascitic fluid and wounds (60.0% and 58.8% respectively).

To differentiate the intrinsic-resistant MRSA from other isolates, MIC determination and agar screen plate method were used. Among isolates examined in this study, there was an absolute correlation between the results obtained by these two methods. All isolates with oxacillin MIC> 8 µ g/ml grew on agar screen plate and all isolates growing on agar screen plate had an oxacillin MIC >8 µ g/ml.

To evaluate the role of sodium chloride in the expression of resistance trait, MICs were determined with and without addition of NaCl. In 87(82.0%) isolates, NaCl had no effect on MIC . Fifteen (22.7%) of sensitive and four (11.4%) of the resistant isolates, however, demonstrated some increase in MIC (one or two dilutions) in the presence of sodium chloride. None of the isolates showed any decrease in MIC in the presence of NaCl. Thus, it seems that sodium chloride enhances resistance to oxacillin in both methicillin-sensitive and resistant isolates *(P<0.05)*. The results of antibiotic sensitivity tests are shown in Table 4. Since the pattern of antibiotic sensitivity of borderline-resistant isolates was similar to sensitive isolates, they are regarded as "methicillin-sensitive" in the susceptibility test results.

All methicillin sensitive *S.aureus* isolates were highly sensitive to the first generation cephalosporins and gentamicin, although approximately all MRSA isolates were resistant to these agents (cephalothin 91.5%, cephalexin 94.3%, and gentamicin 91.5%). The aminoglycoside antibiotic, amikacin, though more effective than gentamicin against MRSA, showed poor activity against methicillin-sensitive isolates which was second only to penicillin.

Vancomycin and rifampin were the most active antibiotics used in this study and all isolates were uniformly susceptible to both agents regardless of methicillin sensitivity or resistance (vancomycin 100%, rifampin 100%).

Chloramphenicol represented the third most active agent. Only 6 (5.6%) isolates, all methicillin-resistant with very high MICs (>128 μ g/ml) were resistant to chloramphenicol. Clindamycin and lincomycin also showed an acceptable activity against both MRSA and MSSA. In the case of MRSA, clindamycin seemed to be superior to lincomycin, but the difference was not statistically significant*(P= 0.45)*. Ciprofloxacin and TMP-SMZ also seem to be relatively good anti-staphylococcal agents. The overall number of resistance to these agents were 14 (13.2%) and 16 (15.0%) respectively. No resistant strain was seen in the MSSA group, although in the case of MRSA, there was a relatively high rate of resistance (40.0% and 45.8% respectively) to both agents. Erythromycin showed very poor activity against MRSA and 33 (94.2%) out of 35 isolates were resistant, but in the case of MSSA, only 3 (4.2%) resistant isolates were seen and most isolates were sensitive to erythromycin. Among antibiotics used in this study, penicillin showed the least anti-staphylococcal activity and over 91.5% of MSSA and 100% of MRSA isolates were resistant to this agent.

Discussion

Among *S. aureus* isolates collected over a one-year period in this study, 33% were classified as "intrinsic-resistant MRSA" by laboratory analysis. It appears that MRSA has emerged as an important endemic pathogens in our hospitals. It is especially problematic in burn unit patients, where the rate of wound infection caused by MRSA was as high as 90%. The reason is multi-factorial such as widespread use of antibiotics (especially β -lactams), prolonged hospital stay, and absence of any control measures and screening procedures to find carrier state among staff and personnel, all contribute to the emergence and spread of MRSA in our burn unit.

The prevalence of MRSA in patients admitted in other departments was 27%, analogous to the reports from high prevalence countries in Europe (Austria 21.6%, Belgium 25.1%, Spain 30.3%, and France 33.6%).[14]

Although the specimens collected in this survey were isolated from large university affiliated hospitals, including a burn unit, such a high prevalence of resistance should not be overlooked.

All borderline-resistant isolates in this study belonged to the "acquired-resistant" class of MRSA. None of the isolates showed the characteristics of "MOD-type resistance". The clinical significance of borderline-resistance has not been clearly understood and remains to be determined.

An interesting observation was the very low frequency (0.9%) of "tolerance" among the isolates. Compared to the very high rate of "tolerance" reported in some surveys (up to one half to two thirds of clinical isolates,[15] the reason for such a low prevalence of "tolerance" among our staphylococcal isolates is not clear.

The accurate detection of MRSA isolates is also a major problem for most clinical microbiology laboratories because of the heterogeneous nature of methicillin-resistance. Because the presence of PBP-2a is synonymous with the concept of" methicillin-resistance", detection of its genetic determinant (*mec*) provides an accurate method for identification of MRSA, independent of environmental conditions that may affect the phenotypic expression of resistance.[16] In view of the fact that this procedure and its necessary supplies are not currently available in most microbiological laboratories, particularly those in

the developing countries the only practical methods for detection of MRSA are MIC determination and agar screen plate method.[16] Among the studied staphylococcal isolates, there was an absolute correlation between the results of agar screen plate and MIC determination i.e., all isolates growing on agar screen plate had oxacillin MIC>8 μ g/ml and vice versa. Thus, with regard to the difficult and time consuming procedures associated with the MIC method, the agar screen plate seems to be the practical method of choice for laboratory identification of the methicillin-resistant *S. aureus*. It is very simple and inexpensive and can be easily performed in most clinical microbiology laboratories. The effect of sodium chloride in the phenomenon of methicillin-resistance was ascertained by an increase in MIC in about 11% of resistant isolates in the presence of NaCl. MIC of some sensitive and borderline-resistant isolates showed only a modest increase with the addition of sodium chloride, probably due to enhancement of β -lactamase production. As already described, sodium chloride exerts its effect by stimulating PBP2 a production and so enhances methicillin-resistance. Although, none of our resistant isolates passed into the "borderline-resistant" or sensitive group in the absence of NaCl, it theoretically may occur. The addition of sodium chloride seems to increase the sensitivity of the test and so is necessary if we are to precisely differentiate methicillin-resistant from sensitive isolates. The pattern of antibiotic sensitivity of *S. aureus* differs widely between methicillin-sensitive and resistant isolates. Except for penicillin and to some extent amikacin, most methicillin-sensitive isolates were susceptible to nearly all antibacterial agents used in this study. In contrast, in the case of MRSA, multiple-drug resistance was the rule and only few antibiotics were active against methicillin-resistant isolates. All cases of MRSA, even those with very high oxacillin MIC (>128 μ g/ml), were uniformly susceptible to vancomycin and rifampin. Chloramphenicol (82.8%) also showed excellent activity against methicillin-resistant isolates, although the results of clinical trials that used chloramphenicol for treatment of MRSA infections were disappointing.[17] Clindamycin was also active against most MSSA and MRSA isolates (97.1% and 71.4% respectively). Opinions vary on the efficacy of clindamycin as an alternative agent for treatment of infections caused by *S. aureus*. Early clinical studies attested to its efficacy in treatment of *S. aureus* infections but problems with toxicity[18] and reports of emergence of resistance during therapy[19] dampened initial enthusiasm for clindamycin as an alternative therapy for infections caused by *S. aureus*.[11] There are few clinical reports on the use of ciprofloxacin for treatment of infections caused by MRSA.[20] In this study we have found relatively high rate of resistance (40%) to ciprofloxacin among methicillin-resistant isolates. This is disconcerting because the antibiotic has a novel structure and has only recently entered clinical use. MSSA isolates, on the other hand, were uniformly sensitive to the antibiotic. TMP-SMZ, like ciprofloxacin, was active against all MSSA isolates. The rate of resistance among MRSA isolates to TMP SMZ was high (45.8%), however, it is one of the few antibiotics with clinically documented efficacy for treatment of infections caus