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Chromosomal and plasmid DNA mediated antibiotic resistance among *Staphylococcus aureus* obtained from mastitic milk of cattle in Jaipur, Rajasthan

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ABSTRACT

The present investigation was aimed to isolate and identify the *Staphylococcus aureus* isolates from mastitic milk sample of cattle along with studies of phenotypic and genotypic antibiotic resistance pattern. Among 100 mastitic milk samples of cattle, total 20 (20.0%) isolates were identified and confirmed as *S. aureus*. Out of the total identified isolates, 17 (85.0%) isolates having multidrug resistance (MDR) property including 11 (55.0%) isolates had 0.2 or more than 0.2 MAR index value with high-risk potential source of spread MDR. In antibiotic sensitivity studies, Gentamicin, Doxycycline Hydrochloride and Tetracycline antibiotics were most effective with 85.0%, 65.0% and 60.0% efficacy, respectively while highest resistance was recorded against Penicillin–G (80.0%). In reference to specific resistance mechanisms, six (30.0%) isolates were detected as MRSA and one (C58) isolate had High- level mupirocin resistance. None of the isolate was detected as VRSA along with average 1.37mcg/ml MIC for vancomycin. Both chromosomal as well as plasmid DNA were evaluated for detection of antibiotic resistance genes *i.e.* eighteen isolates (90.0%) were carrying *blaZ* gene (517bp) in chromosomal DNA, while twelve isolate (60.0%) having same gene in plasmid DNA. Only one (C69) isolate was positive for *mecA* (533bp) gene in both chromosomal and plasmid DNA and none of the isolate was found positive for *vanA* (560bp) and *vanX* (480bp) gene.

Keywords: Cattle, Mastitis, MRSA, *Staphylococcus aureus*, VRSA

India is an agriculture-based country in which animal husbandry plays an important role by maintaining the livestock under heterogeneous climate, and among different species of livestock the dairy cattle rearing is preferred by the rural farmers for their economic wellbeing. With the increment in the milk production potential of cattle, disease resistance becomes decreased, which makes them more susceptible to the infections such as mastitis. Bovine mastitis has resulted in huge economic losses from decreased milk yield and poor animal health (Sumathi *et al.* 2008, Brahma *et al.* 2022). *Staphylococcus aureus* is one of the most common bacteria along with wide variety of pathogens associated with cattle suffering from mastitis (Reksen *et al*. 2006, Wernicki *et al.* 2014). *Staphylococcus aureus* is a versatile pathogen along with the highly variable biochemical and cultural properties. Although, it can be identified by conventional methods, due to high precision, sensitivity and specificity of PCR based molecular methods, the *nuc* gene sequence-based method is more suited for confirmation of *S. aureus* (Sasaki *et al.* 2010, Wang *et al*. 2018).

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Extensive and indiscriminate use of antibiotics in human and veterinary practices has led to the development of antibiotic resistance. It is of public health concern which has imparted to the increase in morbidity and mortality of infectious diseases with serious socio-economic losses (Alvan *et al.* 2011). *S. aureus* is very apt to acquire antibiotic resistance in a short span of time after their exposure to the antimicrobial agents (Virdis *et al.* 2010, Alian *et al.* 2012) with various resistance mechanisms i.e. horizontal gene transfer, mutations in chromosomal DNA and vertical or natural resistance mechanism. Out of these, β-lactamases production, methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA) phenotype are of important concern (Wootton *et al.* 2002, Cuny *et al*. 2010).

MRSA has been listed as a priority pathogen by the World Health Organization (Shrestha *et al.* 2021). MRSA strains are not only resistant to β-lactam antibiotics, but also resistant to a wide range of antibiotics, including cephalosporins and aminoglycosides (Klingenberg *et al*. 2004, Ardic *et al.* 2006). Vancomycin is frequently used to treat the infections caused by MRSA but the emergence of vancomycin resistance (VRSA) and hetero-resistance in *S. aureus* has resulted in wide-reaching implications for the animal and human health (Wootton *et al.* 2002, Tiwari and Sen 2006, Bhattacharyya *et al.* 2016, Kumar

et al. 2017, Mahanti *et al.* 2020). The antibiotic resistance mechanisms in *S. aureus* have various genetic bases such as β-lactamases production, methicillin resistant *S. aureus* (MRSA) phenotype and vancomycin resistant *S. aureus* (VRSA) governs by *blaZ, mecA, vanA* and *vanX,* respectively. The *mecA* gene is considered as molecular marker for methicillin resistance staphylococci and the detection of this gene by the polymerase chain reaction (PCR) is considered to be as the "gold standard" method (Hiramatsu *et al.* 2014, Ventola 2015). Looking to the above facts, the present study was designed to explore occurrence of *S. aureus* in bovine mastitic milk samples with special reference to phenotypic and genotypic detection of specific antibiotic resistance mechanisms.

MATERIALS AND METHODS

Sample collection: Total of 100 mastitic milk samples from cattle were collected in a randomized manner irrespective to age, sex and breed as describe in Table 1. Written consent was obtained from the animal owners prior to sample collection. These sample sources belonged to five different localities of Jaipur (Rajasthan). The samples were collected in sterile vials in the morning and were immediately taken thereafter to the laboratory on ice for further processing. Isolation and identification of *Staphylococcus aureus* is conducted as per standard protocols on Mannitol Salt Agar (MSA) and Baird Parker Agar (BPA). The morphological characterization of the isolates was done by Gram staining and biochemical characterization by catalase, oxidase and motility tests as per prescribed by Quinn *et al* (1994).

Molecular identification of Staphylococcus aureus: Isolation of bacterial chromosomal and plasmid DNA was carried out using HiPurA® Bacterial Genomic DNA Purification Kit (Himedia) and HiPurA® Plasmid DNA Miniprep Purification Kit (Himedia) subsequently as per given instructions. All *S. aureus* isolates were tested for carriage of species-specific *nuc* (thermonuclease [TNase]) gene-based primers with 359bp size amplicon as described by Sasaki *et al.* (2010). Eluted DNA and plasmid concentrations and quality were measured using a Bio Spectrometer (Eppendorf, Hamburg, Germany) and stored at −20°C for further analysis.

Antibiogram of Staphylococcus aureus isolates: All the confirmed *S. aureus* isolates were checked for their resistance or susceptibility against different antibiotics by Kirby Bauer disc diffusion method. Antibiotics, which are commonly used in the area of study were selected as mentioned in CLSI guidelines (2020) as mentioned in Supplementary Table 1 are used for antibiogram study.

Phenotypic detection of MRSA, high- level mupirocin resistance and VRSA: The standard CLSI guidelines (2020) were used for the identification of MRSA and high- level mupirocin resistance and VRSA phenotypes. Cefoxitin (30 µg) and mupirocin (200 µg) were placed on the activated growth of *S. aureus* (0.5 McFarland) on Muller Hinton agar plates for MRSA and high- level mupirocin resistance, respectively. The isolates showing resistance towards cefoxitin discs were declared MRSA and those who were found sensitive were considered methicillin-sensitive *S. aureus* (MSSA). Similarly, if any zone of inhibition is shown with mupirocin, the isolate was considered as absence of high-level mupirocin resistance (negative) while if no zone of inhibition is shown than considered as high-level mupirocin resistance (positive).

VRSA was determined by vancomycin agar screen method and vancomycin minimum inhibitory concentration (MIC) method. If isolate grows in the presenceof 6.0 µg/mL vancomycin agar then it will be considered as VRSA while absence of growth in presence of 6.0 µg/mL vancomycin considered as negative as per CLSI guidelines (2020). In vancomycin MIC method, ≤ 2.0 mcg/mL was considered as sensitive, 4-8 mcg/mL as intermediate (VISA) and if MIC is ≥ 16.0 mcg/mL, then the isolate was considered as VRSA positive.

Multiple antibiotic resistance index (MAR) values: All multidrug-resistant isolates were evaluated using Multiple Antibiotic Resistance (MAR) index for risk assessment of multi drug resistance (MDR). This index was calculated as per the method given by Krumperman (1983). MAR index of single isolate = a/b, where a represents the number of antibiotics to which the isolate was resistant and b represents the number of antibiotics to which the isolate was exposed.

Genotypic detection of antibiotic resistance genes: All the confirmed *S. aureus* isolates were tested for the presence of Antibiotic resistance genes *mecA, blaZ, vanA, vanX* among bacterial chromosomal and plasmid DNA with various methodology and PCR conditions. The primers and thermo cyclic conditions of PCR amplification are given in table 2.

RESULTS AND DISCUSSION

In the present investigation, twenty isolates were recovered from 100 mastitic milk samples of cattle from

Table 1. Place wise distribution of *Staphylococcus aureus* isolates

Place of Sampling (Jaipur)	Source (Number of Samples)	Isolate I.D.	No. of Isolate	Percentage (%)
Govindgarh	Mastitic Milk (28)	C4, C18, C27, C29, C34, C36, C47	07	25.0
Jahota	Mastitic Milk (29)	C48, C50, C53, C54, C57	05	17.24
Naga baba goshala Hathnoda	Mastitic Milk (13)	C58, C61, C68	03	23.07
Rampura	Mastitic Milk (13)	C69, C73	02	15.38
Cattle farm Chomu	Mastitic Milk (17)	C74, C80, C93	03	17.64
Total	100	20		20.0

Table 2. Details of primers used in the present study

Gene	Forward primer sequence (5′ to 3′)	Size	Annealing Temp. (°C)	Reference
nuc	F-5' TCG CTT GCT ATG ATT GTG G 3' R-5' GCC AAT GTT CTA CCA TAG C3'	359bp	55°C	(Sasaki *et al.* 2010)
mecA	F-5'-AAA ATC GAT GGT AAA GGT TGG C-3' R-5'-AGT TCT GCA GTA CCG GAT TTT GC-3'	533bp	52°C	(Murakami *et al.* 1991)
blaZ	F-5'-AAG AGA TTT GCC TAT GCT TC-3' R-5'-GCT TGA CCA CTT TTA TCA GC-3'	517bp	50°C	(Sawant *et al.* 2009)
vanA	F-5'-AAC AAC TTA CGC GGC ACT-3' R-5'-AAA GTG CGA AAA ACC TTG C-3'	560bp	52°C	(Aarestrup 1996)
vanX	F-5'-TGC GAT TTT GCG CTT CAT TG-3' R-5'-ACT TGG GAT AAT TTC ACC GG-3'	480bp	52°C	(Aarestrup 1996)

five different localities of Jaipur (Rajasthan). All isolates were characterized on Mannitol Salt Agar (MSA) with characteristic golden yellow colonies (Supplementary Fig. 1a) and lecithinase production (Supplementary Fig. 1b) with black colony surrounded by clear opaque zone on Baird Parker Agar (BPA). Further, morphological characterization was done by Gram staining, where the organisms were Gram-positive cocci (bunches of grapes), oxidase negative, catalase positive and non-motile in motility test. All phenotypically characterized isolates were confirmed and identified by PCR amplification by using species-specific nuc (thermonuclease TNase) gene-based primers with 359 bp size amplicon (Supplementary Fig. 2) according to the method of Sasaki *et al*. (2010). Almost similar to the findings of the present study, 22.5% recovery rate was reported by Hamid *et al*. (2017) from 160 mastitic milk samples of cattle while lower prevalence 9.73% (55/565) of *S. aureus* was reported by Neelam *et al*. (2022). Similarly, slightly higher (26.60%) prevalence was reported by Moustafa *et al*. (2021) from cattle and 28.0% prevalence was reported by Nazir *et al*. (2017) from bovine mastitis milk samples. Similar findings were also reported from Udaipur (Rajasthan) by Kalwaniya *et al*. (2024) where 46% prevalence was recorded. On other hand, Nathiya *et al.* (2018) reported higher (47.45%) recovery of *S. aureus* isolates from milk of cattle with clinical mastitis and similarly, Sharma *et al*. (2015) reported 40.63% prevalence in milk samples in Jaipur (Rajasthan). Variable prevalence of *S. aureus* might be due to multiple factors i.e. environment, season, area of sampling, milk yield of cattle and processing of samples hence, it is suggested to explore multiple factors to assess prevalence of *S. aureus* from mastitic milk samples.

Antibiotic sensitivity pattern of *S. aureus* was obtained against fourteen different antibiotics belongs to different groups and classes. Out of the total studied isolates, 11 isolates were detected as multi drug resistant (MDR) isolates which were resistant to 04 to 12 antibiotics. The isolate number C47 and C58 were resistant to 12 screened antibiotics and one isolate (C36) were resistant to 10 antibiotics and 6 isolates were resistant to more than 6 antibiotics indicating the ability to acquire and transfer MDR in *S. aureus*. Overall antibiotic resistance pattern revealed that gentamicin, doxycycline hydrochloride and tetracycline were most effective antibiotic with 85.0%, 65.0% and 60.0% efficacy respectively followed by minocycline and chloramphenicol with 50.0% efficacy and clarithromycin with 45% efficacy. Highest resistance was recorded against Penicillin–G (80.0%), followed by levofloxacin and linezolid, (a second line antibiotic recommended as per CLSI 2020) with 60.0% resistance (Supplementary Table 1). Similar to the present study, Nathawat *et al.* (2013) also detected that most effective antibiotics were gentamicin against mastitic milk origin *S. aureus* isolates and similarly, high resistance to penicillin G (83.64%), (83.3%) was observed by Awad *et al.* (2017) and Neelam *et al.* (2022) against *S. aureus* isolates from clinical mastitis in cattle. Contrary to this investigation, 100% susceptibility to azithromycin was recorded by Bhati *et al.* (2013) in *S. aureus* isolates from subclinical mastitis. In accordance to our results, Yadav *et al.* (2015) reported similar antibiogram against *S. aureus,* who observed that doxycycline, gentamicin, methicillin and tobramycin were more effective against all isolates. Ahmed and Saeed (2020) reported linezolid as a sensitive drug against *S. aureus* isolates from mastitic cattle in Sudan. In the present study, only one isolate (C58) show resistance towards mupirocin. In agreement to our study, Khazaie and Ahmadi (2021) studied 95 *S. aureus* isolates from subclinical bovine mastitis milk samples and reported that mupirocin is the most effective drug against MRSA isolates.

In the present investigation, total 30.0% isolates were detected as MRSA while none of the isolate was VRSA along with average MIC of 1.37 mcg/mL in both vancomycin agar screen and vancomycin minimum inhibitory concentration (MIC) method (Table 3). Similar to the present study, Adhikari *et al.* (2017) have also reported 35.50% MRSA with no VRSA in cattle mastitic milk samples. Memon *et al.* (2013) also recorded 29.0% MRSA in case of bovine subclinical mastitis while in contrary to the present findings, higher prevalence (71.79%) of MRSA was reported by Rahi *et al.* (2020) from raw milk samples.

Multiple Antibiotic Resistance (MAR) Index typing was carried out to assess the potential risk associated with each isolate in terms of acquiring and transmitting drug resistance to other bacterial pathogens. It was found that

Table 3. Detection of MRSA, high-level mupirocin resistance and VRSA among *Staphylococcus aureus*

Detection Method	Isolate I.D.		Remark
	Positive	Negative	
Methicillin Resistance / Oxacillin Resistance (MRSA)			
Cefoxitin disk Method	(06/30%) C29, C47, C48, C54, C58 and C69	(14/70%)C4, C18, C27, C34, C36, C50, C53, C57, C61, C68, C73, C74, C80 and C93	Cefoxitin (30µg) disk Method ≤21mm: *mecA* positive ≥22mm: *mecA* negative
High-Level Mupirocin Resistance			
Disk Diffusion method	C58	C4, C18, C27, C29, C34, C36, C47, C48, C50, C53, C54, C57, C61, C68, C69, C73, C74, C80, C93	Disk diffusion method (200-µg mupirocin disk) No zone: high-level mupirocin resistance. Any zone: the absence of high-level mupirocin resistance.
Vancomycin Resistant Staphylococcus aureus (VRSA)			
Vancomycin Agar Screen and MIC Method	Nil	C4, C18, C27, C29, C34, C36, C47, C48, C50, C53, C54, C57, C58, C61, C68, C69, C73, C74, C80 and C93	Vancomycin Agar Screen and MIC Method Single colony on 6 µg/mL Vancomycin: positive Not a single colony on 6 µg/mL Vancomycin: negative

95% of the isolates exhibited a MAR index exceeding 0.2% which highlighting their substantial capability to disseminate multiple antibiotic resistance to other pathogens (Table 4). Elevated MAR index shows that isolates are subjected to diverse antibiotics at the human environment interface level. This finding sheds a light on the uncontrolled use of antibiotics in animal husbandry. Consequently, this underscores the imperative need for implementing a One Health approach to combat antibiotic resistance in Staphylococcus aureus-like organisms, which have the potential to impact both humans and animals, in addition to being present in the environment (Krumperman 1983, Sharma *et al.* 2020).

To detect genetic aspects of antibiotic resistance chromosomal and plasmid DNA of *S. aureus* isolates was explored and detection of *blaZ (*β-lactamase*), mecA* (MRSA), *vanA* and *vanX* (VRSA) genes was attempted. Out of the total isolates of chromosomal DNA, eighteen isolates (90.0%) (Supplementary table 2) were positive for *blaZ* gene while twelve isolate (60.0%) were positive for plasmid of *blaZ* gene (Fig. 1a and 1b) with 517 bp specific amplicon size. Further, in the present investigation, only one isolate C69 were positive for *mecA* gene in both chromosomal DNA and plasmid with 533bp size specific amplicon (Fig. 2a and Fig. 2b). During exploration of both chromosomal and plasmid DNA, none of the isolate was detected positive for *vanA* (560bp) and *vanX* (480bp) gene. In consent to our results Akpaka *et al.* (2017) have also observed that none of the isolate in study has carried vancomycin gene (vanA) and the *blaZ* gene, which is the most common β-lactam (Penicillinase) resistance mechanism for *S. aureus*. Similarly, Brahma *et al.* (2022) isolated 80 *S. aureus* isolates and reported that Sixteen isolates were positive for the *mecA* gene; however, none of the isolates were positive for *mecC* and *vanA* genes. Close to our results, Yang *et al.* (2015) detected *blaZ* gene in 94.6% of penicillin resistant *S. aureus* isolates from bovine mastitis cases. Several studies have confirmed the most prevalent antibiotic resistance gene is *blaZ*, followed by *mecA* among *S. aureus* isolates from mastitis milk samples of cow. In agreement to the present study, Neelam *et al.* (2022) have also reported that 92.73% isolates carry

Table 4. Detection and distribution of Multiple Antibiotic Resistance Index (MAR) value among *Staphylococcus aureus* isolates

MAR Index Type	Isolate I.D.	No. of Isolate	No. of antibiotic for which the isolate was resistant*	MAR Index	Significance
MAR1	C47, C58	02	12	0.85	11 (55.0%) isolates had 0.2 or more than 0.2 MAR index with high potential risk for the spread of multi drug resistance.
MAR2	C36	01	10	0.50	
MAR3	C53	01	09	0.45	
MAR4	C48, C57	02	07	0.35	
MAR5	C68	01	06	0.30	
MAR6	C54	01	05	0.25	
MAR7	C18, C69, C80	03	04	0.20	
MAR8	C4, C27, C29	03	03	0.15	09 (45.0%) isolates had less than 0.2 MAR index with less risk source of multi drug resistance.
MAR9	C50, C61, C93, C73, C74	05	02	0.10	
MAR10	C34	01	01	0.05	

***** *S. aureus* isolates were screened for total 14 antibiotics

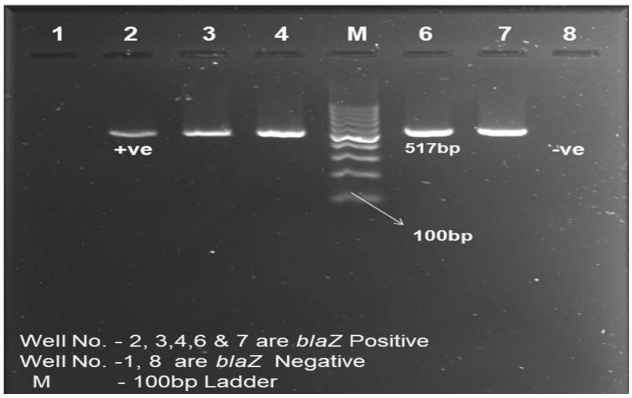

Fig. 1(a). Detection of *blaZ* gene among chromosomal DNA *Staphylococcus aureus* isolates (amplicon size: 517bp) Well No. 2,3,4,5,6 and 7 are *blaZ* Positive, Well No.1,8 are *blaZ* Negative, M: 100bp Ladder

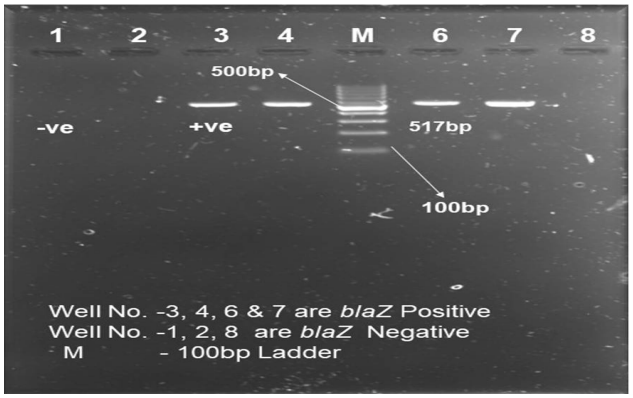

Fig. 1(b). Detection of *blaZ* gene in plasmid of *Staphylococcus aureus* isolates PCR (amplicon size 517bp) Well No. 3,4,6 and 7 are *blaZ* Positive, Well No.1,2,8 are *blaZ* Negative, M:100bp Ladder

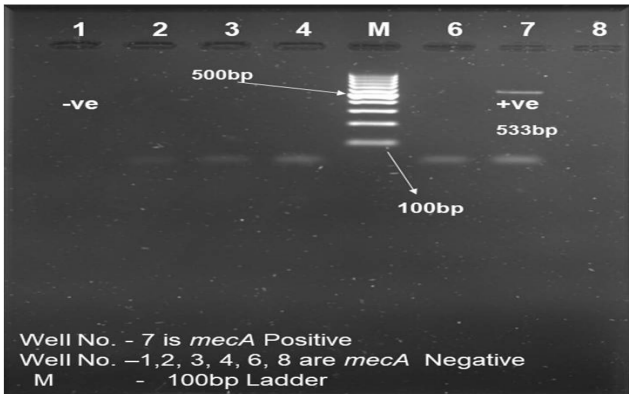

Fig. 2 (a). Detection of *mecA*, gene among chromosomal DNA *Staphylococcus aureus* isolates by PCR (amplicon size: 533bp**)** Well No. 7 is *mecA* Positive, Well No. 1,2,3,4,6,8 are *mecA* Negative, M: 100bp Ladder

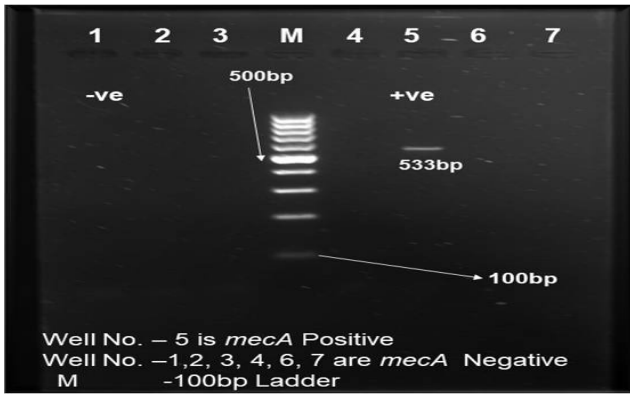

Fig. 2(b). Detection of *mecA*, gene in plasmid of *Staphylococcus aureus* isolates by PCR (amplicon size: 533bp) Well No.5 is *mecA* Positive, Well No. 1,2,3,4,6,7 are *mecA* Negative, M:100bp Ladder

blaZ gene. In agreement to this study, Rychshanova *et al.* (2022) studied 64 *S. aureus* isolates from cattle mastitis and reported that the presence of the *mecA* gene was found in only one isolate resistant to *β*-lactams (ampicillin). In study of *S. aureus* strains from mastitis, Bhattacharyya *et al.* (2016) have also reported that none of the isolate carry vancomycin resistance gene but contrary to present investigation, they found that all MRSA isolates were positive for *mecA* gene.

Although it is very difficult to establish the specific linkage of spread of specific antibiotic resistance mechanism in *S. aureus* infections but it is very well known that horizontal gene transfer is a major source for acquisition of antibiotic resistance genes. Hence in the present study, exploration of both chromosomal and plasmid DNA for presence of resistance genes is very important step to find epidemiology of particular resistance gene. This study evaluated both vertical and horizontal mechanisms governing DNA's transfer and concluded that *blaZ* gene mediated resistance mechanism is the most common to spread MRSA and VRSA as vancomycin resistant gene (*vanA* and *vanX*) is absent in Jaipur region of Rajasthan. It is also highlighted that higher MAR index and presence of resistance gene in plasmid of *S. aureus* isolates claims that isolates previously exposed to various antibiotics have evolved the capability of spreading antibiotic resistance to others.

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