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Molecular and Clinical Features of Heterogeneous Vancomycin Intermediate *Staphylococcus aureus* in Tertiary Care Hospitals of South India

Sreejisha M,[1] Shalini Shenoy M,[1] Suchitra Shenoy M,[1] Dhanashree B,[1] Chakrapani M,[2] *Gopalakrishna Bhat K[1]

Departments of [1]Microbiology and [2]Medicine, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, Karnataka, India
**Corresponding Author's e-mail: gkbhat999@gmail.com*

Abstract

Objectives: This study aimed to detect heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) among methicillin resistant *S. aureus* (MRSA) isolated from healthcare-associated infections and identify staphylococcal cassette chromosome *mec* (SCC*mec*) types. ***Methods:*** Isolation and identification of MRSA were done using standard bacteriological methods. Antimicrobial susceptibility testing was done using Kirby-Bauer disc diffusion and macrolide-lincosamide-streptogramin B (MLS_B) phenotypes identified using D test. The minimum inhibitory concentration (MIC) of vancomycin was determined using agar dilution. hVISA were confirmed by modified population analysis profile-area under the curve (PAP-AUC) test. SCC*mec* types and Panton-Valentine leukocidin (*pvl*) were detected using multiplex PCR. ***Results:*** Out of 220 MRSA stains, 14 (6.4%) were hVISA. None of the MRSA isolate was vancomycin intermediate or resistant. All hVISA were susceptible to linezolid and teicoplanin. Macrolide-streptogramin B ($MS_{B)}$ phenotype was present in 42.9% hVISA. 92.9% hVISA strains had vancomycin MIC in the range 1-2 µg/mL. Majority of hVISA and vancomycin susceptible MRSA were isolated from skin and soft tissue infections. SCC*mec* III and IV were present in 50% and 35.7% hVISA respectively. 14.3% hVISA harboured SCC*mec* V. ***Conclusion:*** The rate of hVISA among MRSA was 6.4%. MRSA

strains should be tested for hVISA before starting vancomycin treatment. None of the isolates was vancomycin intermediate or resistant. All the hVISA strains were susceptible to linezolid and teicoplanin. The majority of hVISA were isolated from skin and soft tissue infections. The majority hVISA harboured SCC*mec* III and IV.

Keywords: MRSA; Hospital infection; Molecular typing; Vancomycin

Advances in Knowledge

- To the best of our knowledge, this is the first report of heterogeneous vancomycin intermediate *Staphylococcus aureus* (hVISA) infections in tertiary care hospitals of coastal Karnataka, South India.
- This study showed high frequency of staphylococcal cassette chromosome *mec* (SCC*mec*) types III and IV among hVISA.

Application to Patient Care

- Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from clinical specimens should be tested for the presence of hVISA before starting vancomycin treatment.
- Susceptibility of all hVISA strains to linezolid and teicoplanin.

Introduction

Methicillin resistant S*taphylococcus aureus* (MRSA) continues to be an important pathogen that can cause a variety of healthcare-associated and community-associated infections.[1] Although, vancomycin was the drug of choice for severe MRSA infections after its introduction, the emergence of organisms with reduced susceptibility and complete resistance has been a challenge in the treatment of such cases.[2] MRSA with reduced susceptibility to vancomycin includes heterogeneous vancomycin intermediate *S. aureus* (hVISA) and vancomycin intermediate *S. aureus* (VISA), both first reported in Japan in 1997.[3] The Clinical and Laboratory Standards Institute (CLSI) defines VISA as *S. aureus* with vancomycin minimum inhibitory concentration (MIC) 4-8 µg/mL.[4] hVISA shows MIC of vancomycin in the susceptible range (≤ 2 µg/mL) but, contains a subpopulation at the rate 10^{-5} to 10^{-6} with vancomycin MIC in the intermediate range (4-8 µg/mL).[5] The prevalence of hVISA and VISA has increased worldwide from 4.68% and 2.05% in 2006 to 7.01% and 7.93% in 2014.[6] A recent study from South India showed the prevalence of hVISA at 12.4%.[7]

Mutations of genes associated with the cell wall, thickened cell wall, slow growth, and reduced autolysis are believed to be responsible for reduced susceptibility of hVISA/VISA phenotypes to vancomycin.[8] Mutations in the *wal*KR (sensor protein kinase/regulator), *gra*SR (glycopeptide resistance–associated sensor/regulator), and *vraSR* (vancomycin resistance associated sensor/regulator) and genes are considered important.[2,9,10] Prolonged exposure to vancomycin could induce these mutations.[11]

Vancomycin therapy has been shown to be ineffective for infections caused by hVISA.[2] Therefore, detection of hVISA in the clinical specimens is essential before starting vancomycin treatment. Detection of hVISA among MRSA is a challenge for clinical microbiologists, because it exhibits vancomycin MIC in the susceptible range.[2,5] The antimicrobial susceptibility tests such as Kirby-Bauer disk diffusion, broth dilution, agar dilution, and automated methods fail to detect hVISA.[5] Screening tests such as macro E-test (MET), vancomycin screen agar, and glycopeptide resistance detection (GRD) E-test vary in their sensitivity and specificity.[10,12] Population analysis profile-area under the curve (PAP-AUC), which is considered a reference method is labour intensive, expensive and inappropriate for the routine clinical microbiology laboratories.[12]

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is being used for understanding the epidemiology of MRSA infections. Healthcare-associated MRSA (HA-MRSA) normally harbours SCC*mec* I, II and III. Whereas, community-associated MRSA (CA-MRSA) harbours SCC*mec* IV, V and Panton-Valentine leukocidin gene (*pvl*).[1,13] Panton- Valentine leukocidin is an important virulence factor in CA-MRSA.[13] Several recent studies have reported overlapping of SCC*mec* types between HA-MRSA and CA-MRSA.[14,15] Studies conducted in Europe, USA, Australia and Japan have shown presence of SCC*mec* II, III, and IV among hVISA.[6] However, reports from India have shown predominance of SCC*mec* V in hVISA.[10,16] Therefore, there are differences in the SCC*mec* types harboured by MRSA in different parts of the world. The objectives of the present study were to determine the rate of hVISA among MRSA isolated from healthcare-associated infections (HAIs) and to identify the SCC*mec* types present in these strains.

Methods

The present cross-sectional study was conducted on nonrepetitive MRSA strains isolated from patients admitted in four tertiary care hospitals attached to a private Medical College in Coastal Karnataka South India during the period from February 2019 to March 2020. HAIs were identified using Centers for Disease Control and Prevention (CDC) guidelines.[17]

Isolation and identification of *S. aureus* was done using standard bacteriological methods.[18] Methicillin resistance was detected using cefoxitin (30 µg) disk diffusion method[4] and confirmed by detecting *mecA* gene using PCR.[19] *S. aureus* ATCC 43300 and *S. aureus* ATCC 25923 were used as positive and negative controls respectively. Antimicrobial susceptibility testing was done using the Kirby-Bauer disk diffusion. 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 (1.25µg/ 23.75 µg). Results were interpreted as per CLSI guidelines.[4] *S. aureus* ATCC 25923 was used as the control.

Identification of macrolide lincosamide streptogramin B (MLS_B) was done using D test.[4] Mueller-Hinton agar (MHA) (HiMedia laboratories, Mumbai, India) plates were lawn cultured with test bacterial inoculum with turbidity matching McFarland 0.5 standard (bacterial count 1.5 x 10^8 CFU/mL). Clindamycin (2 µg) and erythromycin (15 µg) disks placed at a distance of 15 mm edge to edge on the inoculated plate. The plates were incubated at 35°C for 16-18 h and the results were interpreted according to CLSI guidelines.[4]

The MIC of vancomycin to MRSA was determined using agar dilution method.[4] MHA agar plates with range of vancomycin (Sigma-Aldrich Corporation, St. Louis, US) concentrations (32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/mL) were prepared. Two to three colonies of the test organism grown on blood agar plate were inoculated into Mueller-Hinton broth (HiMedia laboratories, Mumbai, India) and incubated at 37°C for 4 to 6 h until the turbidity was matched with McFarland 0.5 standard. The broth culture was diluted 10^{-1} to prepare the working inoculum (1.5 x 10^7 CFU/mL). 2 µL was spot inoculated on each plate. The plates were incubated at 35°C for 24 h and observed for growth. The minimum concentration of vancomycin inhibiting the bacterial growth was considered as MIC and the results were interpreted as per CLSI guidelines.[4] MRSA isolates with MIC of vancomycin ≤ 2 µg/mL, 4-8

µg/mL and ≥ 16 µg/mL were considered VSSA, VISA and VRSA respectively.[4] *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 29213 were used as vancomycin susceptible controls. *E. faecalis* ATCC 51299 was vancomycin resistant control.

Screening of MRSA for hVISA was done using brain heart infusion agar (BHIA) (HiMedia laboratories, Mumbai, India) containing 16 g/L pancreatic digestion of casein and 4 µg/mL vancomycin.[12] The test organisms were grown in brain heart infusion broth till the turbidity matched with McFarland 0.5 and 2.0 standard. Four 10 µL drops from each suspension were spot inoculated on BHI screen agar plates and allowed to dry for 10 minutes. The plates were incubated at 35°C for 48 h and observed for bacterial growth. An isolate was considered hVISA if at least one drop had 2 or more colonies.[12] *S. aureus* ATCC 700698 (Mu3 strain of hVISA) and *S. aureus* ATCC 29213 were used as a positive and negative controls respectively.

Confirmation of hVISA was done using the modified population analysis profile- area under the curve (PAP-AUC) method.[20] In brief, the test and control (Mu3) were grown at 35°C for 4-6 hours in brain heart infusion broth, and the turbidity was matched with McFarland 0.5 standard. (1.5×10^8 CFU/mL). The broth culture was further diluted 10^{-4} to achieve viable bacterial count of 10^4 CFU/mL and used for inoculation.[5] A 10 µL bacterial inoculum was spread on BHI agar plates with a range of vancomycin concentrations (16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL). The inoculated plates were incubated at 35°C for 48 h and colonies were counted. The $\log_{10}$ number of colonies was plotted against the concentrations of vancomycin and the area under the curve (AUC) was determined using GraphPad Prism software version 9.0 (Graphpad Software USA).[20] AUC_{test}/AUC_{Mu3} ratio was calculated and used for the confirmation of hVISA. MRSA strains with AUC_{test}/AUC_{Mu3} ratio 0.9-1.3 were considered hVISA [Figure 1] and strains with AUC ratio > 1.3 were considered VISA.[5] Mu3 strain of hVISA (*S. aureus* ATCC 700698) and *S. aureus* ATCC 292l3 (VSSA) were used as positive and negative controls respectively.

SCC*mec* types I-V and *pvl* in the test organisms were identified using multiplex PCR with specific primers and controls.[19,21] DNA was extracted using Qiagen DNA extraction kit as per manufacturer's instructions. The principle of present multiplex PCR performed was based on a previous study by Zhang et al.[19] Multiplex PCR kit was purchased from Qiagen, Hilden,

Germany. The list of primers used for the molecular detection and characterization of HA-MRSA isolates are listed in Table 1.

A 50 µL PCR mixture containing 25 µL multiplex master mix (Containing Taq DNA polymerase, dNTPs and 1X Qiagen Multiplex PCR buffer with 6 mM $MgCl_2$), 5 µL 10X primer mix, 15 µL water and 5 µL DNA extract was prepared in 0.2 mL PCR tubes. Multiplex PCR reaction was performed for 1 cycle of initial denaturation at 97°C for 5 minutes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 54°C and 90 seconds at 72°C, with a final extension for 10 minutes at 72°C. The amplicons were analysed using 2% agarose gel electrophoresis in 1X Tris-Acetate EDTA (TAE) buffer. The electrophoresis was carried out at 120 V for 90 minutes, and the gel was stained with ethidium bromide staining solution for 30 minutes. The gel was then visualized under an ultraviolet (UV) illuminator, and the size of the bands was compared with the 100 base pair ladder (Bangalore Genei Private Limited, Bengaluru, India).

Sensitivity and specificity analyses were done to evaluate the performance of vancomycin agar screen. The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 29.0 (IBM Corp., Chicago, Illinois, USA). The rate of hVISA among MRSA was expressed in percentage. The results were analysed using Fisher's Exact test. P value of $\leq$ 0.05 was considered statistically significant.

This study had the approval of the Institutional Ethics Committee, Kasturba Medical College, Mangalore. The isolates for the present study were collected from the clinical specimens received at the laboratory for investigation. The samples were anonymized and the patient details were not disclosed. Therefore, informed consent was not obtained in the present investigation.

Results

Out of 220 nonrepetitive strains of MRSA isolated form healthcare associated infections, 14 (6.4%) were confirmed hVISA by PAP-AUC and the remaining 206 (93.6%) were vancomycin susceptible. Vancomycin screen agar using both McFarland 0.5 and 2.0 standard inoculum density detected hVISA in 21(9.5%) MRSA isolates. This included 14 isolates confirmed by PAP-AUC. The sensitivity and specificity of the screening method were 100%

and 96.6% respectively. However, the end point (minimum 2 colonies) was clear in the screening method using McFarland 2.0 standard inoculum. None of the isolates was VISA or VRSA. Out of 14 hVISA, 10 (71.4%) and 4 (28.6%) were isolated from male and female patient respectively. In case of 206 vancomycin susceptible MRSA, 133 (64.6%) and 73 (35.4%) were isolated from male and female patients respectively. The majority of hVISA (6/14; 42.9%) were isolated from patients belonging to age group 61-70 years whereas majority of vancomycin susceptible MRSA (48/206; 23.3%) were isolated from patients belong to age group 41-50 years.

Out of 14 patients infected with hVISA, 11 (78.6%) were diabetic, 13 (92.9%) were previously hospitalized, 8 (57.1%) received previous vancomycin treatment and 8 (57.1%) underwent surgery previously. The majority of hVISA and vancomycin susceptible MRSA were isolated from skin and soft tissue infection. 21.4% of hVISA and 10.7% of vancomycin susceptible MRSA were isolated from cases of bacteremia [Table 2].

Table 3 shows the antimicrobial resistance profile of the test organisms. Compared with vancomycin susceptible MRSA more number of hVISA were resistant to antimicrobial agents except trimethoprim-sulphamethoxazole. All the test organisms were susceptible to linezolid and teicoplanin. More than 80.0% of the isolates were resistant to ciprofloxacin and erythromycin. MS_B phenotype was more common in both hVISA (6/14; 42.9%) and vancomycin susceptible MRSA (82/206; 39.8%). 92.9 % hVISA had vancomycin MIC ranging from 1 to 2 µg/mL [Table 4]. For both hVISA and vancomycin susceptible MRSA MIC_{50} and MIC_{90} were 1 µg/mL and 2 µg/mL, respectively.

Results of SCC*mec* typing are presented in Table 5 and Figure 2. The majority of hVISA and vancomycin susceptible MRSA carried SCC*mec* III and IV. There was no significant difference between hVISA and vancomycin susceptible MRSA with regards to SCC*mec* type. 6.8% of vancomycin susceptible MRSA were nontypeable. *pvl* gene was detected in 2/14 (14.3%) hVISA and 57/206 (27.7%) of vancomycin susceptible MRSA isolates.

Discussion

In this study we present the prevalence and molecular features of hVISA in four tertiary care hospitals of coastal Karnataka, south India. The hVISA phenotype was detected among 6.4%

of MRSA strains isolated from healthcare-associated infections. A recent systematic review and meta-analysis has reported the rate of hVISA around the world.[22] The hVISA phenotype was reported in 82 studies on a total of 47,721 strains with an average prevalence of 4.6%. This study showed that the prevalence of hVISA has increased over the last few years in different parts of the world.[22] Three previous studies from India have reported the prevalence of hVISA ranging from 2 to 12.4%.[7,23,24] The differences in the prevalence of hVISA could be due to geographical area of the study, sample size, patient population and testing methods. Increase in the rate of hVISA is a matter of concern. Further, since hVISA is considered as the precursor stage of VISA,[2,3] we may expect an increase in the rate of VISA in the future.

In this study there was no association between hVISA and type of infections. Factors such as age, extended hospital stay, previous vancomycin treatment, diabetes mellitus, instrumentation and surgery may increase in the risk of hVISA infections.[2] In the present study, more than 50 per cent of the patients infected with hVISA had risk factors such as diabetes mellitus, previous hospitalization and vancomycin treatment. The clinical profile of *pvl* positive cases was not different from the negative ones.

Vancomycin treatment of hVISA infections may result in persistence of infection, greater risk of complications and treatment failure.[2,25] Some researchers believe that hVISA arises as a consequence of prolonged vancomycin treatment.[25] Studies have demonstrated that area under curve/MIC of vancomycin > 400 can bring about effective treatment.[26] This can be achieved if vancomycin MIC is ≤ 1 µg/mL. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) classifies *S. aureus* with vancomycin MIC >2 µg/mL as vancomycin resistant.[27] A previous study reported higher mortality among patients with hVISA infection admitted in intensive care unit.[28] In the present study, patients with hVISA deep infections responded for vancomycin treatment. However, in cases where vancomycin toxicity developed, vancomycin was replaced with teicoplanin.

Identification of hVISA phenotype among MRSA is difficult.[2,12] The screening methods vary in sensitivity, specificity and validity. Vancomycin screen agar method used in the present study had sensitivity and specificity of 100% and 96.6% respectively. The PAP-AUC, which is the reference method for the confirmation of hVISA is laborious.[12] It may be difficult to test all MRSA strains for hVISA. In this study, 92.9% of hVISA had vancomycin MIC

ranging from 1 to 2 µg/mL. Similar observations were made by other researchers too.[10,29] Therefore, we suggest MRSA strains with MIC range 1-2 µg/mL could be chosen for detection of hVISA phenotype. In critically ill patients with MRSA infection, hVISA identification may have to be done upfront. In non-critical conditions, hVISA identification may be carried out if clinical response is sub-optimal.

In this study, none of the MRSA was vancomycin intermediate or resistant. All hVISA and vancomycin susceptible MRSA were susceptible to linezolid and teicoplanin. MS_B phenotype was most common followed by $iMLS_B$ (inducible clindamycin resistance). In routine disk diffusion test, MRSA exhibiting inducible clindamycin appears resistant to erythromycin but susceptible to clindamycin. If clindamycin is wrongly used for the treatment of infections caused by such organisms, treatment failure occurs. Therefore, hVISA strains resistant to erythromycin and susceptible to clindamycin should be subjected to D test to detect the possibility of inducible clindamycin resistance.

In this study, the majority of hVISA harboured SCC*mec* III and IV. This in contrast to the previous Indian studies which reported high frequency of SCC*mec* V among hVISA.[7,10,16] Presence of hVISA harbouring SCC*mec* IV, V and *pvl* in the present study is suggestive of entry of CA-MRSA into hospitals. This also shows that molecular differences between HA-MRSA and CA-MRSA is blurring. Although all hVISA strains in the present study could be typeable, 6.8% vancomycin susceptible MRSA were nontypeable. It is possible that these strains could harbour SCC*mec* types not included in the present study. A recent study from South India also reported nontypeable strains among clinical isolates of MRSA.[30]

The present study had some limitations. It is difficult to draw general conclusions based on investigations conducted on small number of hVISA. A larger sample size would have given better understanding of hVISA infections. Multiplex PCR was designed for the detection of SCC*mec* types I-V only. Additional genetic and molecular tests could have helped in better understanding of the epidemiology hVISA.

Conclusion

The rate of hVISA among MRSA was 6.4%. MRSA strains should be tested for hVISA phenotype before starting vancomycin treatment. Vancomycin agar screen with 4 µg/mL

vancomycin and McFarland 2.0 inoculum could be used for screening of MRSA for hVISA. However, confirmation needs PAP-AUC. None of the isolates was vancomycin intermediate or resistant. All hVISA strains were susceptible to linezolid and teicoplanin. The majority of hVISA were isolated from skin and soft tissue infections. SCC*mec* III and IV were predominant among hVISA and vancomycin susceptible MRSA.

Conflicts of Interest

The authors declare no conflict of interests.

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Authors' Contribution

SM collected and organized data, performed the experiments, carried out the statistical analysis of the results, and wrote the initial draft of the article. GBK conceived and designed the study, reviewed the results, analysed and interpreted the data, wrote the initial and final draft of the article, and supervised the study. SM and GBK acquired financial support for the project and participated in the literature review, methods, and discussion. SSM (second author), SSM, and GBK planning and execution of the research activity. SSM provided logistic support and provided research materials. SSM (second author), SSM, DB, and CM designed the study, analysed and interpreted the data, participated in the literature review, methods, and discussion**,** participated in the final writing, and provided supervision. All authors approved the final version of the manuscript.

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Table 1: Primer sequence, control strains with their respective genes used for multiplex PCR and size of amplicon (base pair) post amplification

Genes	Sequence	Control strain	Amplicon size (bp)
mecA	F- GTG AAG ATA TAC CAA GTG ATT R- ATG CGC TAT AGA TTG AAA GGA	MRSA ATCC 43300	147
SCC*mec* I	F- GCT TTA AAG AGT GTC GTT ACA GG R- GTT CTC TCA TAG TAT GAC GTC C	MRSA NCTC 10442	613
SCC*mec* II	F-CGTTGAAGATGATGAAGCG R-CGAAATCAATGGTTAATGGACC	MRSA N315	398
SCC*mec* III	F-CCATATTGTGTACGATGCG R-CCTTAGTTGTCGTAACAG ATCG	MRSA 85/2082	280
SCC*mec* IVa	F-GCCTTATTCGAAGAAACCG R-CTACTCTTCTGAAAAGCGTCG	MRSA JCSC 4744	776
SCC*mec* IVb	F-TCTGGAATTACTTCAGCTGC R-AAACAATATTGCTCTCCCTC	MRSA JCSC 2172	493
	F-ACAATATTTGTATTATCGGAGAGC		200

SCC*mec* IVc	R-TTGGTATGAGGTATTGCTGG	MRSA MR 108	
SCC*mec* IVd	F-CTCAAAATACGGACCCCAATACA R-TGCTCCAGTAATTGCTAAAG	MRSA JCSC 4469	881
SCC*mec* V	F-GAACATTGTTACTTAAATGAGCG R-TGAAAGTTGTACCCTTGACACC	MRSA JCSC 4469	325
Pvl	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R-GCATCAAGTGTATTGGATAGCAAAAGC	MRSA MR108	433

*MRSA= Methicillin resistant Staphylococcus aureus***;** *SCCmec= Staphylococcal cassette chromosome mec;*

Table 2: Isolation of heterogeneous vancomycin intermediate *Staphylococcus aureus* and vancomycin susceptible methicillin resistant *Staphylococcus aureus*

Type of infections (Number)	hVISA (N=14) Number (%)	Vancomycin susceptible MRSA (N=206) Number (%)	*P* value
Surgical site infection (87)	4 (28.6)	83 (40.3)	0.385
Wound infection (63)	3 (21.4)	60 (29.1)	0.762
Bacteremia (25)	3 (21.4)	22 (10.7)	0.220
Abscess (18)	1 (7.1)	17 (8.3)	0.883
Cellulitis (6)	1 (7.1)	5 (2.4)	0.295
Osteomyelitis (6)	0 (0.0)	6 (2.9)	0.517
Carbuncle (5)	0 (0.0)	5 (2.4)	0.555
Gangrene (3)	1 (7.1)	2 (1.0)	0.054
Septic arthritis (2)	0 (0.0)	2 (1.0)	0.711
Umbilical site infection (2)	0 (0.0)	2 (1.0)	0.711
Necrotising fascitis (2)	0 (0.0)	2 (1.0)	0.711
Sepsis (1)	1 (7.1)	0 (0.0)	0.064

hVISA= Heterogeneous vancomycin intermediate Staphylococcus aureus;
MRSA= Methicillin resistant Staphylococcus aureus

Table 3: Antimicrobial resistance profile of heterogeneous vancomycin intermediate *Staphylococcus aureus* and vancomycin susceptible methicillin resistant *Staphylococcus aureus*

Antimicrobial agents	hVISA (N=14) Number (%) resistant	Vancomycin susceptible MRSA (N=206) Number (%) resistant	*P* value
Ciprofloxacin	14 (100.0)	179 (86.9)	0.227

Clindamycin	3 (21.4)	32 (15.5)	0.472
Erythromycin	13 (92.9)	173 (84.0)	0.701
Gentamicin	8 (57.1)	102 (49.5)	0.784
Linezolid	0 (0.0)	0 (0.0)	-
Rifampicin	6 (42.9)	11 (5.3)	<0.001*
Teicoplanin	0 (0.0)	0 (0.0)	-
Tetracycline	5 (35.7)	63 (30.6)	0.767
Trimethoprim-sulphamethoxazole	4 (28.6)	101 (49.0)	0.172
MLS_B phenotypes			
$iMLS_B$	4 (28.6%)	59 (28.6%)	1.000
$cMLS_B$	3 (21.4%)	32 (15.5%)	0.472
MS_B	6 (42.9%)	82 (39.8%)	1.000

**P value ≤ 0.05 statistically significant*

$cMLS_B$= Constitutive clindamycin resistance; hVISA= Heterogeneous vancomycin intermediate Staphylococcus aureus; $iMLS_B$= Inducible clindamycin resistance; MLS_B= Macrolide lincosamide streptogramins B; MS_B= Macrolide streptogramins B; MRSA= Methicillin resistant Staphylococcus aureus

Table 4: Minimum inhibitory concentration of vancomycin to heterogeneous vancomycin intermediate *Staphylococcus aureus* and vancomycin susceptible methicillin resistant *Staphylococcus aureus*

Vancomycin MIC (µg/mL)	hVISA (N=14) Number (%)	Vancomycin susceptible MRSA (N=206) Number (%)
0.125	0 (0.0)	0 (0.0)
0.25	0 (0.0)	5 (2.4)
0.5	1 (7.1)	55 (26.7)
1	8 (57.1)	93 (45.1)
2	5 (35.7)	53 (25.7)
4	0 (0.0)	0 (0.0)
8	0 (0.0)	0 (0.0)
16	0 (0.0)	0 (0.0)
32	0 (0.0)	0 (0.0)
MIC_{50}[a]	1 µg/mL	1 µg/mL
MIC_{90}[b]	2 µg/mL	2 µg/mL

hVISA= Heterogeneous vancomycin intermediate Staphylococcus aureus; MIC= Minimum inhibitory concentration; MRSA= Methicillin resistant Staphylococcus aureus

[a]MIC_{50} = MIC value at which growth was inhibited in 50% of isolates; [b]MIC_{90} = MIC values at which growth was inhibited in 90% of isolates

Table 5: Staphylococcal cassette chromosome *mec* types of vancomycin to heterogeneous vancomycin intermediate *Staphylococcus aureus* and vancomycin susceptible methicillin resistant *Staphylococcus aureus*

SCC*mec* types	hVISA (N=14) Number (%)	Vancomycin susceptible MRSA (N=206) Number (%)	*P* value
SCC*mec* I	0 (0.0)	0 (0.0)	-
SCC*mec* II	0 (0.0)	3 (1.5)	0.649
SCC*mec* III	7 (50.0)	73 (35.4)	0.389
SCC*mec* IVa	4 (28.6)	47 (22.8)	0.621
SCC*mec* IVb	0 (0.0)	0 (0.0)	-
SCC*mec* IVc	0 (0.0)	12 (5.8)	0.353
SCC*mec* IVd	1 (7.1)	20 (9.7)	0.752
SCC*mec* V	2 (14.3)	37 (18.0)	0.727

hVISA= Heterogeneous vancomycin intermediate Staphylococcus aureus; SCCmec= Staphylococcal cassette chromosome mec; MRSA= Methicillin resistant Staphylococcus aureus

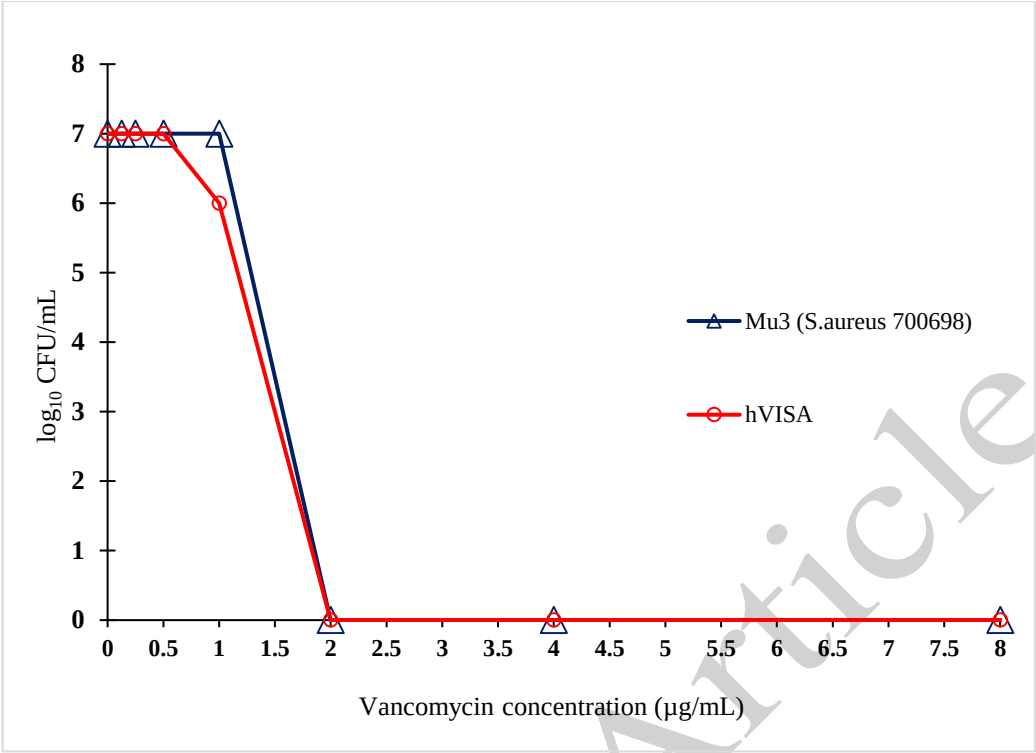

Figure 1: Confirmation of hVISA using modified PAP-AUC

Mu3- hVISA reference strain (*S. aureus* ATCC 700698)

AUC_{test} = 9.750; AUC_{Mu3} =10.50; AUC_{test}/AUC_{Mu3} ratio = 0.93 (hVISA)

AUC= area under the curve; CFU= colony forming unit; MRSA= methicillin resistant Staphylococcus aureus; hVISA= heterogeneous vancomycin intermediate Staphylococcus aureus; PAP-AUC= population analysis profile-area under the curve

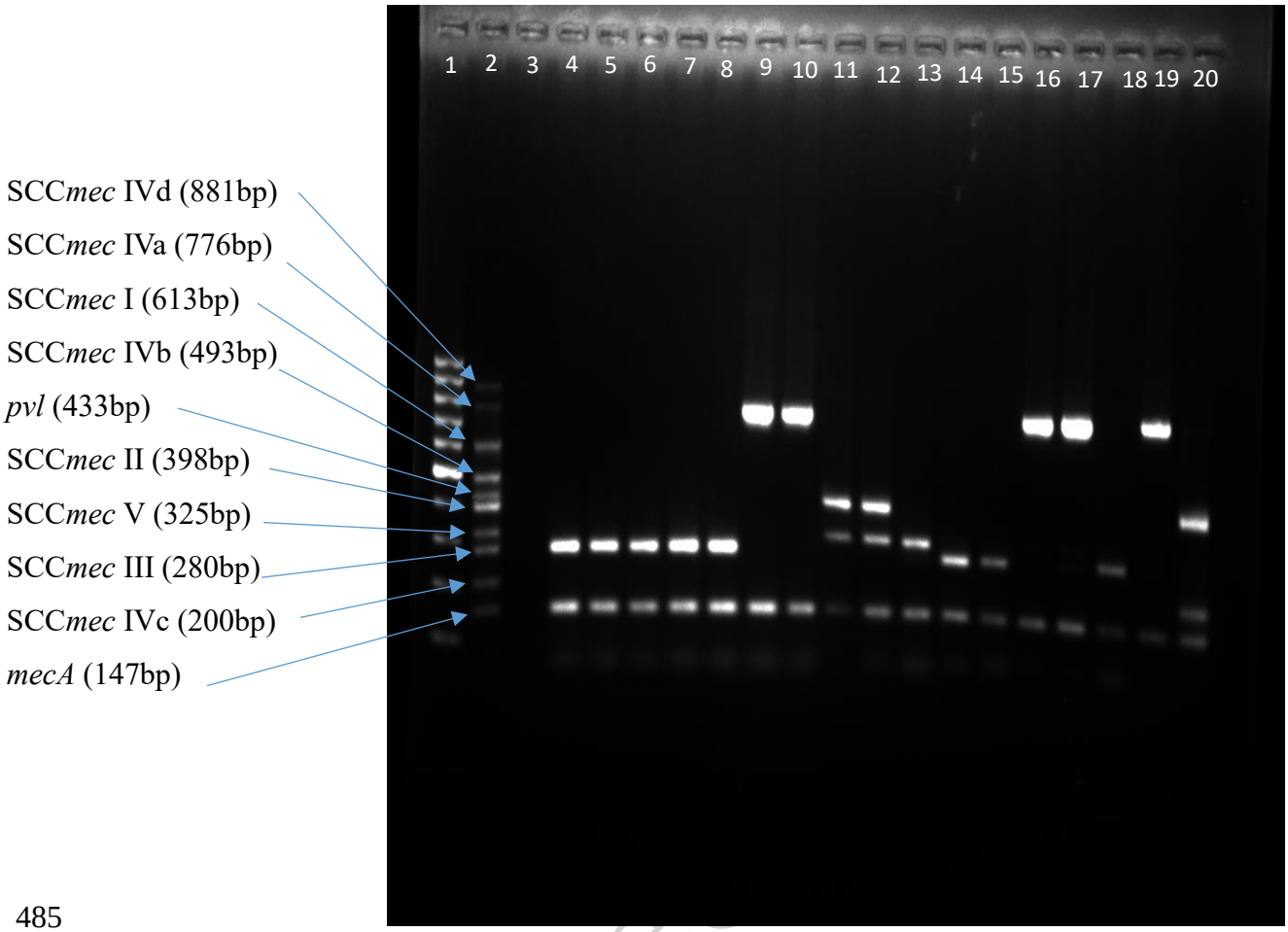

Figure 2: Gel electrophoresis of multiplex PCR for the detection of *mecA,* SCC*mec* types 1-V and *pvl* gene

Lane 1: 100 bp DNA ladder; **Lane 2**: positive controls; **lane 3**: negative control (master mix and nuclease-free water); **Lane 4-8, 14, 15 and 18**: Vancomycin susceptible MRSA isolates positive for *mecA*, and SCC*mec* III; **Lane 9, 10, 17, 19:** Vancomycin susceptible MRSA isolates positive for *mecA*, and SCC*mec* IVa; **Lane 16**: hVISA isolate positive for *mecA*, and SCC*mec* IVa, **Lane 11 and 12**: Vancomycin susceptible MRSA isolates positive for *mecA*, SCC*mec* V, and *pvl*; **Lane 13**: Vancomycin susceptible MRSA isolate positive for *mecA*, and *SCCmec* V; **Lane 20**: Vancomycin susceptible MRSA isolate positive for *mecA*, SCC*mec* IVc, and *pvl*

SCCmec= Staphylococcal cassette chromosome mec; hVISA= heterogeneous vancomycin intermediate Staphylococcus aureus; pvl= Panton-Valentine leukocidin gene