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Prevalence of Heterogeneous Glycopeptide Intermediate Resistance in Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Multidrug resistant Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community acquired infections and is on the rise. The aim of this investigation was to explore the prevalence of MRSA and heterogeneous Glycopeptide Intermediate *Staphylococcus aureus* (hGISA) in various clinical samples, to investigate the various antibiotic resistant determinant genes among these strains collected from north and west Indian hospitals and to evaluate the response of various drugs to these strains. A total of 413 clinical specimens collected from different hospitals were processed for the screening of *S. aureus* and MRSA. All the MRSA strains were further screened for hGISA on Mueller-Hinton agar containing 8 μg mL^{-1} teicoplanin or 6 μg mL^{-1} vancomycin. hGISA confirmed by the E-test method with a dense inoculum and a simplified method of population analysis. Susceptibility study was conducted according to the Clinical and Laboratory Standards Institute (CLSI) methods. Among 211/413 *S. aureus* clinical isolates, 61.6% (130/211) of the isolates were confirmed to be MRSA which included maximum isolates from pus, blood, urine, wound swab and ear swab samples in decreasing order. hGISA strains were found in 8/130 (6.1%) isolates. Vancoplus, a novel antibiotic adjuvant entity was found to be susceptible in 96.1 to 97.8% MRSA strains and showed intermediate response in 2.2 to 3.8% of isolates. Linezolid appeared to be second most active antibiotic with 48.0 to 81.2% susceptibility, followed by teicoplanin (41.3 to 56.2% susceptibility). There was 8.7 to 9.6% resistance observed in Linezolid which was increased to 48% in teicoplanin, to >60% in daptomycin and >75% in vancomycin. Interestingly, none of the isolates were susceptible to ceftriaxone and cefoperazone plus sulbactam. From the above study it can be concluded that prevelance of MRSA has reached a significant level and Vancoplus is the most effective drug in MRSA as well as hGISA organisms in comparison to comparator drugs.

Keywords: Methicillin-Resistant *Staphylococcus aureus*, Heterogeneous Glycopeptide Intermediate *Staphylococcus aureus*, Vancoplus

1. INTRODUCTION

Staphylococcus aureus (SA) is a commensal organism and is present in up to 80% of healthy individuals. It is one of the common causative agents of hospital and community acquired infections, particularly skin infections, pneumonia, surgical site infections, chronic bone infections and blood stream infections (Kreienbuehl *et al*., 2011; Perichon and Courvalin, 2009). The antibiotics such as methicillin, oxacillin and nafcillin, macrolides, tetracycline and aminoglycosides which are being used to treat these infections, however are getting resistant (Thati *et al*., 2011).

Glycopeptides have been used worldwide against Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections (Pletz *et al*., 2010), but unfortunately, it is

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losing potency against MRSA (Benjamin *et al*., 2010) and leading the emergence of glycopeptide-resistant *S. aureus* (GRSA; vancomycin/teicoplanin MIC >8 μg mL^{-1}), glycopeptide-intermediate *S. aureus* (GISA; vancomycin/teicoplanin MIC 8 μg mL^{-1}) the expression of which is frequently heterogeneous across bacterial populations (hGISA) (Perichon and Courvalin, 2009; Andrew, 2009). In the past few years, GRSA, GISA and hGISA have increasingly been reported (Kirby *et al*., 2010). hGISA strains are defined as for which vancomycin MICs are 1 to 4 μg mL^{-1} but which contain subpopulations that can grow on agar plates supplemented with 4 mg of vancomycin per liter (Satola *et al*., 2011).

The prevalence of hGISA has been indicated in various countries including Japan, United States, France and Korea which varied from 0.6 to 74% (Dhand and Sakoulas, 2012; Howden *et al*., 2010). The clinical significance of hGISA strains is controversial. In few reports, hGISA infections were found to be associated with a high rate of failure of vancomycin therapy (Howden *et al*., 2010), whereas in other studies hGISA infections were successfully treated with vancomycin (Kim *et al*., 2002).

A number of recent studies showed that vancomycin treatment failure rate has exceeded 40% (Aston *et al*., 2010). Similarly, the rate of non-susceptibility of penicillin-resistant *Streptococcus pneumoniae* strains to cephalosporins particularly ceftriaxone is increasing significantly (Karunakaran *et al*., 2012). Several reports from India recorded the emergence of various degree of vancomycin resistance (Veer *et al*., 2010; Thati *et al*., 2011). Other parts of the world including France (Perichon and Courvalin, 2009), United Kingdom (Reynolds *et al*., 2012) and Korea (Jang *et al*., 2012) also reported the prevalence of vancomycin resistance, indicating vancomycin resistance is a global issue but this is not properly recognized as VRSA. The growing problem in the Indian scenario is that MRSA prevalence has increased from 25% in western part of India to 50% in South part of India (Joshi *et al*., 2013) which is now becoming resistant to Vancomycin hence can be termed as VISA or VRSA or GISA or GRSA.

The increase in vancomycin resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity. In view of the growing consequences of resistance to commonly used drugs, Venus Medicine Research Centre, India has developed a new combination product which is named as Vancoplus (US patent no;7960337, Japan patent no: 4918502). Vancoplus is a novel antibiotic adjuvant entity comprised of a β-lactam moiety (ceftriaxone) plus a glycopeptide (vancomycin) along with a non antibiotic adjuvant VRP1020. Clinically, this antibiotic combination has been designed for the treatment of infections caused by multi drug-resistant gram-positive and gram-negative organisms. The aim of this investigation was to explore the prevalence of MRSA and heterogeneous glycopeptide intermediate *Staphylococcus aureus* (hGISA) in various clinical samples, to investigate the various antibiotic resistant determinant genes among these strains collected from north and west Indian hospitals and to evaluate the response of various drugs to these strains.

2. MATERIALS AND METHODS

2.1. Clinical Isolates Collection

A total of 413 clinical specimens were collected from pus (165), blood (76), urine (43), wound swab (83) and ear swabs (46) from ICUs patients from various hospitals of Haryana, Utter-Pradesh, Rajasthan, Madhya-Pradesh and Maharastra. The names of hospitals can not be disclosed because of confidentiality agreement.

2.2. Media and Culture Condition

All clinical samples except urine were first inoculated onto blood agar (Hi-Media, India) whereas urine samples were inocoulated on CLED agar (Hi-Media, India) plates. All the plates were incubated at 35°C for 24-48 h.

2.3. Growth on Mannitol Salt Agar

Mannitol salt agar (Himedia) was used as a selective medium for *S. aureus*. All staphylococcal isolates were re-inoculated onto mannitol salt agar (Hi-Media, India) and plates were incubated at 37°C for 24-48 h. Mannitol fermenting yellow colored colony is selected, subcultured and subjected to identification based on Gram's stain morphology, colony characteristics and positive catalase and coagulase tests according to standard methods (Akbar *et al*., 2013).

2.4. Screen Test for MRSA

A suspension equivalent to 0.5 MacFerland was prepared from each strain. A swab was dipped and streaked over an area of approximately 2×2.5 cm on the surface of a Mueller-Hinton agar supplemented with 4% NaCl and 6 μg mL^{-1} Methicillin (Sigma-Aldrich). Plates were incubated overnight at 37°C. A growth indicates that the strain is methicillin resistant.

2.5. Glycopeptide Susceptibility Testing

2.5.1. Agar Incorporation Method

All *S. aureus* isolates were processed for reduced glycopeptide susceptibility by an agar incorporation

method. 10 µL of a 0.5 Macfarland suspension was inoculated onto brain heart infusion agar (BHIA, Hi-Media, India) containing 8 µg mL^{-1} teicoplanin or 6 µg mL^{-1} vancomycin. The plates were incubated at 37°C for 48 h. The isolates growing on these agar plates were tested by the Macro Etest method.

2.6. Macro Etest Method

BHIA was inoculated with a 250 µL of a 2.0 Macfarland suspension of *S. aureus* with vancomycin and teicoplanin Etest strips (Hi-Media, India) and were placed on the agar plates and incubated at 37°C for 48 h. Isolates with a Macro Etest reading of 8 µg mL^{-1} to vancomycin and teicoplanin 12 µg mL^{-1} to teicoplanin were considered as having reduced glycopeptide susceptibility. The reduced susceptibility method detection includes hGISA, GISA and GRSA. To define the type of susceptibility, Etest MIC were determined.

2.7. Etest MIC Method

Etest MICs were determined according to the manufacturer's instructions (Hi-Media, India). An isolate with reduced glycopeptide susceptibility by the Macro Etest method, but on Etest MIC of teicoplanin or vancomycin of 4-8 µg mL^{-1} was considered to be hGISA/GISA, respectively (Andrew, 2009).

2.8. Population Analysis Method

This study was done according to the method described by Satola *et al*. (2011). In brief, 100 µL of cell suspension at 0.5 Macfarland suspension and serial 10 fold dilution of the suspension in BHI broth were spread on to BHI agar plates containing vancomycin at concentration ranging from1 to 10 µg mL^{-1}. After incubation at 37°C for 48 h, the number of viable cells were counted growing on the different concentration of vancomycin and plotted on a semi-logrithmic scale. Any screen positive strain that contained subpopulations with MIC >4 µg mL^{-1} for vancomycin at a frequency of 1 in 10^6 cfu/ml or higher was defined as hVISA.

2.9. DNA Isolation

DNA from each clinical isolates as well as positive control was extracted using the methods described earlier (Chaudhary and Payasi, 2013a; 2013b).

2.10. PCR

The PCR amplification of the *mecA* and *vanA* were done using the Eppendorf thermocycler (Germany). The primers and the PCR conditions were as described earlier (Chaudhary and Payasi, 2012). For PCR amplifications, about 200 pg of DNA was added to 20 µL mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 3.0 U of Taq polymerase (Banglore Genei) in 1x PCR buffer. The amplified products were separated in 1.5% agarose gel containing ethidium bromide. The gel images were taken under ultraviolet light using gel documentation system (Bio-Rad, USA). A 100 bp ladder molecular weight marker (Banglore genie) was used to measure the molecular weights of amplified products.

2.11. MIC and MBC Determination

Minimal Inhibitory Concentration (MIC) of each antibacterial agent was determined by agar dilution method using CLSI guidelines (CLSI, 2011). The MIC90 value represents the lowest dilution at which bacteria fail to grow at 37°C after 18 to 24 h of incubation. To determine Minimum Bactericidal Concentration (MBC), 100 µL was aspirated from wells where there was no visible growth of planktonic bacterial population in the MIC90 experiment and spread onto MHBA plates and incubated overnight at 37°C in incubator. MBC was read as the lowest antibiotic concentration to kill 99.9% of the initial inoculum.

2.12. AST Study

AST study was carried out by the disc diffusion method with a final inoculum size of 10^6 cfu/mL according to CLSI guidelines (CLSI, 2011) using different antimicrobial agents;. Vancoplus (30:15 µg), Vancomycin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), teicoplanin (30 µg), linezolid (30 µg) and cefoperazone plus sulbactam (75:30 µg). The standard *S. aureus* ATCC-43300 (mec A positive) was used as positive control. The zone of inhibition around the wells was measured in mm (millimeter), averaged and the mean values were recorded.

3. RESULTS

3.1. Screening of MRSA and hGISA

In a total of 413 clinical specimens, 232 isolates were identified to be of Staphylococcus species (56.2%) of which 90.9% (211/232) specimens were of *S.aureus*.

The prevelance of *S.aureus* was maximum in pus isolates (97.1%) followed by blood (87.5%), ear swab (85.7%), wound swab (76.5%) and urine samples (75.0%). These *S. aureus* were when further subjected to MRSA screening 61.6% (130/211) were confirmed to be MRSA isoaltes (**Table 1**).

Table 1. Prevalence of MRSA in various clinical specimens

Name of specimens	Total no of specimen	Staphylococcus species (%)	*S. aureus* isolates (%)	MRSA isolates (%)	hGISA screening test		(%) Population analysis profile
					BHI-V4	Macro E test	
Pus	165	137/165 (83.0)	133/137 (97.1)	99/133 (74.4)	22/99 (22.2)	8/99 (8.0)	5/99 (5.0)
Blood	76	32/76 (42.1)	28/32 (87.5)	12/28 (42.8)	2/12 (16.6)	1/12 (8.33)	1/12 (8.33)
Urine	43	8/43 (18.6)	6/8 (75.0)	2/6 (33.3)	0/2 (0)	0/2 (0)	0/2 (0)
Wound swab	83	34/83 (40.9)	26/34 (76.5)	9/26 (34.6)	1/9 (11.1)	1/9 (11.11)	1/9 (11.11)
Ear swab	46	21/46 (45.6)	18/21 (85.7)	8/18 (44.4)	1/8 (112.5)	1/8 (12.5)	1/8 (12.5)
Total	413	232/413 (56.17)	211/232 (90.9)	130/211 (61.6)	26/130 (20.0)	11/130 (8.5)	8/130 (6.1)

Table 2. MIC90 study of Vancoplus in *mecA* and *vanA* positive clinical isolates of MRSA

Total no. of isolates with antibiotic resistant	MIC $_{90}$ Values in µg mL^{-1}						
	A	B	C	D	E	F	G
mecA (46)	0.25-4	2-32	16-32	2-8	4-16	2-16	32-256
mecA+VanA (52)	0.5-4	8-64	16-128	2-16	8-32	4-32	128-512
VanA (32)	0.25-4	4-32	16-64	1-8	4-16	4-16	32-128
S. aureus ATCC43300	0.25	8	16	1	2	1	32

Where, A = Vancoplus; B = Vancomycin; C = Ceftriaxone; D = Linezolid; E = Daptomycin; F = Teicoplanin; G = Cefoperazone + sulbactam

Out of the 130 clinical isolates of MRSA, 26 (20.0 %) isolates grew countable number (1-30) on BHI-V4 plates within 48 h. Further screening of these isolates using heavy inoculum (2 Mcfarland) macro E test, 11 (8.5%) isolates showed reduced susceptibilities to vancomycin (MIC 6-12). The detailed distribution of these isolates in various clinical specimens are depicted in **Table 1**. The population analysis method confirmed, 8 isolates out of the 11 isolates suspected of having reduced susceptibility to vancomycin by macro E test as hGISA as these isolates produced subpopulations with MIC of vancomycin > 4 µg mL^{-1} (5-8 µg mL^{-1}) at a frequency of 10^{-6} or higher (figure not shown).

3.2. Genotyping of MRSA

Out of the 130 clinical isolates of MRSA, 35.4% (46/130) were found to be mecA positive and 24.6% (32/130) isolates were *vanA* positive. Interestingly, we observed 40% (52/130) isolates were both mecA plus vanA positive (**Table 2**).

3.3. MIC and MBC Identification

As shown in the **Table 2**, Vancoplus emerged as the most active antibacterial against MRSA with MIC values 0.25 to 4 µg mL^{-1}. The second most active antibacterial agent was linezolid with MIC values 2 to 16 µg mL^{-1}. The MIC of teicoplanin and daptomycin ranged from 2-32 µg mL^{-1}, whereas vancomycin MIC ranged 2-64 µg mL^{-1}. The MIC of ceftriaxone and cefoperazone plus sulbactam ranged from 16-128 and 32-512 µg mL^{-1}, respectively. Overall, MBC of all tested drugs was 3 to 4 times higher of their MIC (**Table 3**).

3.4. Antimicrobial Susceptibilities of Clinical Isolates

Antimicrobial susceptibility data are presented in **Table 4**. Vancoplus was susceptible in >95% cases (96.1% to 97.8%) and showed intermediate response in 2.2 % to 3.8% to MRSA isolates. Linezolid appeared to be second most active antibiotic with 81.2% susceptibility in alone Van A gene and upto only 48.0% susceptibility to MRSA strains where both *mecA* and *VanA* genes were present, followed by teicoplanin where <60% susceptibility was observed. Other comparator drugs showed very low susceptibility to all three types of genes observed in MRSA strains (daptomycin (19.2 to 28.2% susceptibility) and vancomycin (13.0 to 15.6% susceptibility). Interestingly, none of the isolates were susceptible to ceftriaxone and cefoperazone plus sulbactam. However, intermediate response varied from 10.9 to 18.7% for ceftriaxone (**Table 4**).

Table 3. MBC study of Vancoplus in *mecA* and *vanA* positive clinical isolates of MRSA

	MBC Values in µg mL^{-1}						
Total No. of isolates with antibiotic resistant	A	B	C	D	E	F	G
mecA (46)	1-32	16-128	128-256	8-64	32-128	8-64	128-512
mecA+VanA (52)	2-32	64-512	128-512	8-128	64-128	16-128	>1028
VanA (32)	1-32	32-256	64-512	8-64	32-128	32-128	128-1024
S. aureus ATCC43300	2	64	128	8	8	8	256

Where, A = Vancoplus; B = Vancomycin; C = Ceftriaxone; D = Linezolid; E = Daptomycin; F = Teicoplanin; G = Cefoperazone + sulbactam

Table 4. Antimicrobial susceptibility of various drugs against MRSA

	Susceptibility %																				
Total No. of isolates with	A			B			C			D			E			F			G		
antibiotic resistant	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
mecA (46)	97.8	2.2	0	13.0	21.7	65.2	-	10.9	89.1	28.2	15.2	56.5	41.3	19.5	39.1	73.9	17.4	8.7	-	-	100
mecA+VanA (52)	96.1	3.8	0	5.7	17.3	76.9	-	-	100	19.2	19.2	61.5	28.8	23.0	48.0	48.0	42.3	9.6	-	-	100
VanA (32)	96.8	3.1	0	15.6	21.8	62.5	-	18.7	81.2	28.1	25.0	46.8	56.2	15.6	28.1	81.2	9.3	9.3	-	-	100
S. aureus ATCC43300	100	-	-	-	-	100	-	-	100	-	100	-	-	100	-	-	100	-	-	-	100

Where, A = Vancoplus; B = Vancomycin; C = Ceftriaxone; D = Linezolid; E = Daptomycin; F = Teicoplanin; G = Cefoperazone + sulbactam

4. DISCUSSION

MRSA has appeared as a dynamic human pathogen since the first MRSA isoaltion in USA. Since then, MRSA has spread across the world and has become a global problem. The detection of GISA and hGISA strains is an important factor to understand the prevalence and clinical importance of GISA, specially hGISA, because heterogeneous resistance might be more clinically relevant in patients with a severely underlying conditions. There are much confusions about the methods being used to detect heterogenous glycopeptide resistance (Casapao *et al*., 2013). In the current investigation, three different methods were used to identify hGISA. The strains positive with agar screening method were subjected to macro Etest and subpopulation analysis. These methods are reliable and sensitive to detect hGISA (Kirby *et al*., 2010).

The incidence of prevalence of MRSA in India ranges from 30-70% (Singh *et al*., 2012; Muralidharan, 2009). The prevalence rate of MRSA in various clinical specimens obtained from different clinical subjects were determined. Overall, our results showed prevalence rate of MRSA in north and western India is about 61.6%. These findings are similar to studies from Eastern U.P. and AIIMS in New Delhi which also reported prevalence of MRSA 54.85 and 44% respectively (Manchanda *et al*., 2013). In our study the maximum MRSA was from pus (74.4%) samples. However, low prevalence of MRSA in pus and wound swab (52.5%) were reported (Manchanda *et al*., 2013). In the blood MRSA was obtained 42.8% which similar to the study of Singh *et al.* (2012). In urine sample, 33.3% MRSA was detected, which was lower than previous reports (Manchanda *et al*., 2013). With regard to wound swab and ear swab 34.6 % and 44.4% MRSA had been reported. Moreover, further genotypic characterization of the MRSA revealed the prevalence of mecA plus vanA was 40%, followed by mecA (35.4%) and vanA (24.6%).

In our results the prevalence of hGISA among MRSA strains collected from various clinical specimens was found to be 6.1% (8/130), which was comparable to that reported in other Asian coutries, USA, Israel which was 4.5 to 7.5% (VanHall *et al*., 2011). Moreover, the prevalence of hGISA may vary according to the source of the strains from hospital or community acquired one.

The antibiotic sensitivity pattern of MRSA isolated from clinical specimens were found to be highly variable. In the last decade dramatic changes have occurred in the epidemiology of MRSA infections. The susceptibility study indicated that among 130 (61.6%) MRSA screened from clinical specimens, cefoperazone sulbactam and ceftriaxone were found to be 100% resistant. The emergence of high level of resistance in patients has important implications for the use of these drugs as potential therapeutic agents. Vancomycin showed >60%

resistance followed by Daptomycin >45%, teicoplanin >25% and linezolid >9%. Vancoplus was not found to be resistant to any MRSA strain. Interestingly, the resistance rates were oberved to be the highest amongst those MRSA strains which were found to have both mecA and VanA genes. An intermediate or low-level resistance, ascribed to innate glycopeptide resistance, arises both *in vivo* and *in vitro* in *S. aureus* following prolonged drug exposure. In our study, 8.7 to 9.6% isolates were resistant to linezolid in contrast to a study conducted by Thool *et al*. (2012) where 23.5% were resistant to linezolid. Resistance to linezolid is recognized to be associated with mutations in the central loop of domain V of 23S rRNA of the 50S ribosomal subunit and these changes alter the linezolid-binding sites of the bacteria (Long *et al*., 2010). The present study shows that there is a significant rise of reduced susceptibility of glycopeptide (vancomycin, teicoplanin) and lipopeptide (daptomycin). The emergence of the glycopeptide resistance is of great concern in treatment of gram positive infections particluarly MRSA/VISA/VRSA/GISA. Several other studies have also demonstrated reduced susceptibility of MRSA against glycopeptide and lipopeptide (Estes and Derendorf, 2010; Judge *et al*., 2012). The reduced susceptibility of glycopeptide is due to trapping of glycopeptide in the peptidoglycan of the bacterial cell, thus allowing decreased number of glycopeptide reaching the cytoplasmic membrane where the targets of glycopeptides are located (Westerlind, 2012). Nonsusceptibility to daptomycin has also been associated with changes in the structure and function of the cell envelope and surface charge. Linezolid resistance is associated with mutations in the 23S rRNA, although acquired resistance has now been documented (Nannini *et al*., 2010).

Our results demonstrated that, 96.8 to 97.8% of MRSA screened were sensitive to Vancoplus, the novel antibiotic adjuvant entity. The enhanced susceptibility of Vancoplus is because of synergistic activity. When ceftriaxone and vancomycin are used in combination there are less chances of trapping of these molecules in the peptidoglycan as a result enhanced susceptibility.

5. CONCLUSION

There is a significant increase in level of resistance among MRSA and glycopeptides (vancomycin and teicoplanin) are still the current mainstay of therapy for infections caused by MRSA. Excessive use of antimicrobial agents have worsened the sensitivity of the glycopeptide drugs. There is a lack of simple, reliable assays for detecting hGISA phenotypes, because detection of reduced susceptibility to teicoplanin is far easier, but cross-resistance to vancomycin and linezolid is difficult. It is likely that multiple genetic changes occur during acquisition and maintenance of the resistance phenotypes which need to be dealt with new mechnism to fight resistance. The rate at which *S. aureus* can develop or acquire resistance to antibiotics seems to be higher than the rate at which new antibiotics are discovered and developed. Use of antibiotic adjuvant entities is one of the few alternatives that are being developed for use in the treatment of infections due to multidrug-resistant organisms and a number of studies have demonstrated its effectiveness. From the above study it can be concluded that Vancoplus has broad spectrum activity and is more effective in MRSA as well as hGISA organisms. In nut shell prescribing combination antibiotics with a different modes of action combined with adjuvant, effective surveillance, rational use of antibiotics and appropriateness in antimicrobial therapy could reduce the increasing resistance to MRSA.

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