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Full Length Research Paper

Investigation of reduced glycopeptid susceptibility among methicillin resistant staphylococci

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Incidence of vancomycin intermediate *Staphylococcus aureus* (VISA) and vancomycin hetero-intermediate *Staphylococcus aureus* (h-VISA) has been increasing in the world and our country. We aimed to determine whether there is any difference between the minimal inhibitory concentration (MIC) of glycopeptide antibiotics against meticillin susceptible and resistant staphylococci and investigate the prevalence of VISA and h-VISA among *Staphylococcus aureus* strains. 60 MRSA, MSSA, MRCNS and MSCNS isolated in our laboratory between April 2007-2008, were included in this study. Bacteria were inoculated onto a BHI agar supplemented with 6 µg/ml vancomycin, strains which have shown growth on screen agar were evaluated for being VISA/hVISA by determining PAP-AUC ratio. Vancomycin and teicoplanin MIC values of the strains were determined by microdilution and E-test methods. Two of MRSA strains which grew on screen agar were excepted as suspicious VISA/h-VISA and they were confirmed as h-VISA by PAP-AUC method. Among all strains, methicillin resistant strains showed higher MIC values for glycopeptides than methicillin susceptible strains. We suggested that clinicians should be aware of h-VISA and VISA strains which are being increasingly reported in the world. More clinically supported multisentric studies must be performed in our country to clarify these prevalence rates and determine the clinical relevance of this resistance profile.

Key words: Staphylococci, meticillin resistance, glycopeptid susceptibility, vancomycin intermediate *Staphylococcus aureus* (VISA), vancomycin hetero-intermediate *Staphylococcus aureus* (h-VISA).

INTRODUCTION

Antibiotic resistance problem in staphylococci started with penicillin in 1940s and continued with the development of resistance to methicillin in 1960s, awhile after this antibiotic was used for treatment. Methicillin resistant *Staphylococcus aureus* (MRSA) strains have been widely identified since 1970s and as these bacteria were resistant to other groups of drugs than antistaphylococcic penicillin, vancomycin provided a hope for the treatment of these infections (Ayliffe, 1997). Although this was the case, widespread use of vancomycin brought forward the concern that developing resistance to glycopeptide antibiotics could as well be a possibility; in 1997, Hiramatsu reported the first strain with "decreased vancomycin susceptibility" from Japan (Hiramatsu, Hanaki et al., 1997). This strain had a minimal inhibitory concentration (MIC) value of 8 µg/ml for vancomycin and it was named Mu50; staphylococci with decreased susceptibility to vancomycin were defined as 'VISA'. Following this, in 1997 Hiramatsu and colleagues again reported a new type of resistance to vancomycin. This new strain was called Mu3, although its MIC values were within the susceptible range for vancomycin, $1/10^{5-6}$ bacteria in the population could grow at 4-9 µg/ml concentrations of vancomycin and therefore they were found to have intermediate susceptibility to vancomycin. These strains were defined as heterogeneous-VISA (h-VISA) (Hiramatsu et al., 1997). During the following years, several studies from various countries reported decreased vancomycin susceptibility; these studies pointed out to the fact that vancomycin resistance in staphylococci was a common problem worldwide (Hood,

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et al., 2000; Kim et al., 2000; Ploy et al., 1998; Smith et al., 1999). Vancomycin resistant *S. aureus* (VRSA) strains were first reported in 2002 and in the coming years, despite being in limited numbers, they confronted us as causes of bacteriemia and infective endocarditis, thereby increasing the concerns about this issue significantly (Centers for Disease Control and Prevention, 2002, 2004).

High MIC levels demonstrated by clinical isolates of staphylococci are known to lead to unfavorable clinical results during treatment with glycopeptides, however studies performed during recent years reported that MIC level increases below that of the susceptibility range could also change the clinical efficacy of these drugs (Gould, 2008). With an incubation of 24 h, disc diffusion method is insufficient in identifying strains with decreased susceptibility to vancomycin from those that are susceptible. Clinical and Laboratory Standards Institute (CLSI) stated that vancomycin resistant *S. aureus* strains can only be reliably identified by reference liquid microdilution method and that by utilizing screening agar plates containing 6 µg/ml vancomycin, the sensitivity in identifying vancomycin intermediate susceptible and resistant *S. aureus* strains can be increased (Clinical and Laboratory Standards Institute, 2009). In standard conventional methods like microdilution and disc diffusion that abide by CLSI standards, as the inoculum density is low as 5×10^5 cfu/mL, it is difficult to identify h-VISA subgroups that include $1/10^{5-6}$ of the whole population with these tests, population analysis that analyzes 10^{7-9} cfu bacteria is considered as the standard method for identifying h-VISA (Hanaki et al., 2001; Walsh et al., 2001). In this study, we worked on staphylococci isolated from hospitalized patients, and assessed vancomycin MIC values with microdilution and E-test methods. Among methicillin resistant and vancomycin susceptible isolates, we aimed at identifying the prevalence of those isolates with decreased susceptibility to vancomycin. We investigated the prevalences of VISA and h-VISA by using population analysis profile on *S. aureus* strains growing on brain heart infusion (BHI) agar containing 6 µg/ml vancomycin.

MATERIALS AND METHODS

This study was conducted at Süleyman Demirel University School of Medicine Microbiology and Clinical Microbiology Laboratories from April 2007 to April 2008. Different clinical samples were employed (114 blood, 59 wound swabs, 33 urine, 14 deep tracheal aspirates, 7 sterile bodily fluids, 6 catheter tips, 6 sputum, 1 vaginal smear). 60 meticilline resistant *S. aureus* (MRSA), meticilline susceptible *S. aureus* (MSSA), meticilline resistant coagulase negative staphylococci (MRCNS) and meticilline susceptible coagulase negative staphylococci (MSCNS) strains were analyzed. To identify the methicillin susceptibilities of staphylococci isolates, cephoxitine disc (30 µg) was used with disc diffusion method based on CLSI recommendations (Clinical and Laboratory Standards Institute, 2009). Bacteria were kept in 10% glycerol bouillon at –80 ^{0}C until the time of the study. MIC values of isolates for vancomycin and teicoplanin were identified with microdilution and standard E-test methods based on CLSI criteria, for identifying isolates with decreased vancomycin susceptibility, BHI agar screening plates containing 6 µg/ml vancomycin that were prepared in line with CLSI recommendations were used (Clinical and Laboratory Standards Institute, 2009). For this purpose, fresh colonies kept in blood agar were suspended into a volume of 10 µl after adjustment to 0.5 McFarland turbidity. These were transferred onto BHI agar containing 6 µg/ml vancomycin. At 24 and 48 h, the plates were examined with magnifiers for the presence of any growth. *S. aureus* strains growing on screening agar were further analyzed for VISA and h-VISA by keeping them in media not containing vancomycin for 9 days. They were reevaluated with population analysis profile by calculating the area under the curve (PAP-AUC).

Standard E test

Of the colonies kept in blood agar plates overnight, bacteria equaling to 0.5 McFarland densities were added into Mueller-Hinton liquid medium; 200 µl of this suspension was transferred to BHI agar plates for superficial culture. After a 10 min drying period, vancomycin and teicoplanin (0.016-256 µg/ml) E test strips (AB Biodisc, Sweden) were placed for incubation at 35°C for 24 h and MIC values were calculated.

Microdilution

Following an overnight stay in blood agar media, the colonies were suspended in liquid Mueller-Hinton medium to yield a 0.5 McFarland density solution. In line with CLSI recommendations, bacteria were added onto individual wells containing twice diluted antibiotic solutions to give a final concentration of $5X10^4$ CFU. Microplates were covered with sterile plates and incubated at 35°C for 24 h. For each strain, the lowest concentration at which there was no growth was evaluated as MIC.

Population analysis profile-area under the curve (PAP-AUC) ratio

PAP-AUC ratio was identified as previously defined by by Wootton and colleagues (Wootton et al., 2001). Logarithmic graphs of colony numbers were prepared in correlation with vancomycin concentrations and area under the curve was calculated for each strain. Mu3, Mu50 and ATCC 25923 (*S. aureus subs. aureus*) were used as control strains. PAP-AUC of each strain was then compared with that of Mu3 control strain. After this comparison, the ones with a value of ≤0.90 were regarded as VSSA, those between 0.90–1.3 as h-VISA and those with values of ≥1.3 as VISA (Walsh et al., 2001; Wootton et al., 2001).

RESULTS

Of the isolates included in the study, 21 MRCNS (35%), 15 MSCNS (25%) 2 MRSA (3.3%) (total 38 strains 15.8%) grew in 6 µg/ml vancomycin containing BHI agar. None of the MSSA group bacteria grew in this medium. Of the bacteria growing and not growing on screening agar plates, MIC values for vancomycin and teicoplanin were identified with microdilution and E test methods and demonstrated on Table 1 and Table 2.

For all the strains that grew on screening agar plates,

Table 1. The distribution of the bacteria growing on screening agar plates based on vancomycin and teicoplanin MIC values as identified by E-test and microdilution methods (strains with moderate susceptibility and resistance are depicted in dark colors).

	E-test					Microdilution				
	Vancomycin MIC (µg/ml)		Teicoplanin MIC (µg/ml)			Vancomycin MIC (µg/ml)		Teicoplanin MIC (µg/ml)		
	6–8	12–16	≤8	16	≥32	8	16	≤8	16	≥32
MRSA n=2	2	-	-	2	-	2	-	-	2	-
MRCNS n=21	16	5	6	9	6	16	5	6	7	8
MSCNS n=15	12	3	11	3	1	12	3	11	2	2
Total n=38	30	8	17	14	7	30	8	16	12	10

Table 2. The distribution of the bacteria not growing on screening agar plates based on vancomycin and teicoplanin MIC values as identified by E-test and microdilution methods (strains with moderate susceptibility and resistance are depicted in dark colors).

	E-test				Microdilution			
	Vancomycin MIC (µg/ml)		Teicoplanin MIC (µg/ml)		Vancomycin MİK (µg/ml)		Teicoplanin MIC (µg/ml)	
	≤2	4	≤8	16	≤2	4	≤8	16
MRSA n=58	16	42	51	7	16	42	51	7
MSSA n=60	45	15	60	-	45	15	60	-
MRCNS n=39	8	31	39	-	8	31	39	-
MSCNS n=45	37	8	45	-	37	8	45	-
Total n=202	106	96	195	7	106	96	195	7

E-test and microdilution methods identified MIC values corresponding to intermediate vancomycin susceptibility. On the other hand, when MIC values identified for teicoplanin were assessed for these strains, 6 of the 21 MRCNS and 11 of the 15 MSCNS that grew on screening plates were identified to have MIC values within the susceptible range with E-test and microdilution methods. Furthermore, when MRCNS growing on screening agar plates were tested with E-test method for teicoplanin, 9 had intermediate susceptibility, 6 were resistant; when tested with microdilution 7 had intermediate susceptibility and 8 were resistant based on their MIC values. With the E-test method, 3 MRCNS had intermediate susceptibility to teicoplanin, 1 had MIC values within resistant range, with the microdilution method, 2 had intermediate susceptibility, 2 had MIC values corresponding to resistance. In short, of the staphylococci growing on vancomycin containing agar plates, MIC values for vancomycin were coherent when tested with E-test and microdilution method. As concerns the MIC values for teicoplanin, the values identified with E-test and microdilution were different from each other. Of the strains that did not grow on screening plates, 42 of the 58 MRSA strains and 15 of the 60 MSSA strains had MIC values in the intermediate susceptibility range for vancomycin when tested with E-test and microdilution methods. For the MRCNS and MSCNS strains that did not grow on screening agar plates, all had MIC values that were susceptible for vancomycin. Except for 7 MRSA strains, all the strains that did not grow on screening plates had MIC values within the susceptibility range for teicoplanin after testing with microdilution and E-test. In this group, the values identified with microdilution and E-test methods were coherent with each other. Of the strains included in the study, MIC values identified with microdilution method for vancomycin and teicoplanin are demonstrated on Table 3.

When the strains included in the study were classified with reference to their methicillin resistance and the MIC values identified with microdilution method for vancomycin and teicoplanin were evaluated; 44 MRSA (73.3%), 15 MSSA (25%), 21 MRKNS (35%) and 15 MSKNS (25%) had MIC values at intermediate susceptibility range for vancomycin. For teicoplanin, 9 MRSA (15%), MRCNS (11.7%) and 2 MSCNS (3.3%) had intermediate susceptibility, 8 MRCNS (13.3%) and 2 MSCNS (3.3%) had MIC values at the resistant range. All MSSA strains were found to have MIC values within the susceptible range for teicoplanin. When a comparison was made for methicillin susceptibility, the strains with methicillin resistance had higher MIC values for vancomycin and teicoplanin as compared to susceptible strains.

MRSA isolates that grew on vancomycin screening agar were evaluated for vancomycin MIC values with microdilution method. The MIC values were identified as8 µg/ml; as a result of the population analysis, the ratio of the area under the curve for these bacteria to Mu3 strain

Table 3. The distribution of the strains according to vancomycin and teicoplanin MIC values identified by microdilution method (strains with moderate susceptibility and resistance are depicted in dark colors).

	MIC (µg/ml)					
Vancomycin	≤2	4	8	16	32	64
MRSA (MIC_{50}: 4, MIC_{90}: 4)	16	42	2	-	-	-
MSSA (MIC_{50}: 2, MIC_{90}: 4)	45	15	-	-	-	-
MRCNS (MIC_{50}: 4, MIC_{90}:8)	8	31	16	5	-	-
MSCNS (MIC_{50}: 2, MIC_{90}:8)	37	8	12	3	-	-
Teicoplanin	≤2	4	8	16	32	64
MRSA (MIC_{50}: 4, MIC_{90}: 16)	9	21	21	9	-	-
MSSA (MIC_{50}: 1, MIC_{90}: 2)	57	3	-	-	-	-
MRCNS (MIC_{50}: 8, MIC_{90}: 32)	15	12	18	7	6	2
MSCNS (MIC_{50}: 1, MIC_{90}:8)	40	4	12	2	1	1

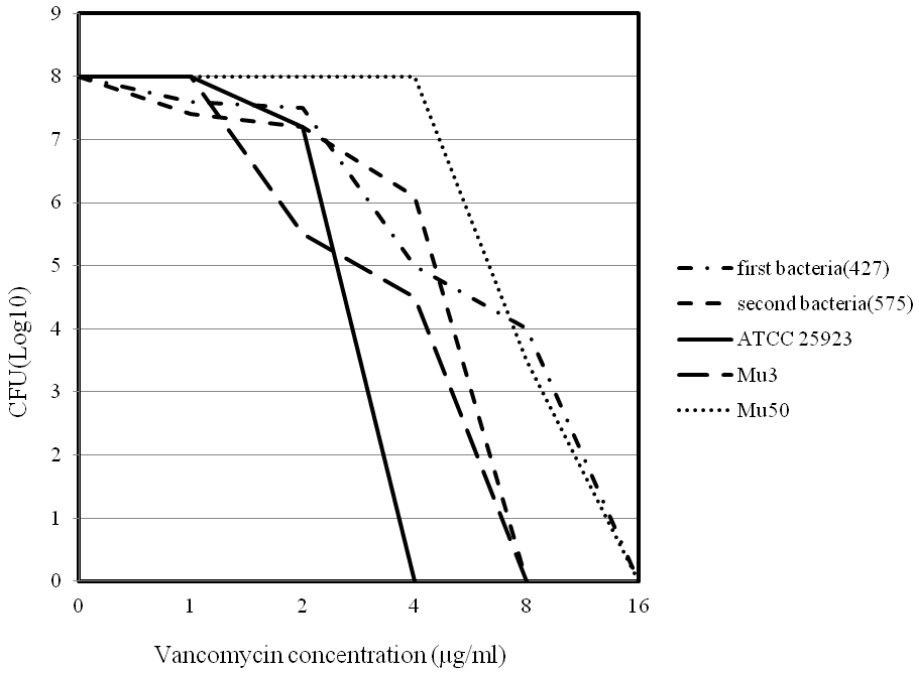

Figure 1. PAP-AUC graphs of the bacteria and standard strains studied by population analysis.

was calculated as 1.01 for the first bacteria and 1.05 for the second leading to a decision of h-VISA for these bacteria. The graphs of the bacteria covered by the population analysis and those of the standard strains are shown on Figure 1.

DISCUSSION

In our country, several studies have been performed to investigate the vancomycin resistance of staphylococci isolated from various hospitals. In some of these studies, all the evaluated strains had MIC values within the susceptibility range (Aktaş et al., 2010; Limoncu et al., 2007; Sünbül et al., 1998); however, during recent years higher, MIC values were identified for vancomycin and despite having MIC values within the sensitive range, isolation rates of strains demonstrating h-VISA characteristics increased (Gülay et al., 1998; Sancak et al., 2005). For example Gülay et al., evaluated MRSA strains in a study and identified decreased resistance to vancomycin in 5.3% of the strains with microdilution method (Gülay, et al. 1998). Sancak et al., reported that hVISA presence in MRSA was 1.6% in 1998 and that this figure increased to 36% in 2001 (Sancak et al., 2005). When all the strains examined in our study were evaluated for vancomycin 145 (60.4%) had MIC values within the susceptible range, 95 (39.6%) had MIC values

that were in the intermediate susceptibility range and of the 120 *S. aureus* strains examined, 2 (1.7%) were identified as hVISA confirming the fact that the susceptibility to vancomycin was gradually decreasing in our country. The fact that vancomycin MIC values of more bacteria than those growing on screening agar plates were within the intermediate susceptibility range can be explained by saying that *S. aureus* isolates with MIC values of below 6 µg/ml would not normally grow on screening agar plates; however, this can be explained by some isolates having MIC values of 4 µg/ml regarded as intermediate susceptibility based on CLSI criteria. None of the isolates growing on screening agar plates had MIC values of more than 4 µg/ml for vancomycin and both of the two isolates growing on agar had MIC values of above 4 µg/ml. This finding was in correlation with the CLSI recommendation suggesting the use of screening media containing 6 µg/ml vancomycin in order to increase the sensitivity in screening for decreased susceptibility to vancomycin. All of the CNS that did not grow on screening agar plates had MIC values within the susceptibility range for vancomycin and teicoplanin. 42 of 58 MRSA (72.4%) and 15 of 60 MSSA (25%) had MIC values at intermediate susceptibility range. We therefore thought that vancomycin agar media yielded better results for CNS than *S. aureus* in identifying the strains with intermediate susceptibility. This also underlined the importance of defining MIC values for accurate evaluation of vancomycin susceptibility in MRSA.

In our study, for the bacteria not growing on screening agar, MIC values identified with E-test and microdilution correlated with each other. Of the isolates growing on vancomycin agar, teicoplanin MIC values of CNS measured with microdilution and E-test method were different from each other. These differences might be related to the higher sensitivity of the E-test system. In microdilutions performed according to CLSI criteria, a value of 16 µg/ml corresponds to intermediate susceptibility whereas a value of ≥32 µg/ml is accepted as resistant. In the E-test system, there is the value of 24 µg/ml between 16 and 32 µg/ml. Bacteria having this MIC value grow at teicoplanin concentrations of 16 µg/ml in microdilution and therefore the upper value of 32 µg/ml is accepted as MIC for them and they are included in the resistant group. However, in the MIC identified with E-test system, these bacteria have a MIC value of 24 µg/ml and are included in the group of intermediate susceptibility.

None of the strains examined in our study had MIC values at resistant range. With microdilution method, 18 strains (7.5%) had intermediate susceptibility to teicoplanin and 10 strains (4.2%) had resistance, these values were higher than the rates reported from around the world, 1-2% (Sloos et al., 1998; Watanakunakorn, 1990). When teicoplanin resistant strains were examined, CNS isolated from patient samples receiving intensive antibiotic treatment were seen. 5 of these strains were isolated from blood cultures; 2 from neurology, 2 from intensive care unit and 1 from geriatrics service; 1 was from urine culture obtained in the oncology department and 1 was from surgical wound culture sent from surgery department. In studies investigating teicoplanin resistance of staphylococci strains, we saw that teicoplanin resistance was 2-6% in our country whereas intermediate susceptibility was around 1-3% (Güleroğlu et al., 2002; Kucukates, 2004; Nakipoğlu et al., 2004; Sünbül et al., 1998). The teicoplanin resistance identified in our study was in correlation with the teicoplanin resistance rates in Turkey in general, whereas intermediate susceptibility value was higher than the general average. Finding a higher intermediate susceptibility value was explained with the fact that teicoplanin use was increasing in our hospital and that staphylococci were rapidly developing resistance to the drug. That is why we thought that the use of this antibiotic should be taken under control in our hospital.

In order to classify a *S. aureus* strain as VISA, CDC recommends incubation of an inoculum with a concentration of 10^6 CFU/ml for 24 h. With liquid microdilution, MIC values for vancomycin should be 8-16 µg/ml, and with E test method, it should be ≥6 µg/ml, the inoculum should grow within 24 h in BHI agar plates containing 6 µg/ml vancomycin (Hiramatsu et al., 1997). As most of the bacteria in the population are within the susceptibility range, it is not generally possible to identify h-VISA with routine laboratory tests. Mu3 is the prototype for this type of resistance and with standard microdilution; vancomycin MIC values are identified as 1–2 µg/ml (Tenover et al., 2001). Today, most of the research in this field focuses on correct identification of h-VISA. Studies conducted so far and CDC recommendations demonstrate us that staphylococci with MIC values of 4-8 µg/ml for vancomycin cannot be reliably defined with disc diffusion method and automated systems. In our study, we first used the screening agar plate method prepared in line with CLSI recommendations (Clinical and Laboratory Standards Institute, 2009). The bacteria growing on this medium were further analyzed with microdilution, standard E-test and population analysis profile-area under the curve (PAP-AUC). Of the 120 *S. aureus* analyzed, 2 h-VISA were identified, h-VISA rate for Süleyman Demirel University Research and Implementation Hospital was calculated as 1.7%; VISA and VRSA could not be found. Although this rate does not seem to be too high for the moment, we have to be attentive and careful. In different studies investigating hVISA and VISA frequencies with different methods, various rates have been reported for our country. Limoncu et al. used BHI agar screening method containing 6-mgr/ml vancomycin together with standard E-test and macro E-test methods As a result of these efforts, they reported that they did not identify any hVISA or VISA isolates (Limoncu et al. 2007). Nakipoğlu et al. used screening agar plates and population analysis methods in their studies, they did not encounter VISA or

h-VISA strains, they reported teicoplanin resistance in 1.2% of *S. aureus* and heterogeneous resistance to vancomycin and teicoplanin in 13% of CNS strains (Nakipoğlu et. al., 2004). Torun and colleagues identified 2 hVISA isolates in their studies and reported that h-VISA had a low rate of 0.2% in their hospital (Torun et al., 2005), Sancak et al. conducted their study with screening agar plates, macro E-test and population analysis methods; they found h-VISA rate as 17.96% and they could not identify any VISA (Sancak et al., 2005). Similarly, prevalence rates reported from around the world differ among regions. In a study covering North and South American countries, a total of 427 MRSA strains were covered, 12 h-VISA were reported out of which 9 were from the USA, 2 were from Argentina and finally, one was from Brazil (Ruef, 2004). Among Asian countries, h-VISA rates were reported as 8.2% in Japan, 6.3% in India, 6.1% in South Korea, 2.3% in Singapore and 2.1% in Thailand (Song et al., 2004). In a metanalysis evaluating 14 studies performed from 1997 to 2001, mean h-VISA prevalence was reported as 1.67% and this was 2.16% for MRSA and 0.05% for MSSA. However, the prevalence rates reported in the individual studies covered in this metanalysis had extreme values like 0 and 74% (Liu and Chambers 2003). One of the most important reasons for these differences seen in the prevalence rates was not using standardized methods in the studies concerned. The need for a reliable and standard method for correct evaluation of h-VISA prevalence and its clinical significance was emphasized.

In conclusion, h-VISA, VISA and VRSA are being reported at higher incidences all around the world, in our country the number of studies conducted on this subject are limited; however, this data provide the first warning signals of the glycopeptide antibiotic resistance that might be encountered in our country in near future. That is why we have to be more selective and careful in the use of these antibiotics and pay attention to the close microbiological follow-up of the patients both before and during the treatment. Until standard methods are defined, preferably all *S. aureus* but mainly those that do not respond to glycopeptide therapies or those isolated from patients bearing risks for VISA development should be analyzed for the presence of this resistance.

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