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Studies on vanA gene loci among Methicillin Sensitive *Staphylococcus aureus* in rural and urban tertiary care centers

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Abstract

The present study was carried out identify Vancomycin A (vanA) gene loci among Methicillin Sensitive *Staphylococcus aureus* (MSSA) isolates collected from rural and urban tertiary care centers. Identification of species was confirmed by Gram staining, Biochemical and PCR methods. In the study, 16665 samples were analyzed out of which 6538 were collected from rural and 10127 from urban regions. In rural, Methicillin Sensitive Staphylococcus aureus (MSSA) constituted 401 (6.1%) and Methicillin Resistant *Staphylococcus aureus* (MRSA) 531 (8.1%) samples, where as in urban MSSA constituted 524 (5.2%) and MRSA 771 (7.6%) samples. Vancomycin Sensitive *Staphylococcus aureus* (VSSA) was isolated by agar dilution method. In rural center was 359 (89.5%), Vancomycin Intermediate Resistant *Staphylococcus aureus* (VISA) was found in 35 (8.7%) and Vancomycin Resistant *Staphylococcus aureus* (VRSA) in 7 (1.8%) samples, but in case of urban center VSSA was isolated in 451 (86.1%), VISA in 52 (9.9%) and VRSA in 21 (4.0%) samples. VISA and VRSA strains obtained from agar dilution method were further confirmed by E-Test, Disc diffusion and PCR methods. In E-Test VSSA was present in 2 (4.7%) and 1 (1.4%); VISA in 33 (78.6%) and 51 (69.8%), VRSA in 7 (16.7%) and 21 (28.8%) samples, whereas disc diffusion results revealed VISA in 31 (73.8%) and 66 (90.4%) samples, but PCR revealed VISA in32 (76.2%) and 46 (63.0%), VRSA in 7 (16.7%) and 21 (28.8%) samples collected from rural and urban centers. Two strains of vanA locus subjected to PCR amplification, Sanger sequencing and sequencing data were analyzed using BLAST NR database of NCBI genbank and results were compatible with Vancomycin-Resistant *Enterococci* (VRE) and Vancomycin-Resistant *Staphylococci aureus* (VRSA). Finally, vanA gene loci could be present in any Gram positive bacteria, which was neither to Methicillin sensitive nor resistant.

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Introduction

Staphylococcus aureus (*S. aureus*) is responsible for a number of infections globally (Enright *et al.,* 2002). Methicillin was the drug of the choice soon after its discovery in 1960's, however after its resistance 1961 an alternative effective anti-Staphylococcal antibiotics such as Vancomyc in was introduced (Rehm *et al*., 2010). Vancomycinis used in the infections caused by Methicillin resistant *Staphylococcus aureus* (MRSA) and often used empirically for the patients in critical-care settings with infections like severe sepsis, pneumonias and those under umbilical catheterization etc. in India. However, Vancomycin resistance *Staphylococcus aureus* (VRSA) was first reported by Hiramatsu *et al*. (1997a; 1997b) in Japan. Since then, report on reduced susceptibility to Vancomycin is available from all over the world, including

India (Tiwari *et al.*, 2006, Howden *et al.*, 2010, Thati *et al.*, 2011).

There is a necessity of surveillance for *S. aureus* with reduced Vancomycin susceptibility (SA-RVS), however there are roadblocks since most Microbiology laboratories perform disc diffusion test for antibiotic susceptibility which is not a reliable test for Vancomycin (Tiwari *et al.*, 2006). A survey revealed by Centers for Diseases Control and Prevention (CDC, 2000), indicated that many laboratories participating in Emerging Infections Program were not using methods that can detect SA-RVS (CLSI-2009). Clinical and Laboratory Standards Institutes and European Committee on Antimicrobial Susceptibility Testing guidelines for diagnosis of Vancomycin Susceptible *S. aureus* (VSSA), Vancomycin Intermediate *S. aureus* (VISA) and Vancomycin ResistantS. aureus (VRSA) are based on minimum inhibitory concentration (MIC) by micro-dilution or agar dilution methods and molecular methods (Howden *et al.*, 2010; EUCAST, 2013).

Leading to fear of wide spread Vancomycin resistance in *S. aureusthere* was emergence of Vancomycin resistance enterococci in 1980s. The vanA (Vancomycin resistance) gene was plasmid mediated (Leclerq *et al*., 1988) transfers horizontally in gram-positive bacterias. Chaudhari *et al*, in 2014 revealed vanA gene transference fromVancomycin-Resistant *Enterococcus faecalis* to *S. aureus.*

VanA-type resistance was first to be elucidated and characterized by high level of resistance to glycopeptides. In the absence of an antibiotic in the medium the resistance pathway is not expressed, whereas in the presence of an inducer (Vancomycin), the resistance mechanism is activated (Bruno *et al.*, 2009). VRSA strains were associated with vanA-type *E. faecalis*. Consistent with these observations, conjugative transfer of an Inc18-like vanA plasmid from Enterococcus species to *S. aureus* was obtained in vitro transfer of mobile genetic elements from other species to *S. aureus* and limit the spread of resistance genes between isolates of different S. aureus lineages (Waldron *et al.*, 2006).

However, few studies have carried out systematically to sequence variations in all these combined regions or carried out genotypic and phenotypic among MRSA isolates. In view of the above, present study was carried out to determine the prevalence of VISA and VRSA among Methicillin Sensitive *Staphylococcus aureus* (MSSA) in rural and urban tertiary care centers by agar dilution, E-test, disc diffusion and PCR methods.

Materials and Methods

The present study was carried out during August 2013 to November 2015 for a period of 2 years 4 months at Saveetha University, Chennai, Tamilnadu on the approval given by the Institutional Ethical Committee. The experiment was conducted at two centers: Ruralcenter or rural tertiary care center, Rajiv Gandhi Institute of Medical Sciences (RIMS), Srikakulam, Andhra Pradesh and Urban tertiary care center, Andhra Medical College, Visakhapatnam, Andhra Pradesh.

A total number of samples 16665 were analyzed, 6538 (39.2%) from rural and 10127 (60.8%) from urban centers. Among these total samples, pus was present in 3106 (47.5%) and 4961 (48.9%) samples of rural and urban centers, respectively. Percentage of *Staphylococcus aureus* isolated from pus samples was more in rural center (14.3%) with predominance of MSSA as compared to the urban center.

All the pus specimens were Gram stained for determining the organisms. Pus samples were then inoculated onto routine and selective medias, and further confirmed by a battery of standard biochemical reactions (Koneman *et al.,*1997). All the confirmed *Staphylococcus aureus* strains were subsequently tested for methicillin Sensitivity based on the recommendations of Clinical Laboratory Standard Institute (CLSI, 2013). Cefoxitin disc (30µg) was obtained from Hi-Media Laboratories and used in the assay. If the zone was more than 22 mm, it was considered as methicillin sensitive *Staphylococcus aureus* (MSSA) (Fig. 1).

As per National Committee for Clinical Laboratory Standards (NCCLS) *Staphylococci* with MIC of Vancomycin<2 µg was considered as susceptible, MIC between 4-8µg was intermediate and those with MIC > 16µg as resistant. Briefly, gradient plates of Mueller-Hinton agar (Hi-media) were prepared with Vancomycin (2-32 mg l^{-1}, Sigma-Aldrich). 0.5 McFarland equivalent inoculum prepared using 18-24 hrs old culture was spotted on to gradient plates. Plates were incubated overnight at 35ºC for 24hrs and assessed for visible growth. Same strains again crossed checked by E-test vancomycin concentration from 0.016 to 256µg and by disc diffusion method vancomycin 30µg.IntermediateResistantand Resistant isolates were inoculated into the semi-solid nutrient agar and stored at -20ºC until further study.

Frozen *Staphylococcus aureus* isolates were thawed and sub cultured into brain-heart infusion broth (Hi-Media. Mumbai) and incubate at 37°C overnight. Genomic DNA was extracted using Macherey-Nagel Nucleospin food kit. gDNA concentration was measured using Thermo Scientific Nano Drop8000.

A Conventional PCR was performed followed standard protocol of PCR analysis proposed by Murugkar *et al.*, (2003). MicroAmp® 96-Well reaction Plate (0.2ml) used, added 3µl buffer, 2µldntps, 0.3µl Taq DNA

Table 1: Isolation of MSSA from all samples (n=401, n=524)

Name of tertiary care centre	Total samples	Pus samples	*Staphylococcus* spp.	*Staphylococcus aureus*	MSSA	MRSA
Rural - Rajiv Gandhi Institute of Medical Sciences Srikakulam, A.P	6538 (100)	3106 (47.5)	1920 (29.4)	932 (14.3)	401 (6.1)	531 (8.1)
Urban - Andhra Medical College Visakhapatnam, A.P	10127 (100)	4961 (49)	2553 (25.2)	1295 (12.8)	524 (5.2)	771 (7.6)

Figures in parentheses are percentages

Table 2: Isolation of MSSA from *Staphylococcus aureus* strains

Name of Tertiary care centre	*Staphylococcus aureus*		MSSA		MRSA	
	No	%	No	%	No	%
Rural - Rajiv Gandhi Institute of Medical Sciences (RIMS) Srikakulam, A.P	932	100	401	43	531	57
Andhra Medical College (AMC) Visakhapatnam, A.P	1295	100	524	40.5	771	59.5

Chi-square = 1.47; Df = 1; P value = 0.226; Not significant

Table 3: Associations of Isolation of VSSA, VISA and VRSA by Agar dilution among Rural and Urban MSSA

Name of centre	Total Strains	VSSA (≤ 2 mcg)		VISA (4 - 8 mcg)		VRSA (≥16 mcg)	
		No	%	No	%	No	%
Rural tertiary care setup	401	359	89.5	35	8.7	7	1.8
Urban tertiary care setup	524	451	86.1	52	9.9	21	4

Chi-square = 4.50; Df = 2; P value =0.106; Not significant

polymerase (NEB, USA), 2µl 5M Betain, template 2µl, 20 picomoles concentration of Primer forward 2µl, primer reverse 2µl and HPLC water 6.7µl and sealed accordingly with the applicator. The ±1030bp product of Vanco gene (vanA) was amplified by using primer set Forward - 5' ATGAATAGAATAAAAGTTGC 3' and Reverse- 5' TCACCCCTTTAACGCTAATA 3' (Eurofins Genomics India Pvt Ltd). Amplification was conducted in Applied Biosystems Veriti 96 well Thermal cycler system. Amplification by Conventional PCR process was started with an initial denaturation step (94 °C, 5 min). Each cycle consisted of three steps (denaturation, annealing, and extension). Each PCR reaction consisted of 35 cycles of amplification (initial 10 cycles was denaturation at 94°C for1 min, annealing at 57 °C for 1 min, and DNA chain extension at 72°C for 1 min, last 25 cycles was denaturation at 94°C for 30sec, annealing at 62°C for 10sec, and DNA chain extension at 72°C for 30sec). A final extension cycle was performed at 72°C for 5 min (Applied Biosystems Veriti Thermal Cycler).

PCR products were detected by using agarose gel electrophoresis. Electrophoresis was performed with 2% agarose gel (Himedia) prestained with 0.5µl $100ml^{-1}$ of ethidium bromide (10mg per ml). Gels were run at 80V using 1X TAE buffer and then photographed under UV illumination by using a Gel documentation system (UVITEC Cambridge).

Sanger Sequencing: Amplified amplicons were purified using QIAquick PCR Purification kit (QIAGEN, Malaysia). The amplicons were sequenced automatically in both directions using BDT v3.1 chemistry, POP7 Polymer on 3730XL Genetic Analyzer. Thermal program was made up of an initial pre-denaturation step at 95ºC for 2 min; followed by 30 cycles consisting of a denaturation step at 95ºC for 10 sec, annealing step at 52ºC for 20 sec and an extension step at 60ºC for 4 min. DNA sequences were analyzed using the sequencher 4.8 software. The Vanco gene sequence obtained was aligned using CLUSTALX software (version 1.83) to obtain the consensus sequence. Statistical analysis: SPSS 16.0 versionused. Chi-square test was applied.

Results and Discussion

In the present study, 16665 samples were analyzed out of which 6538 were collected from rural and 10127 from urban regions. In rural center MSSA were present in 401

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Table 4: Associations of VSSA, VISA and VRSA strains by Agar dilution, E-test, Disc diffusion and PCR forvanA genemethods in rural and urban centres

Rural tertiary care centre	Agar dilution		E-Test		Disc Diffusion (vancomycin 30µg)		PCR for vanA gene	
	(n=42) No	(%)	(n=42) No	(%)	(n=42) No	(%)	(n=42) No	(%)
VSSA	-	-	2	4.7	31	73.8	-	-
VISA	35	83.3	33	78.6	-	-	32	76.2
VRSA	7	16.7	7	16.7	11	26.2	7	16.7
Rural Tertiary care centre	(n=73) No	(%)	(n=73) No	(%)	(n=73) No	(%)	(n=73) No	(%)
VSSA	-	-	1	1.4	66	90.4	-	-
VISA	52	71.2	51	69.8	-	-	46	63
VRSA	21	18.8	21	28.8	7	9.6	21	28.8

chi-square = 2.12; Df = 1; P value =0.145; Not significant; chi-square= 3.06; Df = 2; P value = 0.217; Not significant; chi-square= 17.7; Df = 1; P value = 0.001; Highly significant; chi-square =2.28; Df = 1; P value = 0131; Not significant

Fig.1: Methacholine sensitive *Staphylococcus aureus* (MSSA)≥ 22 mm zone

Fig. 2: Testing of S. aureus isolate by Agar dilution method at 16 µg conc.

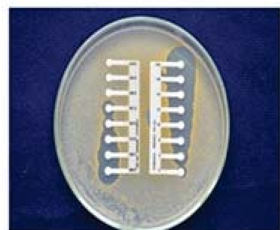

Fig. 3: E-Test showing resistanct to 8 µg (VISA)

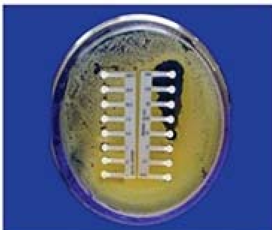

Fig. 4: E-Test showing resistanct to 16 µg (VRSA)

Fig. 5: Van A gene gel picture of RIMS

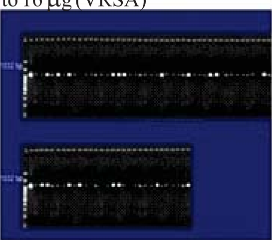

Fig. 6: Van A gene gel picture of AMC

(6.1%) and MRSA in 531 (8.1%) samples, whereas in urban center, MSSA were present in 524 (5.2%) and MRSA 771 (7.6%) samples, respectively (Table 1).

Percentage of Staphylococcus aureus (14.3%) isolated from pus samples was more in rural center with a predominance of MRSA (8.1%) as compared to urban center. Isolation of MSSA were 43%, 40.5% and MRSA 57%, 59.5% in rural and urban centers, respectively, and were found to be insignificant (P>0.05) (Table 2).

In rural center, prevalence of MSSA in 401 samples, VSSA was isolated in 359 (89.5%), VISA in 35 (8.7%) and VRSA in 7 (1.8%) samples (Fig. 2). However, in urban center out of 524 samples of MSSA, VSSA was present in 451 (86.1%), VISA in 52 (9.9%) and VRSA in 21 (4.0%) samples respectively, In urban center, percentage of VISA and VRSA isolated were compared with rural center concentrations of Vancomycin by agar dilution. The distribution of VSSA. VISA and VRSA in both centers were found to be insignificant (P>0.05) showed in Table 3.

The samples were cross checked by E-Test, Disc diffusion and PCR methods for VISA and VRSA strains obtained from agar dilution method. E-Test showed presence of 4.7% and 1.4% VSSA, 78.6% and 69.8% VISA, 16.7%, 28.8% VRSA (Fig 3 and 4).

Disc diffusion assay showed presence of 73.8% and 90.4% VSSA, 26.2% and 9.6% VRSA, while PCRshowed presence of 76.2% and 63% VISA, 16.7%, and 28.8% VRSA in rural and urban centers, respectively (Fig 5 and 6). But none the VRSA isolate were observed from disc diffusion method.Comparison of VISA and VRSA strains by agar dilution, E-test and PCR methods in rural and urban tertiary care centers were found to be insignificant (P>0.05) (Table 4).

In the present study, *S. aureus* was present in 932 (100%) and 1295 (100%) samples; MSSA in 401 (43.03%) and 524 (56.97%); MRSA in 531 (40.46%) and 771 (69.54%) samples of rural and urban centers, respectively. Isolation rate

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of MRSA differed in different geographical areas depending on the climate, hygiene conditions habits and availability of health care facilities. Several studies have reported high prevalance of MRSA in health centres and MESA have been isolated in different clinical and carrier samples (Pantazatou *et al.*, 2003; Lablanc *et al.*, 2007; Mistry *et al.*, 2011; Pai *et al.*, 2010; Singh *et al.,* 2011). Recently, Chaudhari *et al.*(2014) reported antibiotic resistance to commonly used antibiotic by MRSA isolates. MRSA was found to be sensitive towards Vancomycin. Tiwari *et al*. (2011) and Pai *et al*. (2010) reported 100% Vancomycin sensitivity amongst MRSA isolates.

Reduced sensitivity to Vancomycin in *S. aureus* occurs due to several genetic and phenotypic alterations in wild-type bacteria including altered expression of regulatory genetic elements, thickness of cell wall, changes in the penicillin-binding protein profiles and decreased cell wall autolysis (Hanakia *et al.*, 1998). Disc diffusion method revealed VSSA in 66 (90.4%), 31 (73.8%) samples of VRSA in 11 (26.2%), 7 (9.6%), therefore rural and urban centers respectively. It can be concluded that agar dilution, PCR and E-test are standard methods for isolation of VISA and VRSA as compared to disc diffusion method which is not reliable. Similar results were found for VRSA.

Vancomycin resistance (vanA) gene loci in MSSA indicated that there was no relation between Methicillin resistance or sensitivity of bacteria. In generally most of the available literature of vancomycin resistance among MRSA and *Enterococci* spp. not in MSSA. Regarding the Vancomycin intermediate or resistance among MRSA and association of SCCmec (methicillin resistant) gene but the present study exhibited standard methods for detection of VISA and VRSA. VRSA can be detected by any one of the following methods (agar dilution, E- Test or PCR). But in case of VISA standard method for detection was PCR as compared to agar dilution, E-test and disc diffusion methods. In the present study, 9 strains of VISA were phenotypically positive but genotypically (vanA gene loci) negative which might be due to altered expression of regulatory genetic elements, thickness of cell wall, and changes in the penicillin-binding protein (PBP) profile factors by Moreira *et al*. (1997).

One control strain and two VRSA strains from two centers were subjected to PCR amplification and Sanger sequencing. Sanger sequencing data was uploaded in standard nucleotide data bank of Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information. The search portal results were compatible with Vancomycin-Resistant *Enterococci* (VRE) and Vancomycin-Resistant *Staphylococci aureus* (VRSA). Genebank numbers were allotted by NCBI as rural tertiary care center MS-VRSA Banklt1890159 Seq1_SKLM3 KU641697 and urban tertiary care center MS-VRSA Banklt 1890159 Seq1_OMC3 KU641696.

In conclusion, VISA or VRSA can be detected by any one of the method agar dilution, E- Test or PCR but not by disc diffusion method because of its disc content of 30 µg. PCR is the more specific, Sensitive and standard method in detecting even low copy number of organism. VanA gene loci can be identified in Gram positive bacteria, which are not related to methicillin sensitive or resistant.

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