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Detection of Vancomycin susceptibility including h-VISA among Methicillin resistant Staphylococcus aureus and Staphylococcus species isolated from orthopaedic device associated infections in a tertiary care hospital

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Abstract
Globally, Staphylococcus aureus is the major causative organism for medical device associated infections. Of this, the proportion of Methicillin resistant Staphylococcus aureus (MRSA) has worldwide for the last two decades. Treatment of choice for MRSA is vancomycin, however, resistance to vancomycin by Staphylococcus species is being encountered from the midst of 1990's. This study was conducted on 90 Pus samples from the wound of the infected orthopaedic implant, out of that, 75 isolates are pathogens and among that, 59 isolates are identified as Staphylococcus species, of which 31 isolates are Staphylococcus aureus and 28 isolates are Staphylococcus epidermidis. Among 31 Staphylococcus aureus, 19 are MSSA and 12 are MRSA, similarly in 28 Staphylococcus epidermidis 16 are MSSE and 12 are MRSE. Totally 24 isolates were Methicillin resistant Staphylococcus species, of which 14 isolates are showing multidrug resistance. Therefore, these 14 isolates (11 isolates of MRSA and 3 isolates of MRSE) are subjected to vancomycin MIC by using Agar dilution (0.5 & 2 McFarland standards) and Micro-broth dilution (0.5 McFarland standards) methods. Out of 14 tested, only 5 isolates are detected as VISA, and are screened for hVISA also, by using GRD E-test and no hVISA /hGISA are found. Hence, from the 14 isolates, 6 are VSSA, 5 are VISA and 3 are VSSE.

Keywords: *MSSA-Methicillin sensitive Staphylococcus aureus, MRSA- Methicillin resistant Staphylococcus aureus, MSSE- Methicillin sensitive Staphylococcus epidermidis, MRSE- Methicillin resistant Staphylococcus epidermidis, VSSA-Vancomycin susceptible Staphylococcus aureus, VISA- Vancomycin intermediate Staphylococcus aureus, hVISA- Heteroresistant Vancomycin intermediate Staphylococcus aureus, VRSA- Vancomycin resistant Staphylococcus aureus, GRD E-Test-Glycopeptide resistant detection* Epsilometer *Test, CLSI-Clinical and Laboratory standards Institute, MIC-Minimum inhibitory concentration.*

Introduction

Globally, Staphylococcus aureus generally causes various types of skin and soft-tissue infections. This still remains an important cause for mortality and morbidity. Among the Staphylococcus species, Staphylococcus aureus causes both nosocomial and hospital acquired infections. Of this, the proportion of Methicillin resistant Staphylococcus aureus (MRSA) has risen worldwide during the last two decades even in spite of emergence of newer antimicrobial agents. The recommended main stay of treatment for multidrug resistant MRSA is Vancomycin, which is a complex tri-cyclic glycopeptides[7]. But,

however, resistance to new antimicrobials is generally recognized in Staphylococcus aureus soon after they are released for clinical use. It is no way an exception for Vancomycin, which was introduced during midst of 1950's and resistance to it was reported during the midst of 1990's [20].

In the year 1997, the first isolate with reduced or intermediate susceptibility to Vancomycin had emerged in Japan [20]. However, infections with Vancomycin susceptibility Staphylococcus aureus (VSSA) isolates are showing Vancomycin treatment failure [1]. Later, this phenomenon was detected as hVISA which are the precursor of VISA and is defined as the subset of population (1 in 100,000 bacteria)among Staphylococcus aureus isolates which are able to grow in vivo at a relatively higher concentration of Vancomycin (>2 µg/ml) and in vitro Vancomycin containing media[3].

Unraveling the complex genetics and cell wall structural changes conferring low-level Vancomycin resistance in Staphylococcus aureus has proved challenging. However, the recent genomic sequencing had played a key role in determining the breadth of bacterial chromosomal changes linked with resistance. Diverse mutations in a small number of Staphylococcus regulatory genes, particularly walKR, graRS, vraSR and rpo-B genes have been associated with hVISA and VISA. But, however only few experiments have proven their resistance pattern [27].

Transcriptomics studies and analysis of host pathogen interactions indicates the evolution of Vancomycin susceptible Staphylococcus aureus (VSSA) to Vancomycin intermediate Staphylo-coccus aureus (VISA) and this Vancomycin intermediate Staphylococcus aureus (VISA) was associated with persistence of infection. As a result of it, there will be predicted alterations in central metabolism, altered and attenuated expression of virulence factors and alterations in susceptibility to host innate immune responses. Finally altogether there will be reduced susceptibility to other classes of antibiotics by the host as well. [27]

According to CLSI 2016 -M100S guidelines[5], Vancomycin with MIC breakpoints ≤2 µg/ml -VSSA, 4-8 µg/ml -VISA and ≥16 µg/ml -VRSA. The conventionally used disk diffusion method detects only the strains with higher Vancomycin resistance, but fails to detect VISA isolates. Hence MIC (minimum inhibitory concentration) determination by using Agar dilution, Micro broth dilution methods and by using GRD E-Test (a gradient diffusion method employing antibiotic impregnated strips) were recommended for Vancomycin susceptibility testing.

The standard inoculums of ($5x10^4$ per well with MIC broth) will not be able to detect hetero resistance VISA (hVISA). Therefore, most of the times the Vancomycin MIC will fall within the susceptibility range[20]. Hence, Population analysis profile -Area under the curve (PAP- AUC) method is employed, which determines the number of survival cells at increasing antibiotic concentration and is used as the gold standard method to detect hVISA[30]. However this method is technically demanding and it is not suitable for routine clinical microbiology laboratories. In order to overcome this, Glycopeptide resistant detection -GRD E test with standard McFarland's standard has been performed to detect hVISA with a fair degree of accuracy which is helpful for early detection of VISA and hVISA thereby the treatment will be successful [20].

Aim

To detect the vancomycin susceptibility among Methicillin resistant Staphylococcus species in Orthopaedic device associated infections in a tertiary care hospital. (1) By determining the MIC using Agar dilution and Micro-broth dilution methods and 2) To screen for hVISA using GRD E-Test method.

Materials and Methods

The study was conducted in a tertiary care hospital, Chennai in the Department of Microbiology in concurrence with the Department of Orthopaedic surgery, after obtaining ethics

clearance and informed consent. It is a prospective study of cross-sectional type. All age groups and both the genders were included for this study. Totally90 pus samples were collected from the wound of the infected orthopaedic implants. Among these 90 samples, most common organisms i.e. nearly two-third of the isolates were Staphylococcus species (59 isolates) that were identified using standard microbiological method. Out of this 59 isolates, 31 isolates were Staphylococcus aureus and 28 were Staphylococcus epidermidis (CONS).Of these, 12 were Methicillin resistant Staphylococcus aureus (MRSA) and 12 were Methicillin resistant Staphylococcus epidermidis (MRSE) which were identified by using the surrogate marker - Cefoxitin 30µg by disk diffusion method using Staphylococcus aureus ATCC *25923 as a control strain[(7)]. Out of these Methicillin resistant Staphylococcus species strains, 14 isolates were entirely showing resistance to more than two or three classes of antibiotics. So, in this study only those 14 multidrug resistant isolates were included for detecting the vancomycin MIC by agar dilution using 0.5McFarland standard and 2 McFarland standards and micro broth dilution using 0.5McFarland standard based on CLSI 2016-M100S guidelines[(6)].

Vancomycin hydrochloride stock solution was prepared according to the HIMEDIA insert instructions (Ref.No:CMS217-500MG).Based on the CLSI 2016-M100S guidelines [(6)], serial dilutions of Vancomycin was prepared. For Agar dilution, serial concentration of drug in µg/ml is mixed with 24 ml of sterile Muller-Hinton agar and then they are poured into the sterile petri plates. Similarly for Microbroth dilution serial concentration of drug in µg/ml is mixed with sterile Muller-Hinton broth (150µl) in a sterile 96 wells microtitre plates. Overnight (18-24 hours) fresh culture of 14 isolates were taken in peptone water and matched with 0.5 Mcfarland &2 Mcfarland standards for agar dilution and 0.5 Mcfarland for Microbroth dilution[(24)].

For Agar dilution, the sterile petri plates with vancomycin agar in serial dilutions were divided in to 16 quadrants (including suitable controls Staphylococcus aureus ATCC *25923) and are inoculated with 10µl of the respective sample (0.5 McFarland standard) to each quadrant. The plates were incubated aerobically at 37^0C for 24-48 hours. Similarly the above procedure was followed for 2 McFarland standard inoculums also. The plates were incubated aerobically at 37^0C for 24 hours. The MIC readings were taken after 24-48hours and using CLSI guidelines 2016-MS100 the isolates were identified as VSSA, VISA and VRSA and VSSE.

For Micro broth dilution, in sterile 96 wells microtitre plates, 150µl of Muller-Hinton broth with desired drug concentration in µg/ml from lower concentration to higher concentration was taken and mixed with 150µl of the isolates that were inoculated in peptone broth which were matched with 0.5 McFarland standard .The plates were incubated aerobically at 37^0C for 24 hours. The MIC readings were taken after 24 hours using CLSI guidelines 2016-MS100 the isolates were identified as VSSA, VISA, VRSA and VSSE.

The screening test for hVISA was done only for VISA isolates identified by above methods using GRD E-Test (HIMEDIA insert instructions EZY MIC EM111 E STRIP). This test was performed using sterile Muller-Hinton with 5% blood agar plate using standard inoculum of 0.5 McFarland and were incubated aerobically at 37^0 C .The readings were taken after 24 hours and 48hours. If the MIC of Vancomycin or Teicoplanin is $\geq$8µg/ml it was defined as hVISA strain.

Results

The vancomycin MIC which was done by Agar dilution using 0.5 McFarland showed the following results after 24-48 hours of incubation with satisfactory controls ;7 out of 14 isolates of Methicillin resistant Staphylococcus aureus (MRSA) were vancomycin susceptible Staphylococcus aureus (VSSA), 4 out of 14 isolates of Methicillin resistant Staphylococcus

aureus were vancomycin intermediate Staphylococcus aureus(VISA) and 3 out of 14 isolates of Methicillin resistant Staphylococcus epidermidis (MRSE) were vancomycin susceptible Staphylococcus epidermidis (VSSE) . Similarly with 2 McFarland standards, the results that were obtained after 24-48 hours of incubation were as follows;8 out of 14 isolates of Methicillin resistant Staphylococcus aureus (MRSA) were Vvancomycin susceptible Staphylococcus aureus (VSSA), 3 out of 14 isolates of Methicillin resistant Staphylococcus aureus were vancomycin intermediate Staphylococcus aureus (VISA) and 3 out of 14 isolates of Methicillin resistant Staphylococcus epidermidis (MRSE) were vancomycin susceptible Staphylococcus epidermidis (VSSE) .

Table-1: Agar Dilution-0.5 & 2 Mcfarland.

S.NO.	ORGANISM	NO: OF ISOLATES, N=14.	AGAR DILUTION		AGAR DILUTION		
			0.5 McFarland	INFERENCE	NO: OF ISOLATES, N=14.	2 McFarland	INFERENCE
1.	MRSA	7	2 µg/ml	VSSA	8	4 µg/ml	VSSA
2.	MRSA	2	4 µg/ml	VISA	3	8 µg/ml	VISA
3.	MRSA	2	4 µg/ml	VISA	-	-	-
4.	MRSE	3	4 µg/ml	VSSE	3	4 µg/ml	VSSE
TOTAL = 14							

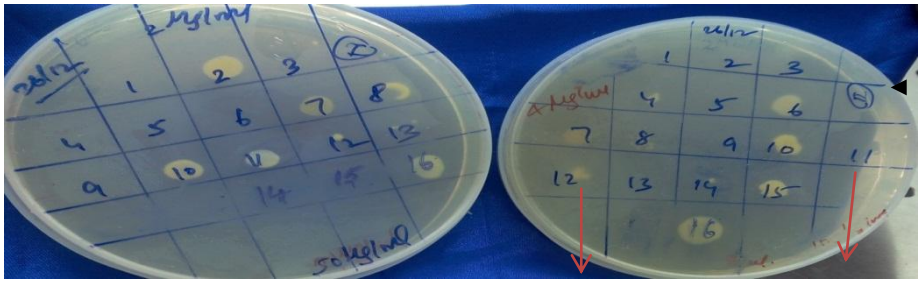
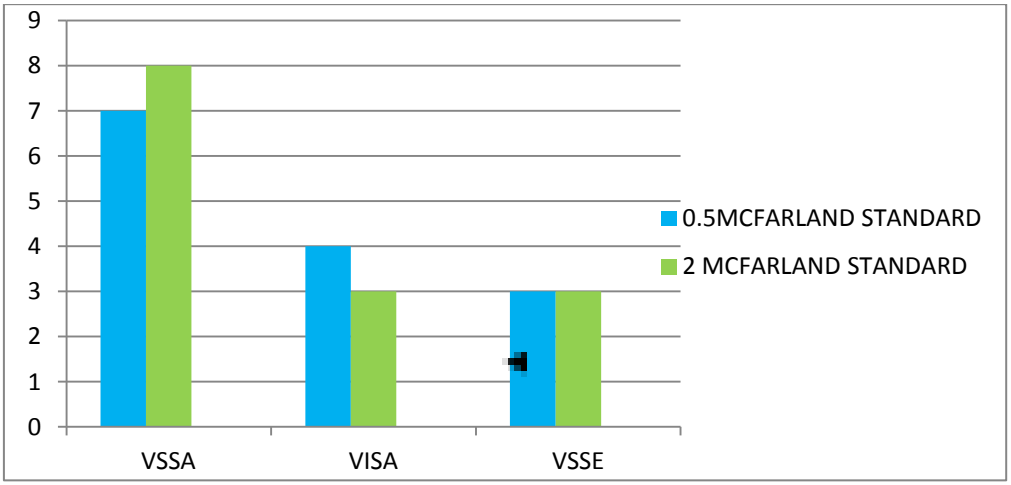


Fig-1 & 2: Agar Dilution by 0.5 McFarland and 2 McFarland Standard

Chart-1: Vancomycin Mic-Agar Dilution (0.5 McFarland Standard & 2 McFarland standards)



The vancomycin MIC by Micro broth dilution using0.5 McFarland standard showed the following results after 24hours of incubation ;6 out of 14 isolates of Methicillin resistant Staphylococcus aureus (MRSA) were vancomycin susceptible Staphylococcus aureus (VSSA), 5out of 14 isolates of Methicillin resistant Staphylococcus aureus were vancomycin

intermediate Staphylococcus aureus(VISA) and 3 out of 14 isolates of Methicillin resistant Staphylococcus epidermidis (MRSE) were vancomycin susceptible Staphylococcus epidermidis (VSSE).

Table-2: Micro-Broth Dilution Method-0.5 McFarland

S.NO.	ORGANISMS	NO: OF ISOLATES, N=14.	MICRO-BROTH DILUTION	INFERENCE
			0.5 McFarland	
1.	(MR)Staphylococcus aureus	6	2	VSSA
2.	(MR)Staphylococcus aureus	3	8	VISA
3.	(MR)Staphylococcus aureus	2	4	VISA
4.	(MRCONS)Staphylococcus epidermidis	3	4	VSSE
TOTAL=		14		

Fig-3: Micro-Broth Dilution Method

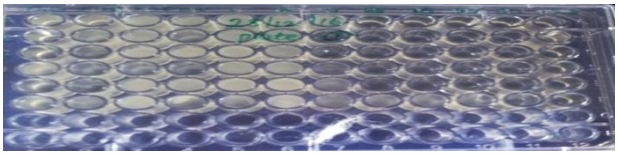
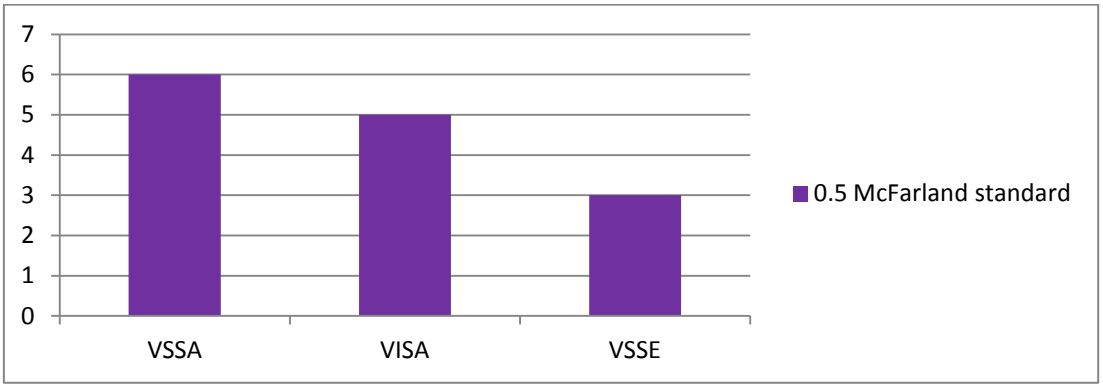


Chart-2: Vancomycin Mic-Micro Broth Dilution (0.5 McFarland Standard)



The results of GRD E-test (which is a double strip, combining both vancomycin and teicoplanin with a nutritional supplement), after incubating aerobically at 37^0C for 24 hours were as follows; all the 5 VISA isolates were showing vancomycin MIC of 2µg/ml, teicoplanin MIC of 1µg/ml and after 48 hours incubation also, the readings were the same. Hence this confirms that there were no hVISA isolate (or hGISA) in this study and only VSSA and VISA were found.

Fig-4: Detection of hVISA by GRD E –Test:

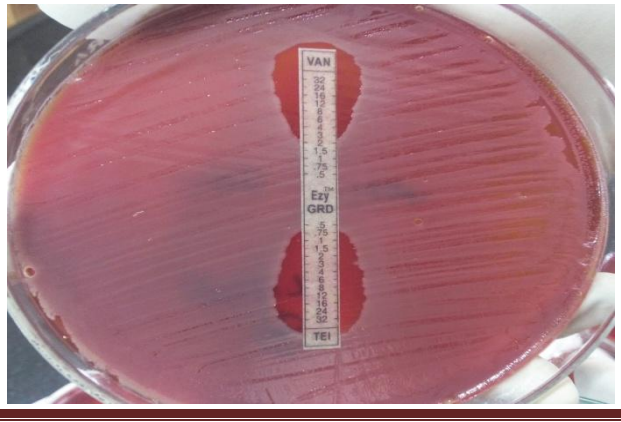
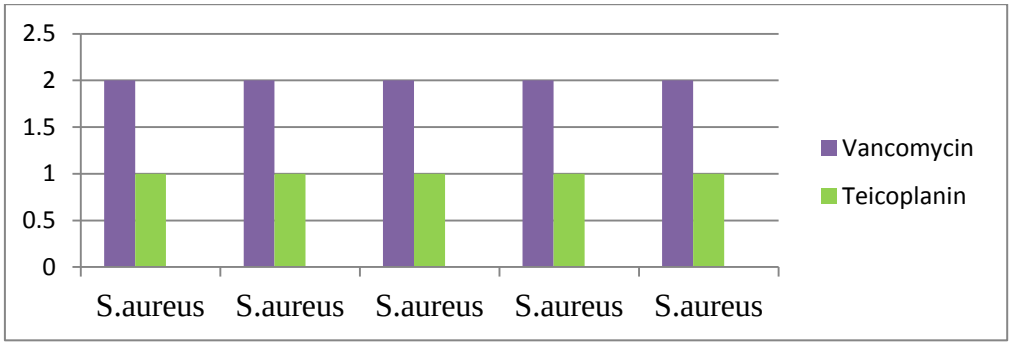


Chart-3: Detection of hVISA by GRD E –Test



Discussion

The main aim of this study was to detect Vancomycin MIC for Staphylococcus species by using Agar dilution and Micro-broth dilution, thereby to screen for heteroresistant vancomycin intermediate Staphylococcus aureus (hVISA) by using GRD-E test. In recent years, there was an increasing trend in the prevalence of Methicillin resistant Staphylococcus aureus (MRSA), for which vancomycin remains the treatment of choice. But, however with the emergence of Staphylococcus aureus with reduced vancomycin susceptibility, there is an increase in treatment failure and mortality.

In this study by Agar dilution using 0.5 McFarland standard, 7 out of 14 (50%) MRSA isolates and 6 out of 14 (42.8%) MRSA isolates by Micro broth dilution (0.5McFarland standard) were VSSA with MIC<to 2 µg/ml. This study corresponds to same as that of Dhaifallah. A. et al[9] where the vancomycin susceptible rate is nearly 60-62% and 0-50% in some studies conducted by Rose et al[25], May et al[21], Sader .HS et al[26], Holmes RL et al[10], Jones RN et al[18], Biedenbach DJ et al[2].

In this current study by Agar dilution (0.5 McFarland standard), 4 out of 14 (28.5%) MRSA isolates and 5 out of 14 (35.7%) MRSA isolates by Microbroth dilution (0.5McFarland standard) were vancomycin intermediate Staphylococcus aureus (VISA) with MIC 4-8 µg/ml[5]. This corresponds to the studies conducted by Harigaya Y et al [11], Auttawit Sirichoat et al[1], Hu J et al,[12] where they suggests that the prevalence of VISA ranges up to 55.2% .

In this present study, by using Agar dilution (2 McFarland standard) 3 out of 14(21.4%) MRSA isolates were showing VISA with MIC of 4-8 µg/ml[5]. This was corresponding to the studies conducted by Harigaya Y et al[11], Auttawit Sirichoat et al[1], Hu J et al,[12] where they suggests that the prevalence of VISA ranges up to 55.2% .Remaining 8 out of 14 (57.1%) MRSA isolates and 3out of 14 (21.4%) MRSA isolates were VSSA and VSSE respectively. This is almost same as that of the study conducted by Dhaifallah. A. et al[9] where the Vancomycin susceptible rate is nearly 60-62%

Based on Musta et al[21],where the vancomycin MIC is between 1.5-3 µg/ml and also according to Mandell Douglas and Bennetts's[20] that there is an increasing evidence that VISA isolates (MIC 4-8 µg/ml)[5] were likely to fail vancomycin therapy and also in more favour of hVISA isolates to have a worst clinical outcome. So the screening method for hVISA was done for all 5 VISA isolates by using E-Test GRD which is a double ended E – Test strip that contains vancomycin and Teicoplanin.

So, in this study based on vancomycin MIC 4-8 µg/ml[5] were considered as VISA and only those 5 isolates were subjected to test for hVISA by using GRD E Test with 0.5 McFarland standard, and none of the isolates were showing hVISA(MIC of > 8 µg/ml for both Vancomycin or Teicoplanin) hence the prevalence rate is 0% . The prevalence of hVISA ranges from 0-50% and this has been reported by many authors Howden et al [13] [14, 20] and similarly low prevalence of hVISA is also reported by Iyer et al [15] [26] i.e. they isolated

one hVISA from 50 MRSA isolates that were tested.

Conclusion

In orthopaedic device associated infections, Staphylococcus aureus is the most common organisms that have been isolated at tertiary care hospital. In this study, the antimicrobial susceptibility pattern of Staphylococcus aureus isolates showed 61% of MSSA and 39% of MRSA. Among the MRSA, 90% were MDR. The study on these MDR isolates showed 42.8% VSSA**,** 35.7% VISA, and 21.4% VSSE and there was no hVISA/hGISA.

Hence routinely, for clinically and laboratory suspected cases of VISA or MDR ,the vancomycin MIC can be done by using agar dilution with 2 McFarland standard .The isolate identified as VISA(MIC-4-8µg/ml)[(5)] can be screened with GRD E-Test using 0.5 McFarland standard and Muller Hinton agar which are routinely used in Clinical Microbiology laboratories. Thereby, we can prevent treatment failures and reduce the mortality due to Staphylococcus aureus infections.

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