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ORIGINAL RESEARCH

Heterogeneous Vancomycin Intermediate *Staphylococcus aureus* Infections in Diabetic and Non-Diabetic Patients – A Hospital-Based Comparative Study

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Purpose: To study the infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) with emphasis on heterogeneous vancomycin intermediate *S. aureus* (hVISA) in diabetic and non-diabetic patients and their comparison.

Patients and Methods: *S. aureus* strains isolated from diabetic and non-diabetic patients admitted in four tertiary care hospitals in Coastal Karnataka, South India, were tested for methicillin resistance and included in the present study. Demographic and clinical data of the patients were collected using structured proforma. Antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method, and MLS_B phenotypes were identified using the D-test. The minimum inhibitory concentration (MIC) of vancomycin was determined using agar dilution. MRSA isolates were tested for hVISA using vancomycin screen agar and population analysis profile – area under the curve (PAP-AUC) test. Statistical analysis of the results was done using the chi-square test. SPSS version 29.0 was used for this purpose.

Results: Out of 665 strains of *S. aureus* isolated, 220 (33.1%) were MRSA. Of these 220 MRSA strains, 122 (55.5%) and 98 (44.5%) were isolated from diabetic and non-diabetic patients, respectively. There was no significant difference in the antimicrobial resistance patterns of MRSA strains isolated from diabetic and non-diabetic patients. Foot infections and osteomyelitis caused by MRSA were significantly more among diabetic patients. Out of 220 strains of MRSA, 14 (6.4%) were hVISA. The rates of hVISA among MRSA isolated from diabetic and non-diabetic were 9.0% and 3.1%, respectively. This difference was statistically not significant.

Conclusion: The rate of hVISA among all MRSA isolates was 6.4%. The risk of hVISA infection was three times more in diabetic patients. The results emphasize the importance of the detection of hVISA among MRSA isolates especially from diabetic patients.

Keywords: methicillin-resistant *S. aureus*, diabetes mellitus, antimicrobial resistance, heterogeneous vancomycin intermediate *S. aureus*

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most common pathogens causing healthcare-associated infections (HAIs).[1] Since MRSA is multidrug resistant (MDR), treatment of infections caused by this organism is a challenge.[2] Vancomycin is being used for the treatment of serious invasive infections caused by MRSA.[3] However, there has been a rise in the rate of MRSA with reduced susceptibility to vancomycin in recent years.[4,5] Vancomycin intermediate *S. aureus* (VISA) and heterogeneous vancomycin intermediate *S. aureus* (hVISA) were described in 1997.[6] The minimum inhibitory concentration (MIC) of vancomycin to VISA is 4–8 µg/mL.[7] Therefore, VISA can be detected by dilution methods.[7] hVISA is a type of *S. aureus* that contains one vancomycin intermediate cell per 10^5 to 10^6 vancomycin-susceptible cells.[5] Vancomycin MIC for hVISA remains in the susceptible range (≤2 µg/mL).[5,8]

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Antimicrobial susceptibility tests conducted routine in clinical microbiology laboratories fail to detect hVISA.[5,8] Special screening methods and confirmatory population analysis-area under the curve (PAP-AUC) test are required to identify hVISA.[8–10] Vancomycin treatment may not be effective for hVISA infections.[11] Therefore, it is essential to detect hVISA before starting vancomycin treatment. The first case of vancomycin resistant *S. aureus* (VRSA) was reported in a diabetic patient in 2002.[12] Although VRSA infections are uncommon, infections caused by hVISA and VISA are being reported from different parts of the world.[4]

Diabetic patients are more susceptible for infectious diseases because of the increase in blood glucose level, impaired phagocytosis, impaired immunity, peripheral neuropathy and peripheral vascular disease.[13] Further, diabetic patients are predisposed to infections with multidrug resistant bacteria due to frequent hospitalizations and long-term use of antimicrobial agents.[14] According to previous research, MRSA is the most common pathogen isolated from diabetic foot infections and ulcers.[13] Further, a recent study from South India reported MRSA as an important pathogen to cause bone infection and the rate of methicillin resistance among *S. aureus* was 37.0%.[15]

hVISA is emerging as an important healthcare-associated pathogen.[4,16] A meta-analysis described the prevalence of VRSA, VISA and hVISA across the globe, 1.5%, 1.7% and 4.6%, respectively.[4] In India, the prevalence of VRSA, VISA and hVISA is 1.6%, 4.6% and 2.5%, respectively.[4] A previous study from India has shown that among MRSA isolates with vancomycin MIC of ≥1 μg/mL, 12.0% were hVISA.[17] Previous MRSA infection, hospitalization and previous vancomycin treatment are common risk factors for hVISA infection.[5]

There is paucity of literature on hVISA infections among diabetic patients in South India. The purpose of the current investigation was to study infections caused by MRSA with emphasis on hVISA in diabetic and non-diabetic patients and their comparison.

Materials and Methods

Study Setting and Design

The present hospital-based cross-sectional study was conducted on MRSA isolated from diabetic and non-diabetic patients admitted in four tertiary care hospitals (2 public and 2 private hospitals) attached to a private medical college in Coastal Karnataka, India. Public tertiary care hospital 1 and 2 have 1000 and 260 beds, respectively. Private tertiary care hospital 1 and 2 have bed strength of 350 and 600, respectively. The present investigation was conducted in the Department of Microbiology between February 2019 and March 2020. Healthcare-associated infections (HAIs) were identified based on Centers for Disease Control and Prevention (CDC) guidelines.[18]

The study had approval of the Institutional Ethics Committee, Kasturba Medical College, Mangalore. The bacterial strains included in the present study were isolated from the clinical specimens received at the laboratory for investigation, and the samples were anonymized. Therefore, informed consent was not required.

Isolation and Identification of Bacteria

Isolation and identification of *S. aureus* was done using the standard bacteriological methods including gram stain, colony morphology, beta hemolysis, pigmentation, catalase test, coagulase test, DNase test, and mannitol fermentation.[19]

Methicillin resistance was detected using the cefoxitin disk (30 μg) diffusion method as suggested by CLSI.[7] A diameter of zone of inhibition ≤ 21mm was considered methicillin resistance. *S. aureus* ATCC 43300 and *S. aureus* ATCC 25923 were used as positive and negative controls, respectively. Methicillin resistance was confirmed by the detection of *mecA* gene using PCR.[20]

Antimicrobial Susceptibility Testing

Antimicrobial Susceptibility Testing Using Kirby-Bauer Disk Diffusion Method

Antimicrobial susceptibility testing was done using the Kirby-Bauer disk diffusion method.[7] The following antibiotics (BD BBL™ Sensi-Disc™ antimicrobial susceptibility test disks) were used: ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), linezolid (30 μg), rifampicin (5 μg), teicoplanin (30 μg) tetracycline (30 μg)

and trimethoprim-sulphamethoxazole (1.25 µg/23.75 µg). The results were interpreted as per CLSI guidelines.[7] *S. aureus* ATCC 25923 was used as control strain.

Detection of Macrolide, Lincosamide and Streptogramin B (MLS_B) Phenotypes

MRSA strains were tested for MLS_B phenotypes using D test.[7] Mueller–Hinton agar (MHA) plates were inoculated with the test bacterial inoculum having turbidity matching with McFarland 0.5 standard (bacterial count 1.5×10^8 CFU/mL). Clindamycin (2 µg) and erythromycin (15 µg) (BD BBL™ Sensi-Disc™ antimicrobial susceptibility test disks) disks were placed on the inoculated plate at a distance of 15 mm edge to edge. The plates were incubated at 35°C for 16–18 h, and the results were interpreted as per CLSI guidelines.[7]

(a) Flattening of the zone of inhibition around the clindamycin disk facing erythromycin disk, producing D-shaped zone of inhibition was considered inducible clindamycin resistance ($iMLS_B$ phenotype).
(b) No zone of inhibition around erythromycin and clindamycin disk was considered constitutive MLS_B ($cMLS_B$).
(c) No zone of inhibition around erythromycin disk but susceptible circular zone around clindamycin disk without flattening was considered MS_B phenotype.

Determination of Minimum Inhibitory Concentration (MIC) of Vancomycin

The MIC of vancomycin to the test organism was determined using agar dilution.[7] MHA plates containing different concentrations (32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/mL) of vancomycin (Sigma chemical) were prepared. Two to three colonies of the test organism grown on blood agar plate were inoculated into Mueller–Hinton broth and incubated at 37°C for 4 to 6 h till the turbidity was matched with McFarland 0.5 standard (1.5×10^8 CFU/mL). The broth culture was diluted 10 folds to prepare the working inoculum (1.5×10^7 CFU/mL). Two microliters of the working inoculum was spot inoculated on the plates. The plates were incubated at 35°C for 24 h. The minimum concentration of vancomycin that inhibited the bacterial growth was considered the MIC and the results were interpreted as per CLSI guidelines.[7] Isolates with vancomycin MIC $\leq$ 2 µg/mL, 4 to 8 µg/mL and $\geq$16 µg/mL were considered VSSA, VISA and VRSA, respectively.[7] *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as vancomycin-susceptible controls. *E. faecalis* ATCC 51299 was used as vancomycin resistant control.

Identification of hVISA

Screening for hVISA

Brain heart infusion agar (BHI, HiMedia, Mumbai) with 16 g/L pancreatic digest of casein (Sigma Chemical) and 4 µg/mL vancomycin (Sigma Chemical) (BHI screen agar) was used for screening of hVISA.[8] Two bacterial inocula matching with McFarland 2.0 and 0.5 standard were used. Four 10 µL drops from each inoculum were placed on the BHI screen agar plate and allowed to dry for 10 minutes. The plates were incubated at 35°C for 48 h and observed for bacterial growth. If at least one drop had two or more colonies, the isolate was considered hVISA.[8] *S. aureus* ATCC 25923 and *S. aureus* ATCC 700698 (Mu3 strain of hVISA) were used as negative and positive controls, respectively.

Confirmation of hVISA

Modified population analysis profile-area under the curve (PAP-AUC) test described previously by Wootton et al was used for the confirmation of hVISA.[9] Briefly, the test organism was grown in brain heart infusion broth at 35°C for 6 h and the turbidity was matched with McFarland 0.5 standard (1.5×10^8 CFU/mL). The broth culture was further diluted 10^{-2} to 10^{-5}. One bacterial dilution with bacterial count 10^4 CFU/mL was used for inoculation.[10] Ten microliters bacterial inoculum was spread on BHI agar with a range of vancomycin concentrations (16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/mL). Reference strain Mu3, hVISA (*S. aureus* ATCC 700698) was used as positive control. The plates were incubated at 35°C for 48 h and observed for bacterial growth. The PAP-AUC ratio was determined using GraphPad Prism software version 9.0 (GraphPad Software, USA). The test isolate with AUC ratio 0.9 to 1.3 was reported as hVISA. Strains with AUC ratio >1.3 considered as VISA.[10]

Statistical Analysis of Results

The rates of MRSA among *S. aureus* and hVISA among MRSA were expressed in percentage. Statistical analysis of the results was done using chi-square test (Statistical Analysis-Supplementary File). P value of ≤0.05 was considered statistically significant. Statistical Package for the Social Sciences (SPSS), Version 29.0 (IBM Corp., Chicago, Illinois, USA) was used for this purpose.

Results

Out of 665 non-repetitive strains of *S. aureus* isolated from hospitalized patients, 357 (53.7%) were from diabetic patients and 308 (46.3%) were from non-diabetic patients. Out of 665 *S. aureus* strains, 220 (33.1%) were methicillin resistant. Of these 220 MRSA strains, 122 (55.5%) were isolated from diabetic patients and 98 (44.5%) were isolated from non-diabetic patients. The mean and median age of diabetic patients were 58 and 57 years, respectively. The mean and median age of non-diabetic patients were 27 and 28 years, respectively. MRSA was isolated from pus, tissue, blood and IV catheter tip.

The rate of methicillin resistance among *S. aureus* strains isolated from the two public and two private tertiary care hospitals was 33.1% (93/281), 31.0% (9/29), 32.7% (54/165) and 33.7% (64/190), respectively. Of the 220 clinical isolates of MRSA, 143 (65.0%) were from male and 75 (35.0%) were from female patients.

Gender distribution and types of MRSA infections in diabetic and non-diabetic patients are presented in Table 1. Compared with non-diabetic male patients, MRSA infections were significantly more in diabetic male patients. Foot infection and osteomyelitis caused by MRSA were significantly more among the diabetic patients.

The antimicrobial resistance pattern of MRSA isolates is shown in Table 2. There was no significant difference in antimicrobial resistance of MRSA strains isolated from diabetic and non-diabetic patients. Over 80.0% of MRSA were resistant to ciprofloxacin and erythromycin. Multidrug resistance was detected among 72.3% of MRSA strains. All the

Table 1 Gender Distribution and Clinical Characteristics of Diabetic and Non-Diabetic Patients with MRSA Infection

Characteristics of MRSA Infected Patients	Diabetic Patients (n=122) Number (%)	Non-Diabetic Patients (n=98) Number (%)	Total n=220	*p* value
Sex				
Male	88 (72.1)	55 (56.1)	143	0.010*
Female	34 (27.9)	43 (43.9)	77	0.010*
Skin and soft tissue infections (n=186)				
Surgical site infection	48 (39.3)	39 (39.8)	87	0.946
Wound infection	2 (1.6)	25 (25.5)	27	<0.001*
Foot infection/ulcers	31 (25.4)	3 (3.1)	34	<0.001*
Abscess	8 (6.5)	10 (10.2)	18	0.327
Cellulitis	4 (3.3)	2 (2.0)	6	0.575
Carbuncle	4 (3.3)	1 (1.0)	5	0.264
Gangrene	2 (1.6)	1 (1.0)	3	0.694
Necrotizing fasciitis	2 (1.6)	0 (0.0)	2	0.203
Umbilical site infection	0 (0.0)	2 (2.0)	2	0.113
Burn wound infection	0 (0.0)	2 (2.0)	2	0.113

(*Continued*)

Table 1 (Continued).

Characteristics of MRSA Infected Patients	Diabetic Patients (n=122) Number (%)	Non-Diabetic Patients (n=98) Number (%)	Total n=220	*p* value
Deep infection (n=34)				
Bacteremia	14 (7.4)	11 (4.1)	25	0.954
Osteomyelitis	6 (4.9)	0 (0.0)	6	0.026*
Septic arthritis	1 (0.8)	1 (1.0)	2	0.876
Sepsis	0 (0.0)	1 (1.0)	1	0.263

Note: *p value ≤ 0.05 significant.
Abbreviation: MRSA, methicillin resistant *Staphylococcus aureus*.

Table 2 Antimicrobial Resistance Profile of MRSA Strains Isolated from Diabetic and Non-Diabetic Patients

Antimicrobial Agents (Potency)	Diabetic Patients (n=122) Number (%)	Non-Diabetic Patients (n=98) Number (%)	Total n=220	*p* value
Ciprofloxacin (5 µg)	110 (90.2)	83 (84.7)	193	0.219
Clindamycin (2 µg)	22 (18.3)	13 (13.5)	35	0.337
Erythromycin (15 µg),	106 (86.8)	80 (81.6)	186	0.284
Gentamicin (10 µg)	62 (50.8)	48 (48.9)	110	0.786
Linezolid (30 µg)	0 (0.0)	0 (0.0)	0	-
Rifampicin (5 µg)	9 (7.4)	8 (8.2)	17	0.828
Teicoplanin (30 µg)	0 (0.0)	0 (0.0)	0	-
Tetracycline (30 µg)	37 (30.3)	31 (31.6)	68	0.835
Trimethoprim-sulphamethoxazole (1.25 µg/23.75 µg)	62 (50.8)	43 (43.8)	105	0.306
MLS_B phenotype				
$iMLS_B$	37 (30.3)	26 (26.5)	63	0.536
MS_B	47 (38.5)	41 (41.8)	88	0.618
$cMLS_B$	22 (18.0)	13 (13.3)	35	0.337
hVISA	11 (9.0)	3 (3.1)	14	0.072

Note: p value ≤ 0.05 significant.
Abbreviations: $cMLS_B$,constitutive clindamycin resistance; hVISA, heterogeneous vancomycin intermediate *Staphylococcus aureus*; $iMLS_B$, inducible clindamycin resistance; MLS_B, macrolide lincosamide streptogramins B; MRSA, methicillin resistant *Staphylococcus aureus*.

isolates were susceptible to linezolid and teicoplanin. All 220 strains of MRSA were susceptible to vancomycin (MIC ≤ 2 µg/mL) by agar dilution. MIC_{90} and MIC_{50} of vancomycin were 2 µg/mL and 1 µg/mL, respectively.

Out of 220 strains of MRSA, 14 (6.4%) were confirmed hVISA by modified PAP-AUC method. None of the isolate was VISA. No statistically significant difference was observed in the rate of hVISA isolated from diabetic and non-diabetic patients (p = 0.072) (Table 2).

Although skin and soft tissue infection was most commonly caused by hVISA in diabetic patients, there were two cases of bacteremia also. Further, 10 out of 11 hVISA isolated from diabetic patients had MIC of vancomycin in the range 1–2 µg/mL (Table 3).

Table 3 Demographic and Clinical Details of Diabetic Patients Infected with hVISA

hVISA Strains	Age of the Patients	Gender	Hospital	Clinical Condition	Vancomycin MIC (μg/mL)	AUC_{test} /AUC_{Mu3} Ratio
1	69	Female	Private hospital 1	Bacteremia	1.0	0.93
2	61	Male	Private hospital 1	Diabetic foot ulcer	1.0	1.0
3	57	Male	Public hospital 1	Surgical site infection	1.0	0.93
4	85	Male	Public hospital 1	Surgical site infection	0.5	0.93
5	62	Male	Private hospital 1	Surgical site infection	1.0	1.0
6	64	Male	Private hospital 1	Wound gaping at site of chemoport	2.0	1.0
7	54	Male	Public hospital 1	Bacteremia	1.0	1.0
8	65	Female	Public hospital 1	Surgical site Infection	1.0	1.0
9	45	Male	Private hospital 1	Gluteal abscess	2.0	1.0
10	75	Female	Private hospital 1	Right T3 gangrene	2.0	1.0
11	70	Male	Private hospital 1	Cellulitis	1.0	0.93

Note: MRSA strain with AUC_{test} /AUC_{Mu3} ratio 0.9 to 1.3 confirmed as hVISA.
Abbreviations: AUC, area under the curve; hVISA, heterogeneous vancomycin intermediate *Staphylococcus aureus;* MIC, minimum inhibitory concentration.

Discussion

The rate of methicillin resistance among *S. aureus* isolated from HAIs was 33.1% in our study. The rate of MRSA infections varies widely across the country.[17,21,22] A previous study conducted in 2016, in the same study setting, showed methicillin resistance at a rate of 30.2%.[23] This shows that the rate of MRSA has increased in the study area. Further, a higher rate of HA-MRSA (38.6%) was reported in a study from Mangalore, India.[21] Another study has reported an increase in the prevalence of MRSA from 28.0% in 2017 to 35.1% in 2019.[24] The prevalence of MRSA infection is often high among diabetics than non-diabetics.[14,25] The results of the present study are consistent with this observation. Patient population, geographical area, previous use of antibiotics, sample size, and testing methods may affect the reported rate of MRSA.[26]

We compared the type of infections caused by MRSA among diabetic and non-diabetic patients. Skin and soft tissue infections were more than deep infections. Diabetic patients, had significantly higher rates of foot infections and osteomyelitis than non-diabetic patients. Our results are consistent with those of Shah and Hux, who reported four-fold higher rate of osteomyelitis among diabetic patients.[27] Furthermore, in a recent investigation from South India, MRSA was a significant pathogen in bone infection.[15] *S. aureus* is the most common pathogen isolated from diabetic foot infections, primarily in Western countries.[28,29]

In the present study, all MRSA isolates were susceptible to linezolid, and teicoplanin, which is in agreement with observations of previous studies.[23,30] Over 80.0% of MRSA isolates were resistant to ciprofloxacin and erythromycin in our study. This is consistent with results of a previous study.[25] No statistically significant difference was observed in antimicrobial resistance profile of MRSA strains isolated from diabetic and non-diabetic patients. About 72.3% of MRSA were multidrug resistant, leaving limited options for treatment.

Macrolide, lincosamide and streptogramin B (MLS_B) are distinct family of antibiotics.[31,32] Clindamycin, a member of the MLS_B family, is effective in the treatment of MRSA skin and soft tissue infections.[32,33] Inducible clindamycin resistance ($iMLS_B$) limits the use of clindamycin as a therapeutic agent.[31,33] These strains appear resistant to erythromycin but susceptible to clindamycin in disk diffusion test and are unnoticed;[31] therefore, MRSA strains resistant to erythromycin but susceptible to clindamycin should be tested by D-test to detect any inducible clindamycin resistance. In

our study, 30.0% of MRSA isolated from diabetic and 26.5% MRSA isolated from non-diabetic patients were D-test positive (Inducible clindamycin resistance detected).

The rate of hVISA among MRSA was 6.4% in our study. Further, the rate of hVISA was three times higher in diabetic patients. The majority of hVISA infections among diabetic patients in our study were related to skin and soft tissue. However, there were two cases of bacteremia. There are reports of vancomycin treatment failure in hVISA infection.[4,5,11,34] Therefore, it is essential to detect hVISA among MRSA isolated from diabetic patients before starting vancomycin treatment.

Although the current study did not reveal the presence of VISA and VRSA, the presence of hVISA among MRSA is a matter of concern. hVISA is considered the precursor of VISA.[5,6] Therefore, we may expect the emergence of VISA in the future. Implementation of antimicrobial stewardship programme and infection control in the hospitals may help prevent rise in the rate of hVISA and its transmission.

Conclusion

The rate of hVISA among all MRSA strains isolated from healthcare-associated infections was 6.4%. The risk of hVISA infection was three times more in diabetic patients. The results emphasize the importance of the detection of hVISA among MRSA isolates especially from diabetic patients.

Abbreviations

ATCC, American Type Culture Collection; CDC, Centers for Disease Control and Prevention; CLSI, Clinical Laboratory Standard Institute; DFI, diabetic foot infection; DFU, diabetic foot ulcer; DM, Diabetes mellitus; HA, Healthcare associated; HAI, Healthcare-associated infection; hVISA, heterogeneous vancomycin intermediate *Staphylococcus aureus*; MDR, Multidrug resistance; MIC, minimum inhibitory concentration; MLS_B, Macrolide lincosamide streptogramins B; MRSA, Methicillin resistant *Staphylococcus aureus*; PAP-AUC, Population analysis profile-area under the curve; VRSA, Vancomycin resistant *Staphylococcus aureus*; VISA, Vancomycin intermediate *Staphylococcus aureus*.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Statement

The study had approval of the Institutional Ethics Committee, Kasturba Medical College, Mangalore, India [Reference No: IEC KMC MLR 03-19/75 Dated 20-03-2019].

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Disclosure

The authors report no conflicts of interest in this work.

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