Study on Vancomycin-Resistant *Staphylococcus aureus* and Identification of VanA Gene in These Strains Isolated from Tabriz Shuhada Hospital Using E-Test and PCR Methods

Sughra Sheikh Muneeri[1], Haedeh Mobaiyen[2], Hamid Mirzaie[3]

1- M.S in Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran
2- Department of Microbiology, Faculty of Medical Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran
3- Department of Food Hygiene, Faculty of Veterinary Medicine, Tabriz Branch, Islamic Azad University, Tabriz, Iran
Email: drhmobaiyen@iaut.ac.ir

Abstract: *Staphylococcus aureus* is one of the main causes of hospital-acquired infections. Vancomycin is widely used in treating infections created by this organism which increases vancomycin-resistance and the spread of *S. aureus* strains with intermediate vancomycin resistance in different parts of the world has created major concerns about the clinical samples. Gathering data on the situation of drug resistance - especially vancomycin-resistance of these organisms not only is considered an effective measure to prevent hospital infections but also as one of research priorities. In this study, the frequency of vancomycin resistance and the presence of vanA gene are evaluated in *S. aureus* strains isolated from Tabriz Shohada hospital with PCR. 73 strains of *Staphylococcus aureus* were isolated from hospitalized patients in Tabriz Shohada Hospital during a period of eight months. All of strains were identified through the routine methods. The resistance to vancomycin was identified through agar disk diffusion method and MIC of vancomycin was measured through E-test. Also, in order to identify vanA genes, specific primers were applied. The frequency of intermediate vancomycin-resistant strains using agar disk diffusion method and E-test are % 26.02 and % 14.43, respectively. The results of PCR indicate that 1.3% of strains are identified as vancomycin-resistant. VanA- positive strains were far more resistant against antibiotics than vanA- negative. During the present study vanA gene was identified in only one strain of isolated *S. aureus* with PCR. Separation of vanA gene in isolated strains rings the alarm for authorities to control infection in the given hospital and other medical centers in Tabriz.

[Sughra Sheikh Muneeri, Haedeh Mobaiyen, Hamid Mirzaie. **Study on Vancomycin-Resistant *Staphylococcus aureus* and Identification of VanA Gene in These Strains Isolated from Tabriz Shuhada Hospital Using E-Test and PCR Methods.** *Life Sci J* 2013;10(1):748-752]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 117

Key words: *Staphylococcus aureus*, Drug Resistance, vanA Gene, PCR, Vancomycin, E-test

1. Introduction

Staphylococcus aureus is one of the most common and important pathogens of nosocomial infections that due to variety of enzymes such as coagulase, hyaluronidase, nuclease, lipase, hemolysin and leukocidin can cause infection anywhere in the body (1). These bacteria are causative agent of wide range of human diseases, including endocarditis, food poisoning, toxic shock syndrome, septicemia, dermal infections, soft tissue infections and bone infections . (2). Because of acquired resistance to drought and increasing resistance to antimicrobial agents, it has been got as a major public health concerns and causes treatment failure in infections due to *S. aureus* (3). The first *S. aureus* isolates with reduced susceptibility to vancomycin (VISA) was reported in Japan in 1997 (4). In 2002, another vancomycin resistant *Staphylococcus aureus* (VRSA) strain was reported from America (5).

In 2002 and later, similar studies have conducted on resistance to vancomycin strains of *S. aureus* in Britain, America, Germany, France, India and other countries that in these reports, intermediate and resistance to vancomycin strains also were observed (6-8). Most of VRSA and VISA strains also are observed in isolates of methicillin-resistant Staphylococcus aureus (9). Genes involved in vancomycin resistance are known van gene which is cause resistant by inducing changes in the cell wall of S. aureus and the most important gene, vanA, which causes strange resistance to vancomycin and teicoplanin (10,11). This gene is located on Tn1546 transposons and also can be found on plasmid or chromosome and causes transfer of vancomycin resistant genes from one strain to another or even from one species to another species (12-14). Vancomycin resistant genes can be transferred from Enterococcus faecium to Staphylococcus aureus through the conjugative transposons (15). Vancomycin-resistant Staphylococcus aureus with VanA genotype are known in urinary tract infections, septicemia, and most infections caused by S. aureus (16-18). Resistance to Vancomycin in S. aureus is due to changes in the cell wall of bacteria. This organism has a thick cell wall that vancomycin binds to receptors occupied on the outer cell wall and inhibits its function (19). Detection

and isolation of VRSA strains can prevent from spread these strains in the hospital environment and reduce the rate of S. aureus infections. In this study, the prevalence of vancomycin-resistant Staphylococcus aureus strains isolated from patients in Shohada Hospital has been evaluated using agar disk diffusion method, E-test and PCR.

2. Materials and methods

Sampling and identification of strains:

In this study, during an 8-month period from 2011 to 2012, 73 strains of Staphylococcus aureus were isolated from clinical samples of Tabriz Shohada hospital. After collecting the samples, we used blood agar and mannitol salt agar media for isolating and purification of genera, and then were incubated for 24 hours in 37˚C (20, 21). We used of complementary tests such as gram staining, catalase test, DNase test and fermentation of mannitol and resistance to novobiocin and polymyxin B for confirmation of isolates. We attempted to make coagulase from isolates using human plasma, so, final step of confirmation was made (22, 23). All strains were numbered and were kept in freezer at -70˚C for next steps.

2.1. Antibiogram using disk diffusion method

Agar disk diffusion test was applied using discs prepared from Mast Co. (24). The antibiotic discs used in this study were linezolid (30 µg), vancomycin (30 µg), methicillin (5 µg), Ceftazidime (30 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg). It must remember, in this study, bacterial suspension equivalent to standard 0.5 McFarland has been used and results of antibiotic sensitivity were reported using the CLSI (Clinical and Laboratory Standards Institute's) criteria and data presented as sensitive, intermediate and resistant (24).

2.2. Determination of minimal inhibitory concentration (MIC) for vancomycin

E-test was used for determining MIC (AB biodisk Solna, Sweden). E-test method is a combination of agar disk diffusion method and determination of MIC. In this method, antibiotic is placed on the paper tape based on the gradient of concentration (25-27). To perform this procedure, the 18-hour bacterial culture on Tryticase Soy Broth (TSB) medium was made and a bacterial suspension equivalent to a standard 0.5 McFarland was prepared and then using a swab bacteria were cultured in Brain Heart Infusion (BHI) Agar. Vancomycin-stained E-test strips with ascending concentrations from 0.16 µg/ml to 256 µg/ml was occupied on BHI agar medium and was incubated at 37˚C for 24 hours. After 24 hours, the onset of inhibition zone is considered as the MIC of the isolate (25-27).

2.3. Extraction of DNA from isolates

Of cultures in BHI broth mediums, 1.5 ml was transferred into a microtube and was centrifuged at 13000 rpm for 10 minute. Then, a little liquid nitrogen was added into the sediment. Then, based on table 1, lysing buffer was added to sediment and was transferred into another microtube and was placed in the thermomixer at 80-85˚C for 1 hour. At the end, DNA was extracted using common method (28).

Table1: concentration of materials used in lysing buffer

Material	Amount /ml
Tris 1 M, PH= 7.5	1
Sodium chloride 5 M	1
EDTA 0.5 M	1
SDS 2%	2
Distilled water	9.5

2.4. Identification of vanA gene using PCR

For executing PCR, we used recommended primers for vanA gene (29, 30) as following sequence:

F 5'-CATGAATAGAATAAAAGTTGCAATA-3'
R 5'-CCCCTTTAACGCTAATACGACGATCAA-3'

Compound used in PCR was 10 mmol of Tris-Hcl PH= 8.3, 50 mmol of KCL, 2mmol of magnesium chloride, 0.2 mmol of each dNTP, 1 unit of Taq polymerase, 10 picomole of each primer and 1µl of DNA sample. This mixture was placed in the thermalcycler and was underwent primary denaturation at 94˚C for 10 minutes, second denaturation at 94˚C for 30 seconds, annealing to target DNA at 50˚C for 45 seconds and extension at 72˚C for 30 seconds. Final extension stage was followed at 72 ˚C for 10 minutes (31).

2.5. Identification of PCR product

For assessment of the product of PCR, 1% agarose gel prepared and 10 µl of the PCR product obtained from S. aureus with 1 µl loading buffer, positive control (plasmid obtained from the Pastor Institute) and negative control (distilled water) were transferred into the wells of gel. Electrophoresis was relayed at the voltage 90-100. Then was stained by ethidium bromide for 30 minute and the results were read by gel document apparatus (32).

3. Results

Results of sampling:

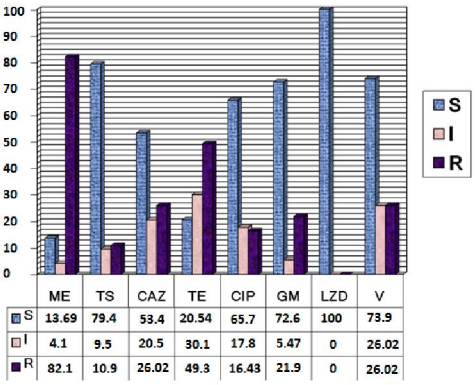
Of 1038 samples referred to laboratory, 73 of them were identified as *S. aureus* that are given in table 2 as detailed.

Table 2: the prevalence of *S.aureus* isolated from clinical samples obtained from patients

Type of sample	No.	%
Wound	14	19.1
Abscesses	9	12.3
Pulmonary discharges	8	10.9
Blood	13	17.8
Synovial liquid	5	6.8
Throat	2	2.7
Urine	11	15.06
CSF	1	1.3
Bone marrow	1	1.3
Femur discharge	3	1.4
Trachea	6	8.2

3.1. Results of Antibiogram

The resistance of isolates is given in the diagram 1.



	ME	TS	CAZ	TE	CIP	GM	LZD	V
S	13.69	79.4	53.4	20.54	65.7	72.6	100	73.9
I	4.1	9.5	20.5	30.1	17.8	5.47	0	26.02
R	82.1	10.9	26.02	49.3	16.43	21.9	0	26.02

Diagram 1: Antibiogram results of S. aureus strains isolated from Shohada Hospital,Tabriz.

3.2. Results of E-test

Considering that Antibiogram is not enough for determination of resistance of isolates to vancomycin, so, using the vancomycin E-test Strips, MIC for strains was calculated. In this survey, MIC≤4 was considered as susceptible strains, MIC4-8 was considered as intermediate strains (VISA) and MIC≥8 was considered as resistant strains (VRSA) (33). Data is given in table 3.

Table 3: results of MIC for vancomycin using E-test in S.aureus isolated from Shohada Hospital, Tabriz.

code of samples	MIC (µg/ml)	No. of strains	%
5-8-9-11-15-17-26-29-33-36-42-48-49-52-55-63-67-70	1.5	18	24.6
1-7-10-14-16-18-25-27-28-30-32-34-40-43-44-53-54-56-69-71	2	20	27.3
2-13-19-41-45-61-62-65-68-73	2.5	10	13.6
12-35-37-46-50-51-56-58-66	3	9	12.3
3	3.5	1	1.3
6	4	1	1.3
4-21-22-23-38-39-47-57-60	4.5	9	12.3
20-31	6	2	2.7
64	8	1	1.3

3.3. Results of assessment of vanA gene by PCR

Considering MIC= 4.5-8 µg/ml as suspect species, number of 12 strain were detected as suspected strain using E-test and were selected for PCR. In the PCR, of 12 samples, only 1 of them showed a band similar to control strain as seen in figure 1. So, 1 strain (1.36%) had vanA gene.

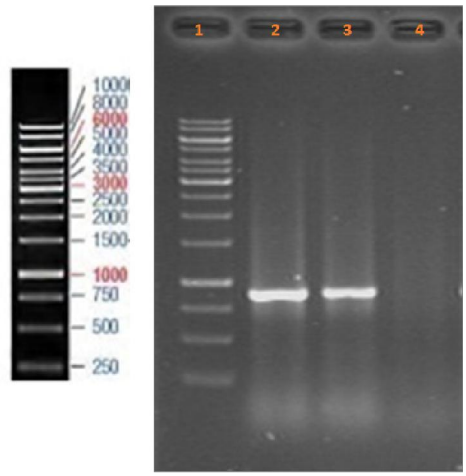


Figure 1: analysis of electrophoresis of PCR product for vanA gene. 1: 1kb ladder (Fermentase Co.), 2: plasmid of standard strain, 3: clinical positive samples, 4: negative sample (distilled water)

4. Discussion

Staphylococcus aureus is one of the important human pathogens that in last decades were as causative agent of community and hospital acquired infections and its resistance is increases against β-lactams antibiotics and vancomycin (34). After the first report of VRSA in Japan in 1997, however prevalence of VISA and VRSA strains remained low, but in many countries is rising as hVISA (35). There are reports that indicate failure of therapy with vancomycin caused to emerging hetrogenous vancomycin and Intermediate *S. aureus* strains (36, 37). In present study, of 73 strains of *S.aureus*, 19 strains (26.02%) showed intermediate resistance to vancomycin in disk diffusion method.

In 2006, Hara Krishna et al., using the disk diffusion method showed that of 783 strains of *S. aureus*, 6 of them (0.7%) had intermediate resistance or was resistant to vancomycin (6). In a study conducted by Shahin Najar Pirae et al., (2009), of 174 strains of *S. aureus*, intermediate or resistant against vancomycin was not reported (38). In the present study, 26.02% of the isolates were reported as VISA or VRSA that in contrast to the above mentioned studies, the most frequency of resistance was shown. Considering that disk diffusion method is used widely in the diagnostic laboratories but it may show false positive and false negative results and also its

sensitivity is low against heterogenic strains. On the other hands, bacterial load that is inoculated, depth of medium and disks used can affect results (38). So, for confirmation we have to use tests recommended by CDC including reference broth, macro-dilution, screening tests in BHI medium with 6 µg/ml concentration and finally E-test (38). In the E-test, 11 of isolates (15.06%) were reported as suspicious for VISA that these strains had MIC 4 µg/ml for vancomycin and 1strain (1.36%) with MIC 8µg/ml was reported as VISA. In this study, for confirmation we used all of the 12 samples for electrophoresis of vanA gene. Hiramatsu et al., (1997), reported the first vancomycin-resistant strains with MIC≥8 µg/ml using the E-test from Japan (9). In a research by Ahmadi et al., (2008), only 2 strains of hVISA with MIC=4 µg/ml was reported (39). The first vancomycin-resistant strains were reported from Japan (9). In another study by Hussainzadeghan et al., (2007) it has been showed that of 64 *S.aureus* isolates, 4 (1.33%) were identified as intermediate-resistant to vancomycin (33). In our study, the E-test result is compatible with their findings. In this study, all strains were shown MIC> 4 µg/ml selected for identification the vanA genes by PCR and only 1 strain (1.36%) showed vanA gene. In a study by Aligholi et al., (2008) in Tehran, 2 cases (1.34%) were identified as vancomycin-resistant Staphylococcus aureus (40). Isolating the VanA genes in isolated strains from Tabriz Shohada Hospital was an alert for hospital authorities.

Acknowledgment

Authors wish to thank from technical supports of Mr. Sadighbayan "MS.c of microbiology, Islamic Azad University, faculty of medicine, Tabriz Branch" Mr. Barzghar "MS.c of microbiology" Dr. Farajli "veterinarian" and honorable staffs of Dr. Mozaffari pathobiology laboratory, and all persons who helped us in this research.

References

1. Anderson N, Pearsall R. Microbiology a human perspective. 3rd ed. Boston: MC Graw Hill; 2001.
2. Koreen L, Ramaswamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN. spa Typing Method for Discriminating among Staphylococcus aureus Isolates: Implications for Use of a Single Marker To Detect Genetic Micro- and Macrovariation. J Clin Microbiol 2004;42(2):792-9.
3. Maleki Z, Anjarani S. Comparison of two antimicrobial susceptibility tests, disk diffusion and E-test for determining oxacillin and vancomycin susceptibility. Med Sci J Islamic Azad Uni-Tehran Medical Branch 2004;26:211-215.
4. Maranan MC, Moreira B, Boyle-Vavra S, Daum RS. Antimicrobial resistance in staphylococci. Epidemiology, molecular mechanisms, and clinical relevance. Infect Dis Clin North Am 1997;11:813-849.
5. Centers for Disease Control and Prevention. Vancomycin resistant Staphylococcus aureus Pensilvania. MMWR Morb Mortal Wkly Rep 2002;51:902.
6. Tsuji BT, Rybak MJ. Etest synergy testing of clinical isolates of Staphylococcus aureus demonstrating heterogeneous resistance to vancomycin. Diagn Microbiol Infect Dis 2006;54(1):73-7.
7. Galani I, Kontopidou F, Souli M, Rekatsina PD, Koratzanis E, Deliolanis J, Giamarellou H. Colistin susceptibility testing by Etest and disk diffusion methods. Int J Antimicrob Agents 2008;31(5):434-9.
8. Denis O, Nonhoff C, Baudouin B, Knoop C, Bobin-Dubreux S, Struelens MJ. Emergence of vancomycin-intermediate Staphylococcus aureus in a Belgian hospital, microbiological and clinical features. J Antimicrob Chemotherapy 2002;50:383-391.
9. Walsh TR, Howe RA. The prevalence and mechanism of vancomycin resistance in Staphylococcus aureus. Annu Rev Microbiol 2002;56:657-75.
10. Ito T, Katayama Y, Hiramatsu K. Cloning and nucleotide sequence determination of the entire mec DNA of pre-methicillin-resistant Staphylococcus aureus N315. Antimicrob Agents Chemother 1999;43(6):1449-58.
11. Chang FY, MacDonald BB, Peacock JE, Musher DM, Triplett P, Mylotte JM, O'Donnell A, Wagener MM. A prospective multicenter study of Staphylococcus aureus bacteremia: incidence of endocarditis, risk factors for mortality, and clinical impact of methicillin resistance. Medicine 2003;82(5):322-32.
12. Maria DA, Citron DM, Kwok R. Evaluation of the velogene genomic assay for detection of vanA and vanB genes in vancomycin-resistant Enterococcus Species. J Clin Microbiol 2004;42:1751-1752.
13. Mendez-Alvarez S, Perez-Hernandez X, Claverie-Martin F. Glycopeptide resistance in Enterococci. Microbiol 2000,7252:71-80.
14. Murray BE.Vancomycin-resistant enterococcal infections. New Eng J Med 2000;32:557-567.
15. Garnier F, Gambarotto K, Denis F, Ploy MC. Molecular study of vancomycin-resistant enterococci isolated from humans and from food in a cattle-rearing area of France. J Antimicrob Chemother 2004;54(1):236-9.
16. Kavindra VS, Weinstock GM, Murray BE, An Enterococcus faecalis ABC Homologue (Lsa) is required for the resistance of this species to Clindamycin and Quinupristin- Dalfopristin. Antimicrob Agents Chemother 2002;46:1845-1850.
17. Van Horn KG, Gedris CA, Rodney KM. Selective isolation of vancomycinresistant Enterococci. J Clin Microbiol 1996;38:924-927.
18. Noble WC, Virani Z, Cree RG. Co-transfer of vancomycin and other resistance genes from Enterococcus faecalis NCTC 12201 to

Staphylococcus aureus. FEMS Microbiol Lett 1992;72:195-198.
19. Sieradzki K, Tomasz A. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of Staphylococcus aureus. J Bacteriol 1997;179:2557-2566.
20. Timothy F. Staphylococcus aureus. 2005. [On Line], Available from http://gsbs.utmb.edu/ microbook/cho012.htm.
21. Jawetz E, Melnick JL, Adelberg EA. Medical Micribiology, 23rd Ed, McGraw-hill, New York 2004;223-229.
22. Duguid JP, Fraser AG, Marmion BP. Staphylococcus: Cluster–Forming Gram-Positive Cocci. In: Collee JG: Practical Medical Microbiology, 13th Ed. Churchill Livingstone, New York 1989;303-316.
23. Bannerman T. Staphylococcus, Micrococcus, and Other catalase-positive Cocci that grow aerobically. In: Murray PA, Baron EJ, Jorgeson JH, Pfaller MA, Yolken RH. Manual of Clinical Microbiology, 8th Ed. ASM Press, Washington 2003;384-402.
24. Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing. 16th International supplement, CLSI document M100-S12, Pennsylvania, USA. 2002;22(1).
25. Manoharan A, Pai R, Sankar V, Thomas K, Lalita MK. Comparison of disk diffusion & E.test methods with agar dilution for antimicrobial susceptibility testing of haemophilusinfluenzae, Indian J Med Res 2003;81-87.
26. Sanchez L, Londono D, Arango AI, Mattar S. In vitro activity of antimicrobial agentsagainst mycobacterium tuberculosis Isolatesfrom Bogota, DC (Colombia) evaluatedby the E. test. Diagn Microbial Infect Dis 1999;35:109-112.
27. Hanberger H, Nilsson LE, Classon B, Karnell I, Larsson P, Rylander M, Svensson E. New species – related MIC break points for early detection of development of resistance among gram-negative bacteria in Swedish intensive care units. J Antimicrob Chemother 1999;44(5):611-619.
28. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning, A Laboratory Manual. 2nd edition. New York: Cold Spring Harbor Laboratory Press 1989; 1.21-1.52, 2.60-2.80, 7.3-7.35, 9.14-9.22.
29. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning: a Laboratory Manual Cold Spring Harbor: Cold Spring Harbor Laboratory Press 1982.
30. Clark NC, Cooksey RC, Hill BC, Swenson JM, Tenover FC. Characterization of glycopeptide-resistant enterococci from U.S. hospitals. Antimicob Agents Chemother 1993;37:2311-2317.
31. Tiwari HK, Sen MR. Emergence of vancomycin resistant Staphylococcus aureus (VRSA) from a tertiary care hospital from northern part of India. BMC Infectious Diseases 2006;6:156.
32. Jamshidian M. Guidelines of veterinary clinical bacteriology and mycology. Shahid Chamran University 1992;148-152.
33. Hussain zadegan H, Mnty Sh, Tarrahi Mohammadi F. Colonization frequency of meticillin and vancomycin-resistant Staphylococcus aureus in the nose, Matthew Shohada Hospital Employee of Khorram Abad. J Lab Sci 2008;2(1):26-32 (in Persian).
34. Marjolein FQ, Vandenbergh EDP, Yzerman F, Alex V, Helene A, Boelens M. Follow-Up of S.aureus NasalCarriage after 8 Years: Redefining the Persistent Carrier State. J Clin Microbiol 1999;37(10):3133-3140.
35. Ariza J, Pujol M, Cabo J. Vancomycin in surgical infections due to methicillin-resistant Staphylococcus aureus with heterogeneous resistance to vancomycin. Lancet 1999;353:1587-8.
36. Moore MR, Perdreau- Remington F, Chambers HF. Vancomycin treatment failure associated with heterogeneous vancomycin intermediate Staphylococcus aureus in a patient with endocarditis and in the rabbit model of endocarditis. Antimicrob Agent Chemother 2003;47:1262-1266.
37. Bert F, Clarissou J, Durannd F, Delefosse D, Chauvet C, Lefebvre P. Prevalence, molecular epidemiology, and clinical significance of heterogeneous glycopeptide intermediate Staphylococcus aureus in liver transplant recipient. J Clin Micribiol 2003;41:5147-5152.
38. Najar Pirayeh Sh, Azimian A, Mostafaei M, Siadat SD. Identification of methicillin-resistant Staphylococcus aureus by disk diffusion method, determination of MIC and PCR for mecA gene. Modares J Med Sci 2009;12(3):61-69 (In Persian).
39. Ahmadi shoar Sh, Nahaei MR, Amir mozafari N. Sensitivity of Staphylococcus aureus strains isolated from clinical specimens against vancomycin by using E-test in Tabriz. Med J Tabriz Uni Med Sci 2008;30(2):17-23 (In Persian).
40. Aligholi M, Emaneini M, Jabalameli F, Shahsavan S, Dabiri H, Sedaght H. Emergence of high-level vancomycin-resistant Staphylococcus aureus in the Imam Khomeini Hospital in Tehran. Med Princ Pract 2008;17:432-434.

12/22/2012