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# PLASMID MEDIATED METHICILLIN AND VANCOMYCIN RESISTANT *Staphylococcus aureus* ISOLATED FROM NORTHERN INDIA

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**ABSTRACT**

A study was conducted to determine plasmid mediated methicillin and vancomycin resistant *Staphylococcus aureus* along with plasmid profiling and detection of vancomycin resistance genes. This study was carried out in the Department of Microbiology and Fermentation Technology, Sam Higginbottom Institute of Agriculture Technology and Sciences, Allahabad, India. The molecular detection of gene was completed in Molecular Diagnostic Research Laboratory, Chandigarh. Clinical specimens were collected from two tertiary care hospitals of Allahabad from September 2012 to May 2013. Altogether 59 *S. aureus* isolates were isolated among which 20 MRSA were screened. Acridine orange in concentration of 0.1mg/ml was used for plasmid curing. Plasmid cured VRSA isolates were subjected to plasmid profiling by agarose gel electrophoresis followed by detection of *vanA* and *vanH* gene. In vitro plasmid curing of 20 methicilin resistant, one vancomycin intermediate (MIC: 8µg/ml) and three vancomycin resistant (MIC: 16-32µg/ml) strains removed resistance markers from eight MRSA and two VRSA isolates, respectively. Vancomycin resistance plasmid was successfully transferred to MSSA. Plasmid borne vancomycin resistant strains harboured 23kbp plasmid. Vancomycin resistance *vanA* and *vanH* gene was identified on molecular basis. It was concluded that plasmid mediated methicillin resistance exists less frequently but in case of VRSA, Both the mechanisms of thickening cell wall and plasmid acquisition imposed equal impact on emergence of VRSA.

**Keywords:** MRSA, (vancomycin resistant *Staphylococcus aureus*) VRSA, Plasmid, *vanA*, *vanH* gene.

## INTRODUCTION

*Staphylococcus aureus* has established itself as a successful pathogen regarding antimicrobial resistance, meantime creating continuous challenges to scientists. In less than a century, it is detected to be resistant against beta lactams followed by penicilinase stable beta lactams and recently vancomycin. Paucible observations regarding drug resitance are available revealing the increased frequency of methicillin resistant *S. aureus* (MRSA) and VRSA by passage of time [1]. Pathogen developed differing mechanism and means to resist against different antibiotics depending on their mechanism of action. Enzymatic degradation of drug, structural modification of target and antibiotic efflux are mere strategies of resistance followed by the bacteria [2]. Drug resistant genes are either chromosomally located or plasmid lodged. However, conjugative plasmid are majorly accused for emerging resistance as it successfully transfer genetic material in both inter and intra-species [3]. This confers a basis for spreading resistance genes among bacterial population quicker than native mutation and vertical evolution.

Two modes of vancomycin resistance have been identified in *S. aureus*. First type encountered in vancomycin intermediate is due to the piling up of additional petidoglycan layer and second type detected in vancomycin resistance strains is resulted by conjugal transfer of responsible gene cluser [4]. Vancomycin molecules are trapped by more D-Ala-D-Ala residues, prohibiting them to reach their target on cytoplasmic membrane. Unlike former one, later is genetically driven replacement of lactate in place of alanaine during synthesis of peptidoglycan precursors. Besides, the organism is capable of synthesizing a sex pheromone, a facilating agent for conjugal transfer. The crucial point is the gene for sex pheromone is harboured in v*anA* plasmid [5].

Plasmid determination is very important in epidemiological studies. Moreover, it has proved to be the earliest DNA-based method implemented for analyzing resistance pattern, frequency and probable future status of the resistance in relation to certain parameter [6]. Molecular identification of genes provides the detail information about the pathways followed for resistance, potency of bacteria to drive this property in long run and genotypic view of resistance whereas curing of plasmid and transfer explains frequency of resistance transferred the extent of emerging resistance in new strains. The study was proceeded with the objectives to determine plasmid mediated MRSA and VRSA along with plasmid profiling and detection of vancomycin resistance genes from VRSA.

## MATERIALS AND METHODS

**Current experiment:** plasmid curing and conjugative plasmid transfer were carried out in Sam Higginbottom Institute of Agriculture, Technology and Sciences, Allahabad. Apart from this, plasmid profiling and detection of *vanA* and *vanH* gene were experimented on Molecular Diagnostic Research Laboratory Pvt. Ltd., Chandigarh, India. It enrolled 20 MRSA isolated from the

hospitals of Allahabad screened by DAD test and Minimum Inhibitory Concentration determination. Among them, one was vancomycin intermediate *S. aureus* (VISA) (MIC=8µg/ml) and remaining three were VRSA (MIC:16-32µg/ml).

**Plasmid curing**

Plasmid curing was accomplished by growing all selected isolates in nutrient broth containing 0.1 mg/ml acridine orange for 24 hours. Then a loopful of broth was inoculated in the oxacillin screening agar (OSA) and vancomycin screening agar (VSA) along with nutrient agar (NA). Growth in both screening agar and nutrient agar shows the chromosomal resistance which is not cured where as growth only in nutrient agar but not in screening agar shows the plasmid borne resistance [7].

**Plasmid profiling**

Bacteria plasmid was extracted by alkaline lysis method, possible RNA dissolved by treating with RNase [8] and further purification by silica membrane spin column method [9]. Purified plasmid DNA contained in TE buffer was stored at $4^0$C. In order to detect the presence of plasmid and to determine its size, sample along with lambda hind digest III (Bioneer) were run in 0.75% agarose gel electrophoresis at 75V for 1hour and 30 minutes. The gel was observed under UV illumination.

**Detection of *vanA* and *vanH* gene**

Sequence of Primers for identification of *vanH* and *vanA* genes (Eurofins Genomics, Bangalore, India)

*vanH*.FP: 5'-ATGAATAACATCGGCATTAC
*vanH*.RP: 5'-CTATTCATGCTCCTGTCTCC
*vanA*.FP: 5'- ATGAATAGAATAAAAGTTGC
*vanA*.RP: 5'- TCACCCCTTTAACGCTAATA

Gene fragment for amplification of v*anH* gene 969bp
Gene fragment for amplification of v*anA* gene 1032bp
(Saha *et al.* 2008 and Chakraborty *et al.* 2011)

Before commencing PCR, the working area in safety cabinet was sterilized. Reaction mixture containing forward–primer, reverse-primer, dNTPs, template DNA, Taq Buffer and Taq polymerase, was set up for a volume of 25µl. Thermocycler (Applied Biosystems' Veriti) parameters were set up as: Initial denaturation at $95^0$C for 3min, followed by 35 cycles of $95^0$C for 30sec, $58^0$C for 30sec and $72^0$C for 30Sec, followed by a last step at $72^0$C for 10min. The amplified products were detected by agarose gel electrophoresis. 10 µl of PCR product was mixed with 5 µl of loading buffer. Then mixture was loaded on 1.8% agarose gel in 1x TAE buffer (pH 8.3). A molecular weight marker of 100 bp DNA Ladder (Applied Biosystems) was used for determination of required size of DNA band. Electrophoresis was carried out at room temperature for one hour at 145V. Finally bands of amplified products were observed under UV transilluminator.

**Plasmid transfer**

Two bacterial strains (*Enterococcus faecalis* and *S. aureus*) were obtained from Microbial culture collection bank of Department of Microbiology and Fermentation Technology, SHIATS. Antibiotic susceptibility patterns (methicillin and vancomycin) of the bacteria were tested by disc agar diffusion test (DAD). Experiment was performed on the basis of protocol mentioned by Lacey, (1972) [10] with some modification. Overnight broth (BHI) culture of donor and recipient bacteria were mixed in the ratio of 1:10 by quick vortexing and incubated at $30^0$C for 20 hours. Broth was diluted to three consecutive dilution then plated in selective agar in stringent condition. The donor and recipient strains were also incubated singly and plated in the same way. Plates were incubated at $37^0$C. After 24 hours of incubation bacterial colonies of both donor and recipient were isolated and antibiotic susceptibility for corresponding antibiotics was checked by DAD test.

**RESULTS AND DISCUSSION**

After curing of plasmid, eight MRSA isolates out of twenty and two of three VRSA isolates were unable to grow in the corresponding screening agar (Table-1). It pointed out that they might contain plasmid responsible for methicillin and vancomycin resistance which was stably lost by incubating organism in media containing mutagen like acridine orange.

**Table-1.** Plasmid curing of MRSA and VRSA.

| Isolates | Resistance pattern | Growth on NA | Growth on antibiotic media OSA/VSA |
|---|---|---|---|
| S-4 | MRSA | Growth | Growth |
| S-8 | MRSA | Growth | Growth |
| S-15 | MRSA | Growth | Growth |
| S-18 | MRSA | Growth | Growth |
| S-19 | MRSA | Growth | Growth |
| S-20 | MRSA | Growth | Growth |
| S-22 | MRSA | Growth | Growth |
| S-27 | MRSA | Growth | No growth |
| S-35 | MRSA/VISA | Growth | Growth |
| S-36 | MRSA/VRSA | Growth | Growth |
| S-37 | MRSA | Growth | No growth |
| S-40 | MRSA/VRSA | Growth | No growth |
| S-42 | MRSA | Growth | Growth |
| S-46 | MRSA | Growth | Growth |
| S-54 | MRSA | Growth | Growth |

| | | | |
|---|---|---|---|
| S-55 | MRSA/VRSA | Growth | No growth |
| S-56 | MRSA | Growth | No growth |
| S-57 | MRSA | Growth | No growth |
| S-58 | MRSA | Growth | No growth |
| S-59 | MRSA | Growth | No growth |

Agarose gel electrophoresis of two VRSA isolates (S-40 and S-55) detected 23kbp plasmid on both the strains with slightly thinner band on S-40 isolate. Plasmid isolation was done from the broth culture in stringent condition of 8 µg/ml vancomycin. Photographic documentation (Figure-1) indicates the single plasmid band in each lane with possibilities of being genetically mediated vancomycin resistance. Responsible genes for vancomycin resistance: *vanA* and *vanH* gene was identified by PCR amplification using corresponding primer (Figure-2).

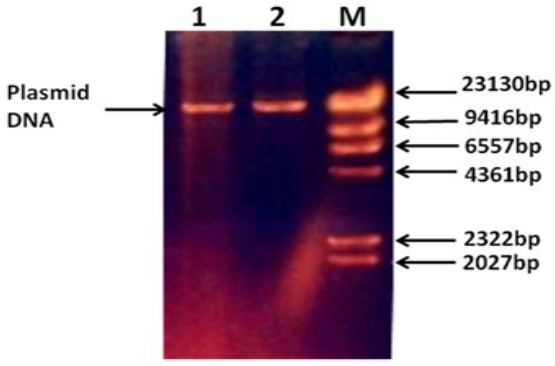


**Figure-1.** 0.75% Agarose gel electrophoresis of plasmid DNA.

Lane 1: Bac_ S-40 (Vancomycin media)
Lane 2: Bac_ S-55 (Vancomycin media)
Lane M: Lambda *Hind III Marker*

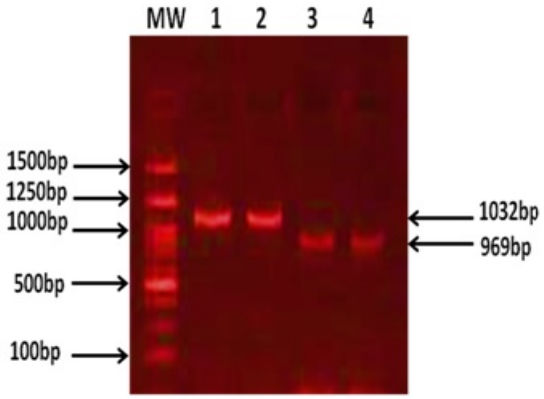


**Figure-2.** Amplification of *vanH* (969bp) and *vanA* (1032bp).

Lane MW: 100bp DNA ladder
Lane 1: *vanA* from Bac_ S-40 (Vancomycin media)
Lane 2: *vanA* from Bac_ S-55 (Vancomycin media)
Lane 3: *vanH* from Bac_S-40 (Vancomycin media)
Lane 4: *vanH* from Bac_ S-55 (Vancomycin media)

Finally between two organisms, *E. fecaelis* and MSSA selected for conjugative plasmid transfer, MSSA was successively acquired resistance while, *E. fecaelis* was unable to acquire (Table-2).

**Table-2.** Invitro study of inter-generic plasmid transfer between gram positive bacteria.

| Donor | Resistant pattern | Recipient | Resistant pattern | Transfer temperature | Resistant pattern after transfer |
|---|---|---|---|---|---|
| S-55 | Methicillin, Vancomycin | *Enterococcus faecalis* | Methicillin | $37^0$C | Methicillin |
| S-55 | Methicillin, vancomycin | *Staphylococcus aureus* | - | $37^0$C | Methicillin, Vancomycin |

Acridine orange intercalates between the bases of DNA inhibiting the replication of plasmid without any interference with chromosomal DNA, which forms the basis of being plasmid curing agent. Thus, to determine whether the observed drug resistance pattern in the isolates was plasmid or chromosomal mediated, the isolates were screened for the presence of conjugative plasmids. On mutagen induced plasmid curing of twenty MRSA and

VISA/VRSA isolates, vancomycin resistance was lost from only two strains whereas methicillin resistance was lost from eight isolates confirming the plasmid borne resistance. Mobile genetic element accounts for most of the antibacterial resistance [3]. Plasmid curing experiment has been performed for drug resistant *S. aureus* isolates with different curing agents by many other scientists [7, 8].This technique was proved as quite helpful in drawing information regarding antibiotic resistance plasmid. It is convenient and feasible in most of the laboratory. If the resistance is confirmed to be plasmid borne then further its spreading limit could be analyzed, whether confined to same species or have broad range recipient so that future epidemiology could be predicted to some extent.

All the three experiments regarding plasmid were supportive for each other and documented its presence. The relative profiles of plasmids were characterized on the basis of their comparative molecular weights. Likewise, one of the previous experiment also revealed that 41.2% of the drug resistant strains were harbouring plasmid however, multiple plasmid of sizes 1.26, 23.13 and 25.12 kbp were lodged by all the strains [11]. Being supportive, other researchers have detected the high molecular weight plasmid accounting for drug resistance in case of *S. aureus* [8].Showing similarity in the present result, plasmid investigation of twenty-four isolates indicated the lodging of large molecular weight single plasmid ranging from (21 – 22) Kbp in all of them [12].

In current study two strain of plasmid mediated VRSA confronted the presence of *vanA* and *vanH* gene. Expression of the *vanA* operon in VRSA has been demonstrated by most of the researcher working on *S. aureus* in almost every parts of globe [13, 14, 15]. In India, First gene encoded vancomycin resistance was reported by Saha *et al*., from west Bengal [16]. With passage of time, genotypic characterization of VRSA was explained from various states and geographical regions of India [17, 18, 1].

Two different mechanisms of vancomycin resistance has been explained in literature. One of them is genetically empowered VRSA and another is due to deposition of extra layer of peptidoglycan. Both are equally reasonable for emergence of resistance. (Ng *et al*., 2011). Thus two genes, *vanH* and *vanA* that cooperate each other to synthesize the D-Ala-D-Lac dipeptide was detected. Former encode D-hydroxy acid dehydrogenase that lead reduction of pyruvate to lactate and later encode ligase which in combination with ATP ligate lactate to alanine forming novel structure D-Ala-D-Lac [2]. Further, due to the replacement of D-alanine by D-glutamic acid, amide bond is overcome by ester bond with decreased tendency of vancomycin to bind, hence fail to work. Even lactate acts as leaving group compared to D-alanine [5].

According to present observation, two isolates were guessed to be non plasmid mediated as failed to cure plasmid. Probably, as mentioned earlier, second type vancomycin resistance might be casuse of it. In favor, there are many experimental researches revealing the VRSA as a result of thickening of cell wall rather than genetically mediated one in case of both native type and around the world. While moving in past, Tiwari and Sen, (2006) mentioned four *S. aureus* strains with 8µg/ml MIC and four strains of 16-64µg/ml MIC of vancomycin from northern India. Not a single isolate showed the presence of *vanA* gene, reason may be of thickening of cell wall like the present study [19]. Similarly Thati *et al.,* found 7 VRSA isolates among which six were genotypically resistant and still one did not show presence of resistant gene [18].

Conjugative plasmid transfer process is spontaneously occurring in different gram-positive species and genera. Though plasmid transfer was observed in MSSA, it was unable to transmit to *E. faecalis*. Invitro analysis of this process documented by related researchers are supportive factor. The experimental transfer of the *vanA* gene cluster from *E. faecalis* to *S. aureus* has appoved the occurrence of such genetically transmitted vancomycin resistance in MRSA [20], still reverse has not been documented. Since the establishment of cell to cell contact is necessary to start the mechanism, donar and receipient cell are mixed together. Limited host range of *S. aureus* might be the possible reason explained in some literature [21].

## CONCLUSION

Chromosomally mediated methicillin resistance gene predominated plasmid lodged one whereas both the mechanisms of thickening cell wall and plasmid mediated emergence of VRSA are found to be equally reasonable. The circulation of strains carrying vancomycin-resistance gene within the species is common rather than inter-species and inter-generic. Although in present context, vancomycin resistance is often less percent encountered in India and globally because of successive plasmid transfer in natural condition, it may create trouble within a decade or two.

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