β-lactamase typing by substrate hydrolysis in clinical isolates of methicillin resistant *Staphylococcus aureus*

Katayoun Borhani, Fereshteh Eftekhar[*], Somayeh Danesh Monfared

Department of Microbiology, Faculty of Biological Science, Shahid Beheshti University, G.C., Tehran, Iran

ABSTRACT

Background: *Staphylococcus aureus* is one of the most important causes of nosocomial infections and most clinical isolates are multidrug resistant. Resistance to β-lactam antibiotics is most often due to bacterial β-lactamase production. Characterization of β-lactamases is important for choosing appropriate antibiotic therapy.

Materials and methods: Thirty methicillin resistant *Staphylococcus aureus* (MRSA) were identified by standard biochemical methods. Antibacterial susceptibility to 9 β-lactam antibiotics was determined. β-lactamase production was shown in all isolates using the colony iodometric test and nitrocefin discs. β-lactamase typing was carried out by measuring the relative substrate hydrolysis rates.

Results: The MRSA isolates were resistant to the majority of β-lactam antibiotics. The results showed that 90% of the isolates displayed type A substrate hydrolysis profile of β-lactamase.

Conclusion: The alarming high level of resistance to β-lactam antibiotics including methicillin and 3rd generation β-lactams show the need for extensive studies on alternative treatment protocols and use of new drugs.

Keywords: *Methicillin resistant Staphylococcus aureus (MRSA), Antibiotic resistance, β-lactamase typing.*
(Iranian Journal of Clinical Infectious Diseases 2009;4(4):202-206).

INTRODUCTION

Staphylococcus aureus is an important cause of food poisoning, pneumonia, postoperative wound infections and nosocomial bacteremia. These organisms are frequently resistant to most of the commonly used antibacterial agents including β-lactam antibiotics such as methicillin (oxacillin) (1). The mechanism of staphylococcal resistance to methicillin is considerably different from other β-lactams. Methicillin resistant *S. aureus* (MRSA) produce a unique penicillin-binding protein, PBP2a, which has a much lower affinity for beta-

lactam antibiotics. On the other hand, the most common mechanism of bacterial resistance to β-lactams is their inactivation by β-lactamases (2-4). Over 90% of *S. aureus* isolates are β-lactamase producers (5). Richmond divided β-lactamases into four serological types, designated A to D. This typing method was lost when the original antisera were exhausted. Kernodle et al. used a kinetic method to type staphylococcal β-lactamases and measured the rate of hydrolysis of certain β-lactams by different β-lactamases (6). In their method, purified β-lactamases were extracted from *S. aureus* isolates and differences in the substrate specificity of the variant enzymes were

demonstrated. Their results corresponded to those previously found by serotyping (5).

The aim of the present study was to identify β-lactamase production in clinical isolates of MRSA and to determine the prevalent β-lactamase type by measuring substrate hydrolysis rates.

MATERIALS and METHODS

Thirty clinical MRSA specimens were selected from a clinical collection of bacteria from two hospitals in Tehran (Shohadae Tajrish and Taleghani). Identification of *S. aureus* isolates was carried out using the standard biochemical tests including catalase and coagulase production, acid production from mannitol, DNase production and resistance to bacitracin discs (0.04 U, Padtan Teb, Tehran, Iran).

Antimicrobial susceptibility test was carried out for ampicillin (10μg), penicillin (10U), oxacillin (1μg), cefazolin (30μg), cefalexin (30μg), ampicillin-sulbactam (30μg), cefotaxime (30μg) (Padtan Teb, Tehran, Iran), ceftazidime (30μg) and amoxiclav (30μg) (Difco, Kansas, USA) by disc diffusion according to the CLSI guidelines (7). Minimum inhibitory concentrations (MIC) were also measured for methicillin (Sigma chemicals, Willetton, Germany) in Mueller-Hinton broth (Merck, Germany) supplemented with 2% NaCl, as recommended for susceptibility testing of MRSA (7-9). A standard culture of *S. aureus* (ATCC 25923) was used as control for all antibiotic susceptibility assays as well as a negative control for β-lactamase production.

The colony iodometric assay was used for β-lactamase detection. This method is based on the detection of penicillanic acid, which results from the action of β-lactamase on penicillin (10,11). Briefly, starch containing paper strips was soaked in a solution of potassium penicillin G (0.01g/ml) in 0.1M phosphate-buffered (PB, pH:7) for 10 min and isolated colonies from overnight cultures were transferred onto paper strips. After 30-40 min incubation at 37ºC, the papers were flooded with Gram's iodine solution. Colorless zones around colonies meant β-lactamase production by the organism and for organisms that did not produce β-lactamase, the paper remained purple.

Chromogenic cephalosporin β-lactamase assay was another test for β-lactamase detection. In this method, all isolates were examined for β-lactamase production using nitrocefin discs (Cefinase discs, Difco, Kansas, USA). The discs were moistened using Phosphate buffer and a loopful of overnight grown bacterial colonies was placed on the discs. Cefinase discs were examined after 1h for a change of color (yellow-to-red) as evidence for β-lactamase production (10,11).

For β-lactamase typing, substrate hydrolysis assay of Kernodle et al. was used (5). Briefly, the isolates were grown for 14h at 37°C on 1% CY agar containing 0.5 μg/ml methicillin. Bacterial suspensions in 0.1 M PB (pH, 6.0) were adjusted to an optical density of 0.4 at 415nm. Hydrolysis rates of cephaloridine (Sigma Chemicals, Willetton, Germany), cephazolin (Loghman Adham, Tehran, Iran) and cefamandole (ACS Dobfar, Italy) were measured by mixing 667μl of the bacterial suspensions with 333μl of 0.3mM antibiotic in 0.1M PB (pH, 6.0) at 37 ºC for 1h. The absorbance was monitored at 254nm for cephaloridine, 272nm for cephazolin and 269nm for cefamandole. Nitrocefin (Calbiochem, California, USA) hydrolysis was monitored at 482nm after mixing 100μl of bacterial suspensions with 900μl of 0.11mM antibiotic solution and incubation at 37ºC for 1h. Spectrophotometric assays were carried out using a Shimadzu UV-120-02 Spectrophotometer (5).

SHV-1 β-lactamase from *E.coli* harboring plasmid pK571 and TEM-1 β-lactamase from *E. coli* J53-2 harboring plasmid pBR322 were used as type A control β-lactamases (12-14). The hydrolysis profile of the control enzymes was carried out in 0.03M Tris- HCl (pH, 8) (15).

RESULTS

All MRSA clinical isolates were ß-lactamase producers. The majority of the isolates were from wound specimens (44%) followed by blood (23%), secretions (17%), urine (7%), pharynx, CSF and abscesses (3% each).

Figure 1 shows the antibacterial susceptibility results. The highest rate of resistance was observed with ampicillin, penicillin and oxacillin (100%). Overall, 44% of the isolates were resistant to all tested ß-lactam antibiotics and 4 different resistance patterns were observed as shown in table 1. The MIC values measured for methicillin were higher than 64μg/ml for all isolates showing 100% resistance.
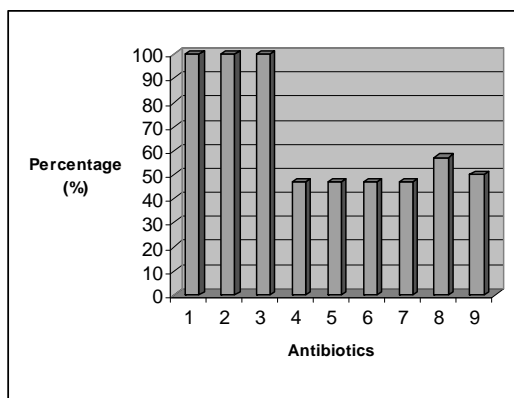

Figure 1. The rate of antibiotic resistance among 30 methicillin resistant clinical isolates of *S. aureu*s.
(1:ampicillin; 2:penicillin; 3:oxacillin; 4:cefazolin; 5:cefalexin; 6:ceftazidime; 7:cefotaxime; 8:amoxiclav; 9:ampicillin-sulbactam)

Table 1. Antibiotic resistance patterns of ß-latamase producing methicillin resistant S. aureus by disc diffusion. Four multidrug resistance patterns are shown with the majority of the isolates belonging to resistance groups 1 and 3.

Antibiotics	Number (%) of isolates	Resistance group
Amp/Pen/Ox/Cfz/Cn/ Caz/Ctx/Amc/Sam	14(47)	1
Amp/Pen/Ox/Amc/Sam	1(3)	2
Amp/Pen/Ox/Amc	2(7)	3
Amp/Pen/Ox	13(43)	4

Amp: ampicillin; **Pen**: penicillin; **Oxa**:oxacillin; **Cfz**:cefazolin; **Cn**:cefalexin; **Caz**:ceftazidime; **Ctx**:cefotaxime; **Amc**:amoxiclav, **Sam**:ampicillin-sulbactam.

Among the ß-lactamase producing isolates, 90% had the substrate hydrolysis profiles resembling the type A ß-lactamases, SHV-1 and TEM-1 as shown in figures 2 and 3. Only 3 isolates (TH$_1$, TH$_{10}$ and SH$_{24}$) showed different ß-lactamase activities (figure 2,3).
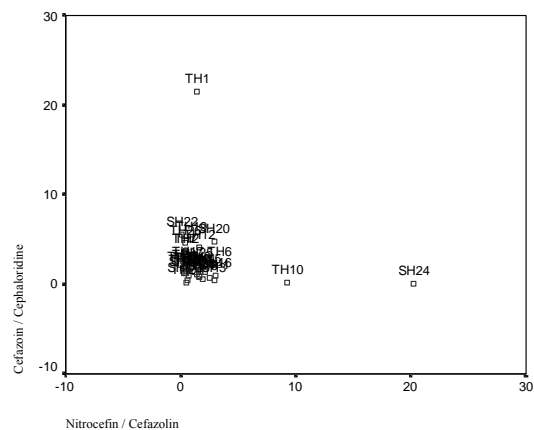
Figure 2. Typing of *S. aureus* β-lactamases by measuring the substrate hydrolysis rates. Hydrolyses of cefazolin, cephaloridine and nitrocefin by cell suspensions were measured and the ratios are shown.
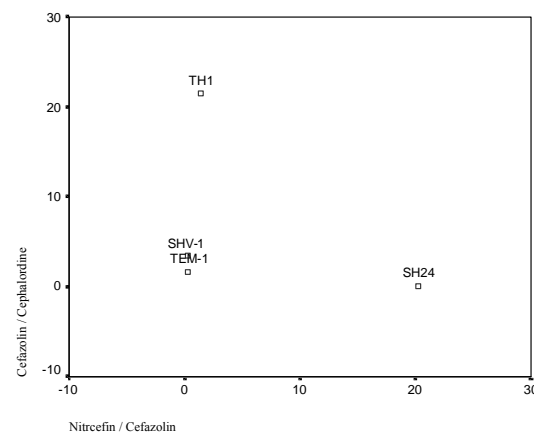
Figure 3. Ratios of the hydrolysis rates measured for SHV-1 and TEM-1 β- lactamases as well as two clinical isolates (TH-1 and SH 24).

DISCUSSION

S. aureus is one of the most common pathogens causing nosocomial infections. Unfortunately, most of the isolates are resistant to methicillin as well as being multidrug resistant (16). Additionally, almost all of the clinical isolates produce β-lactamases

which makes them resistant to new generation penicillins and cephalosporins. In the present study, the majority of the clinical isolates were recovered from wound specimens similar to reports from India and Nigeria (17,18).

The global spread of MRSA has caused one of the most serious challenges to the treatment of *S. aureus* nosocomial infections during the past decade (16,19). The rate of methicillin resistance among human isolates of *S. aureus* was shown to be 50% in USA (1998), 77% in a Taiwanese hospital (2001), 24.6%, over 50% and 61.45%, in Pakistan, Korea and Egypt, respectively (1,16,20,21). In Iran, 35% MRSA was reported in 2002 (22). However, the rate of MRSA in Oman and the Gulhane Military Medical Academy (Istanbul, Turkey) was 95 and 100%, respectively (21). Different distributions of MRSA may be attributed to epidemiological reasons and most importantly, different antibiotic treatment regimes in different societies (18). Mokaddas and Gang reported that most of the MRSA isolates were also multidrug resistant (21).

In the present study, we selected thirty methicillin resistant isolates of *S. aureus* and found that 47% were resistant to all test antibiotics.

The prevalence of β-lactamase production in clinical isolates of *S. aureus* has risen from 5% in 1946 to 50% in 1950 and currently, more than 90% (23). In our study, all isolates produced β-lactamases. Bonfiglio and Kernodle reported that 57% and 48% of their clinical isolates were β-lactamase producers, respectively (5,24).

Measuring the relative rates of substrate hydrolysis for β-lactamase typing, we showed that with the exception of 3 isolates, the rest of the test organisms (91%) were type A β-lactamase which is the most common serological type among β-lactamases (25). Considering that the work of Richmond on serotyping could not be continued due to difficulties in raising larger amounts of specific antibodies, we believe that relative substrate hydrolysis rates can produce the same information for β-lactamase typing.

In conclusion, we found an alarming rate of β-lactamase production in our MRSA clinical isolates of *S. aureus*. Additionally, nearly half of the isolates were resistant to all β-lactam antibiotics tested. This study emphasizes the importance of proper antibiotic selection for treatment of nosocomial staphylococcal infections. It is extremely important to determine the resistance profiles prior to antibiotic therapy, to avoid increased drug resistance in these organisms.

ACKNOWLEDGEMENT

REFERENCES

1. Lee JH. Methicillin (oxacillin) resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Env Microbiol. 2003;69:6489- 94.

2. Phillips OA, Czajkowski DP, Spevak P, Singh MP, Hanehara- Kunugita C, Hyoda A, et al. SYN1012: A New β-lactamase Inhibitor of Penem Skeleton. J Antibiotic. 1997;50:350- 6.

3. Chen CCH, Herzberg O. Inhibition of β-lactamase by clavulanate. J Mol Bio. 1992;224:1103-13.

4. Gardam MA. Is methicillin resistant *Staphylococcus aureus* an emerging community pathogen? A review of literature. Can J Infect Dis. 2000;11:202-11.

5. Catherall EJ, Irwin R, Mizen LW. Efficacy of amoxicillin/clavulanic acid in experimental *Staphylococcus aureus* endocarditis in the rat. J Antimicrob Chemother. 1991;27:117-26.

6. Kernodle DS, Stratton CW, McMurray LW, Chipley JR, McGraw PA. Differentiation of β-lactamase variants of *Staphylococcus aureus* by substrate hydrolysis profile. J Infect Dis. 1989;159:103-8.

7. National Committee for Clinical Laboratory Standards (2000) Performance standards for antimicrobial disc susceptibility tests, Approved Standard. 7th ed. (M2-A7). Villanoa, USA.

8. National Committee for clinical Laboratory Standards (2000) Method for dilution antimicrobial susceptibility tests for bacteria that aerobically, 5th ed. Approved Standards (M7- A5), vol. 20, no. 2. Wayne; Pennsylvania, USA.

9. Franciolli M, Bille J, Glauser MP, Morillon P. β-lactam resistance mechanisms of methicillin- resistant *Staphylococcus aureus*. J Infect Dis. 1991;163:514-23.

10. Doern GV, Tubert TA. Detection of β-lactamase activity among clinical isolates of *Branhamella catarrhalis* with six different β-lactamase assays. J Clin Microbiol. 1987;25:1380-3.

11. Mahon CR, Manuselis G, editors. Textbook of diagnostic microbiology. 2nd edition. Philadelphia:W. B. Saunders; 2000:89-90.

12. Bush K, Jacoby GA, Medeiros AA. A Functional classification Scheme for β-lactamases and its correlation with molecular structure. Abtimicrob Agents Chemather. 1995;39:1211-33.

13. Li Q, Lee JY, Castillo R, Hixon MS, Pujol C, Doppalapudi VR, et al. NB 2001, a novel antibacterial agent with broad–spectrum activity and enhanced potency against β-lactamase–producing strains. Antimicrob Agents Chemother. 2002;46:1256-68.

14. Sabbagh Y, Theriautt E, Sanschagrin F, Voyer N, Palzkill T, Levesque RC. Characterization of a PSE – 4 mutant with different properties in relation to penicillanic acid Sulfones: importance of residues 216 to 218 in class A β-lactamase. Antimicrob Agents Chemother. 1998;42:2319-25.

15. Schaberg DR, Power G, Betzold J, Forbes BA. Conjugative R plasmids in antimicrobial resistance of *Staphylococcus aureus* causing nosocomial infections. J Infect Dis. 1985;152:43-9.

16. Rushdy AA, Salama MS, Othman AS. Detection of methicillin/oxacillin resistant *Staphylococcus aureus* isolated from some clinical hospitals in Cairo using *Meca/Nuc* genes and antibiotic susceptibility profile. Int J Agri Biol. 2007;9:800-6.

17. Orrett FA, Land M. Methicillin resistant *Staphylococcus aureus* prevalence: current susceptibility patterns in Trinidad BMC Infect Dis. 2006;6:1-6.

18. Ikeagwu IJ, Amadi ES, Iroha IR. Antibiotic sensitivity pattern of *Staphylococcus aureus* in Abakaliki, Nigeria. Pac J Med Sci. 2008;24:231-5.

19. Mulla S, Patel M, Shah L, Vaghela G. Study of antibiotic sensitivity pattern of methicillin resistant *Staphylococcus aureus.* Indian J Crit Care Med. 2008;11:99-101.

20. Chambers HF. The changing epidemiology of *Staphylococcus aureus*? Emerg Infect Dis. 2001;7:178-82.

21. Naqvi ZA, HAshmi K, Kharal SA. Methicillin resistant *Staphylococcus aureus* in burn patients. *Pac J Pharma.* 2007;24:7–11.

22. Rahbar M, Yaghooti M, Kia-Darbandsari B. Prevalence of nasal carriage of *Staphylococcus aureus* and susceptibility of isolates to methicillin and mupirocin among healthcare workers in an Iranian hospital. Infect Control Hosp Epidemiol. 2006;27:323-4.

23. Lorian V, editor. Antibiotics in laboratory medicine. 4th edition. New York. William & Wilkins;1996:511-33.

24. Kernodle DS, Mcgraw PA, Stratton CW, Kaiser AB. Use of extracts versus whole-cell bacterial suspensions in the identification of *Staphylococcus aureus* β-lactamase variants. Antimicrob Agents Chemother. 1990;34:420-5.

25. Richmond MH. Wild- type varients of exopenicillinases from *Staphylococcus aureus*. Biochem J. 1965;94:584-93.