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Detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus*: A preliminary report from south India

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***Background & objectives*: Although there are reports of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) across the globe, there is a lack of reliable data on hVISA in India. The present study was undertaken to determine the rate of hVISA among the methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, and to compare the brain heart infusion agar with vancomycin 4 µg/ml (BHIV4) method with population analysis profile-area under the curve (PAP-AUC) method for the detection of hVISA and to study the distribution of mobile genetic element that carries methicillin-resistance gene SCC*mec* (Staphylococcal cassette chromosome *mec)* types among these isolates.**

***Methods*: BHIV4 and PAP-AUC methods were employed to detect hVISA among 500 clinical isolates of MRSA. SCC*mec* typing of these isolates was performed by multiplex polymerase chain reaction. The clinical presentation, treatment with vancomycin and outcome was documented for patients with hVISA.**

***Results*: The rate of hVISA was 12.4 per cent by PAP-AUC method. Sensitivity, specificity, PPV, NPV and kappa agreement of BHIV4 with PAP-AUC was 58.06, 93.15, 54.55, 94.01 per cent and 0.498, respectively. The isolation of hVISA was significantly ($P<0.01$) higher in patients admitted to intensive care units and wards than in patients attending the outpatient departments. Only 38 per cent of the patients received vancomycin as therapy. Majority of the hVISA isolates carried SCC*mec* type V or IV.**

***Interpretation & conclusions*: The rate of hVISA isolation in our study was 12.4 per cent. The sensitivity of the BHIV4 screening test was low, and was in moderate agreement with PAP-AUC test. SCC*mec* type V was the predominant type seen in half of the isolates. More studies need to be done in different parts of the country on a large number of isolates to confirm our findings.**

Key words BHIV4 - hVISA - *mecA* - methicillin-resistant *Staphylococcus aureus* - PAP method - SCC*mec* type - vancomycin

Vancomycin, a glycopeptide antibiotic, was approved by the U.S. Food & Drug Administration in 1958 and has since become the drug of choice for the treatment of serious methicillin-resistant *Staphylococcus aureus* (MRSA) infections. There was no report of vancomycin resistance in *Staphylococcus aureus* for 40 years[1]. The initial reports of MRSA with reduced susceptibility to vancomycin such as

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vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) were from Japan in 1997[2]. After the first report, many cases of MRSA with reduced susceptibility to vancomycin, particularly hVISA were reported with increasing frequency across the globe. Rates varied from 2 to 50 per cent depending on the geographic area and the methods employed[2,3]. Singh *et al*[3] from Lucknow, India, reported a prevalence of 5.8 per cent[3]. hVISA has been associated with treatment failure, prolonged hospitalization and persistent infections which generated major concern in the health-care community[1,2,4-6]. hVISA isolates are difficult to identify in a routine clinical microbiology laboratory as the vancomycin minimum inhibitory concentration (MIC) level is within the susceptible range but with a certain proportion of cell population exhibiting intermediate susceptibility to vancomycin. These subpopulations are present at a low ratio at a frequency of 10^{-5} to 10^{-6}. Population analysis profile-area under the curve (PAP-AUC) is the gold standard method for the detection of hVISA, but it is a laborious and time-consuming procedure[1,2,7]. In addition to PAP-AUC method, there are several screening methods used to detect hVISA, such as brain heart infusion agar with vancomycin 4 µg/ml (BHIV4), macrodilution *E*-test method (MET), glycopeptide resistance detection (GRD) test and gradient plate method with varying sensitivities and specificities. Majority of the studies performed initial screening by BHIV4, MET, GRD test, *etc.*, and only those isolates which were positive by screening test were further confirmed by PAP method. There is a possibility that the exclusion of isolates by initial screening tests might underestimate the exact prevalence rate of hVISA. The distribution of staphylococcal cassette chromosome *mec* (SCC*mec)* types among hVISA isolates has shown difference between the study by Singh *et al*[8] and the rest of the world. While the SCC*mec* types predominantly reported from Europe, USA, Australia and Japan were II, III and IV[4]. Singh *et al*[8] reported a high prevalence of type V in their study.

The present study was undertaken to determine the rate of hVISA among the MRSA isolates, to compare the BHIV4 with PAP-AUC method for the detection of hVISA and to study the distribution of SCC*mec* types among these isolates.

Material & Methods

A total of 500 non-repetitive MRSA isolates obtained from various clinical samples during January 2014-December 2016 in the department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry, India, were included in the study. This included 125 stored isolates each from 2014 to 2015 (maintained in 50% glycerol stock, stored at −80°C) and 250 fresh isolates from 2016. Methicillin resistance was determined by cefoxitin (30 µg, Oxoid, UK) disc diffusion method[9]. All MRSA were genotypically confirmed by *mecA* gene polymerase chain reaction (PCR). The MIC of vancomycin for these isolates ranged from 0.25 to 2 µg/ml by *E*-test (bioMérieux Marcy-l'Étoile, France). The study was approved by the Institutional Ethics Committee (Human Studies).

Brain heart infusion agar with vancomycin 4 µg/ml (BHIV4) method: All the isolates were inoculated on brain heart infusion agar (BHI, HiMedia, Mumbai) with 4 µg/ml of vancomycin (Sigma Chemical Co., USA)[1,7]. Spot inoculation of 10 µl of a 0.5 McFarland bacterial suspension was made onto BHIV4. Growth after 48 h was considered to represent hVISA[3]. ATCC *S. aureus* 25923 was used as negative control and ATCC-700698, Mu3 strain of hVISA as a positive control for each plate.

Population analysis profile-area under the curve (PAP-AUC) method: Briefly, PAP procedure was performed by inoculating a few colonies into BHI broth and incubating overnight at 37°C. Log dilutions were then prepared (10^{-1} to 10^{-6}). 100 µl of 10^{-3} and 10^{-6} dilutions were lawn cultured onto a set of BHI agar containing vancomycin at concentrations ranging from 0 to 8 µg/ml. Using colony forming unit (cfu/ml) values, the AUC was determined by using GraphPad prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). hVISA (ATCC-700698, Mu3), VISA (ATCC-700699) and *S. aureus* (ATCC-29213) were used as control strains. As per PAP method, if the ratio of AUC of test to the AUC of control was between ≥0.9 and ≤1.3, the isolate was considered as hVISA and if it was ≥1.3, it was considered as VISA[10].

Determination of SCCmec types of hVISA isolates: DNA was extracted using Qiagen extraction kit as per the manufacturer's instructions (Mericon DNA Bacteria Plus Kit, Qiagen, Germany). Uniplex PCR was performed for *mec*A gene detection as per the standard protocol[11]. Multiplex PCR was performed for SCC*mec* types (I to V) according to previously published protocol with slight modifications[12,13]. PCR conditions were as follows: initial denaturation at 94°C for five minutes followed by 35 cycles of 94°C for 45 sec, 53°C for

45 sec and 72°C for 45 sec with final extension at 72°C for 10 min. The gel was visualized and documented using ImageLab software (Bio-Rad Laboratories, CA, USA). Previously characterized and confirmed MRSA with different SCC*mec* types from our laboratory were used as controls.

Statistical analysis: The performance characteristics of BHIV4 were analyzed using Graph pad Quickcalcs, online software (*https://www.graphpad.com/quickcalcs/catMenu/*). Chi-square test was used to analyze differences in the rate of hVISA across various MIC values of vancomycin. Pearson Chi-square test was used to analyze the difference in the distribution of hVISA in various locations such as outpatient department (OPD), ward and intensive care unit (ICU). The analysis was done using SPSS v21.0 (IBM Corp., Armonk, NY, USA) and OpenEpi v3.03 (*www.OpenEpi.com*).

Results

Of the total 500 MRSA isolates (363 from wards, 118 from OPD, 19 from ICU), PAP-AUC method detected 62 hVISA while BHIV4 identified 66 hVISA isolates. Thirty six isolates were identified by both methods. There were 30 isolates which were identified as hVISA by the BHIV4 which were negative by PAP analysis while 26 isolates identified as hVISA by PAP analysis were negative by BHIV4 (Table I). The tests were in moderate agreement with each other (k=0.498, P<0.001).

hVISA isolates were significantly more common in MRSA isolates with vancomycin MIC 1-2 µg/ml (53/350, 15%) than those with a MIC <1 µg/ml (9/150, 6%) (P<0.01). The rate of hVISA was significantly (P<0.01) higher among the patients admitted to ICUs and wards (21.5% and 14.3%, respectively) when compared to those attending OPD (5.5%). There was a slight difference in hVISA rates in the different years of study (11.2% in 2014, 9.6% in 2015 and 14.4% in 2016); however, this difference was not significant.

Of the 62 hVISA isolates, SCC*mec* type-V was the predominant type in 50 per cent of the isolates (31/62) followed by type IV [11/62 (17.7%)] and type III [5/62 (8%)]. Three isolates carried multiple SCC*mec* types, III, IV and V in one isolate, types II and IV in the second and types IV and V in the third isolate. Thirteen isolates (20.9%) were non-typable. Among the 62 patients who were infected with hVISA, clinical and treatment details were available only for 50. Majority of the isolates were from skin and soft-tissue infections including surgical site infections while nine patients had bacteraemia. The average hospital stay before isolation of hVISA was 15 days and 38 per cent (n=24) of the patients received vancomycin for an average of seven days while 18 per cent (n=11) received linezolid. History of the previous hospitalization was available for 20 per cent of the patients. All the patients with hVISA survived.

Discussion

A meta-analysis described the varied prevalence of hVISA across the globe, with an average of 6.81 per cent of 64,692 MRSA isolates from 35 studies across Asian countries and 5.6 per cent of 34,350 isolates from 41 studies across Europe and America[4]. Till date, there have been four reports on the prevalence of hVISA from India. The earliest was a multi-country study in 2004 reported from South Korea which included some Indian isolates. Here, the prevalence was found to be 6.3 per cent, the second highest among 12 Asian countries, the highest prevalence being from Japan[14]. This was followed by two other reports[15,16] where the prevalence of hVISA ranged from 2 to 6.9 per cent. However, the sample size in these studies was very small (50 and 58, respectively). The study which tested numbers comparable to our study was published by Singh *et al* from Lucknow[3], where the hVISA prevalence was reported to be 5.8 per cent (Table II).

Although the PAP-AUC is the gold standard method for the detection of hVISA, it is labour intensive for routine use, prompting the introduction of

Table I. Comparison of brain heart infusion agar with vancomycin 4 µg/ml (BHIV4) and population analysis profile methods for the identification of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (n=500)

Test method	PAP-AUC positive	PAP-AUC negative	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa agreement	*P*
BHIV4 positive	36	30	58.06	93.15	54.55	94.01	0.498	<0.001
BHIV4 negative	26	408						

PAP-AUC, population analysis profile-area under the curve; PPV, positive predictive value; NPV, negative predictive value

Table II. Heterogeneous vancomycin-intermediate *Staphylococcus aureus* hVISA among methicillin-resistant *Staphylococcus aureus* isolates in India and comparison of screening methods with population analysis profile-area under the curve (PAP-AUC) for detection of hVISA

Study	Sample size	Screening methods for the detection of hVISA (%)	PAP-AUC (%)	Sensitivity	Specificity	PPV (%)	NPV (%)
Song *et al*[14], 2004; India	80	BHIV4 - 16/80 (20)	5/80 (6.3)	-	-	-	-
Iyer and Hittinahalli[15], 2008; Hyderabad	50	BHIV6 - 4/50 (8)	1/50 (2)	100	93.8	25	-
Chaudhari *et al*[16], 2015; Pune	58	BHIV6 - 5/58 (8.6)	4/58 (6.9)	75	93	43	98
		MET - 5 (8.6)	-	67	94	40	98
Singh *et al*[3], 2015; Lucknow	500	BHIV4 - 63/500 (12.6)	29/500 (5.8)	-	-	46.2	-
		MET - 49/500 (9.8)	-	-	-	59.1	-
		GRD - 55/500 (11)	-	-	-	52	-
		Gradient plate - 53/500 (10.6)	-	-	-	54.7	-
Present study, 2017; Puducherry	500	BHIV4 - 66/500 (13.2)	62 (12.4)	58.1	93.1	54.6	94

BHIV4, brain heart infusion agar with vancomycin 4 µg/ml; BHIV6, BHIV 6 µg/ml; MET, macrodilution *E*-test; GRD, glycopeptide resistance detection; PPV, positive predictive value; NPV, negative predictive value

several screening methods such as BHIV4, MET and GRD test[2,4]. Majority of the studies initially performed various screening tests in combination, and only those positive by screening tests were further confirmed by PAP method. Such selective testing may under-report the true prevalence rate of hVISA. In our study, all the 500 isolates were tested by the two methods. The rate of hVISA was found to be 12.4 per cent (62/500) by PAP-AUC method and 13.2 per cent by BHIV4 (66/500). Although higher positivity was found with BHIV4 method, only 36 isolates were confirmed to be hVISA by PAP method. Of the 434 isolates which were negative by BHIV4 test, 26 (5.8%) were positive by PAP method (46.5% of total hVISA isolates). Zhang *et al*[4], in their meta-analysis commented that a significant number of hVISA might have been missed by not performing PAP method for all isolates.

Several studies have demonstrated that rates of hVISA are greater as the MIC of vancomycin approaches the breakpoint of 2 µg/ml[2,4,17]. This was corroborated in our study as 15 per cent of hVISA had MIC between 1 and 2 µg/ml, in contrast to six per cent where MIC was <1 µg/ml.

The rate of hVISA was significantly higher among MRSA isolates from ICU and wards when compared to those from the OPD patients as vancomycin usage was negligible in the latter group. It has been suggested that hVISA arises *de novo* from a previously sensitive isolate when the patient is on prolonged vancomycin therapy[2]. This is particularly likely to happen if the initial trough values of vancomycin do not exceed 10 µg/ml[18].

In our study, only 38 per cent of hVISA-infected patients were on vancomycin treatment and had an average of 15 days of hospital stay before the isolation of hVISA. It is not essential that all patients with hVISA should have been on vancomycin therapy as successful horizontal transmission of hVISA has been shown among hospitalized patients[19]. In the present study, all patients with hVISA for whom treatment and outcome details were available, survived. Most bacteraemic patients received linezolid in addition to vancomycin, which could have contributed to a better outcome. In a case series of 19 patients infected with MRSA tested for hVISA phenotype by PAP method, hVISA-infected patients were significantly associated with treatment failure (86%) compared to non-hVISA-infected patients (20%)[20]. However, in a few other studies, no increase in mortality was observed in patients with hVISA who were treated with linezolid[21,22].

In the present study, majority of the hVISA isolates carried SCC*mec* type V (50%) and type IV (17.7%) and only eight per cent carried type III. However, three isolates carried combinations of SCC*mec* types. Such a high proportion of hVISA with SCC*mec* type V has not been reported from elsewhere, except from India[8].

This study had some limitations as only one screening method (BHIV4) was used and other

screening methods such as MET, GRD, gradient plate and BHIV6 were not applied.

In conclusion, the rate of hVISA was 12.4 per cent among MRSA isolates by PAP-AUC method. BHIV4 screening test was in moderate agreement with PAP-AUC test. The majority of hVISA isolates carried SCC*mec* type V.

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***Conflicts of Interest*:** None.

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