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Correlation of Biofilm Production and Panton-Valentine Leukocidin Gene with Antibiotic Resistance Pattern of MRSA Isolated from Skin & Soft Tissue Infections

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant source of skin and soft tissue infections (SSTIs), which can develop into serious clinical issues because of drug resistance and virulence characteristics. This study aims to determine the correlation of antibiotic resistance with biofilm producer MRSA and its pathogenicity with panton valentine leukocidin (PVL) gene-positive strains.

Methods: Isolates were processed by standard operating procedures of the bacteriology laboratory. Antibiotic susceptibility test and MRSA determination were done according to Kirby-Bauer disk diffusion method and zone sizes were interpreted based on Clinical Laboratory Standards Institute (CLSI) guidelines. Biofilm production was assessed by the congo-red agar method and detection of PVL gene was through polymerase chain reaction (PCR) followed by gel electrophoresis.

Results: Among the 97 MRSA isolates tested highest resistance was detected for erythromycin (83.51%) followed by ciprofloxacin (69.07%) and cotrimoxazole (68.04%). Biofilm was produced by 38 isolates (39.17%) and PVL gene was detected in 46 isolates. Antibiotic resistance was higher among biofilm producers compared to non-biofilm producers ($p<0.05$). The PVL gene was isolated significantly more from invasive infection which fulminated from SSTIs and it was also related to resistance in some of the antibiotics.

Conclusion: Vancomycin and linezolid remain the drugs of choice for MRSA infection. Biofilm production aids in the resistance mechanism as does PVL gene for a few antibiotics, although it is a virulence marker responsible for the severity of infection. Our findings suggest stringent infection control measures are needed to curb the genesis and spread of resistant microorganisms.

Key-words: Antibiotic Resistance, Biofilm, Methicillin Resistant Staphylococcus aureus, PVL gene

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are becoming a significant public health concern. It can penetrate the body through damaged skin or mucous membranes, leading to skin infections.[1]

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The range of conditions involves necrotizing fasciitis, pneumonia, bacteremia, SSTIs, pyomyositis, sepsis and osteomyelitis. MRSA is most prevalent in wounds, even though it may be isolated from blood, urine, respiratory system and other bodily fluids. They are difficult to treat because of various virulence mechanisms including toxins, enzymes and adhesins.[2] Moreover, its prevalence is also concerning because of emerging multiple drug resistance mechanisms. The ability of MRSA strains to form biofilm increases the incidence of morbidity and mortality by abetting antibiotic resistance.[3] Consequent to biofilm production, MRSA is

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a cause of alarm in patients having surgically implanted polymeric devices.[4]

The extent of *S. aureus* infection corresponds to its capacity to modify the human immune system. Several virulence factors come into play which inhibit the innate immune response and postpone the adaptive immune response, thereby encouraging the spread of *S. aureus* into deep tissues and organs. One such virulence factor is the pore-forming Panton-Valentine leukocidin (PVL) cytotoxin which is an exotoxin primarily targeting leukocytes.[5] The toxin when combined with two secretory proteins called S and F, breaks down white blood cells' membrane by creating holes in the membranes and allowing cell contents to seep through them, ultimately leading to cell death. MRSA that produces PVL often cause moderate infections of the skin or soft tissues, but there have also been reports of severe instances of sepsis and necrotizing pneumonia. Poor outcomes are linked to the presence of the PVL gene in MRSA infections, particularly in patients with necrotizing pneumonia, invasive osteomyelitis and SSTIs.[6,7] The detrimental virulence factors like the PVL in conjunction with biofilm production in MRSA strains causing SSTIs make such infections difficult to treat. This study aims to determine the antibiotic-resistance pattern among the MRSA isolates in SSTIs and their correlation with biofilm production and PVL gene detection.

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology at Santosh Medical College, Ghaziabad and Mayo Institute of Medical Science, Barabanki, UP.

Data collection and Ethical approval- The data was part of our research activity delinked from patient testing after a culture sensitivity report was provided to the patient. All data has been kept anonymous. This study has been approved by the institutional ethics committee.

Inclusion criteria- MRSA isolated from pus specimens of patients with SSTIs were included in this study.

Exclusion criteria- Any other bacterial isolates or MRSA isolates from clinical specimens other than pus were excluded from this study.

Sample collection and Isolation- All pus samples from SSTIs received in the Microbiology laboratory were processed as per standard bacteriological procedures i.e. Gram's staining and further inoculation on Nutrient agar, MacConkey agar and Blood agar. In case culture came out to be positive for *S. aureus* based on colony characteristics; catalase test (Positive), coagulase test (Positive), mannitol (fermented), and DNAse (positive) tests were performed. The culture was further processed for antimicrobial susceptibility testing (AST).

Antimicrobial susceptibility testing and MRSA detection- For AST, turbidity of 0.5 MacFarland was prepared from the fresh culture of each isolate and then testing was done using the Kirby-Bauer disk diffusion method on MHA (Muller Hinton agar) as per CLSI (Clinical and Laboratory Standard Institute) guidelines. MRSA was defined as *S. aureus* isolate showing a zone of inhibition ≤21 mm with cefoxitin disk (30µg).[9]

Biofilm Detection- The components of the congo-red agar medium used were 36 grams of sucrose, 0.8 grams of congo-red powder, and 37 grams/litre of brain heart infusion agar. A loop full of culture growth was inoculated on the congo-red agar plate and after 24 hours of incubation at 37°C, color changes in the colonies were noted. Non-biofilm producers were pink in color while biofilm-producing isolates had black colonies with a dry, crystalline structure.[10]

Molecular method- All the MRSA isolates were inoculated on Luria Bertani broth and DNA was extracted according to manufacturer's instructions. Primers used for PVL gene were F: 5'ATCATTAGGTAAAATGTCTGGA CATGATCCA-3' and R: 5'GCATCAAGTGTATTGGATAGCAA AAGC-3'. A thermal cycler was used to perform 30 cycles of amplification (denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, and extension at 72°C for 30 seconds) and a final extension at 72°C for 2 minutes. This profile was used to carry out DNA amplification. After melting 0.45 grams of agarose in 30 millilitres of diluted 1x TBE solution, 1.5% agarose gel was used to analyze the PCR results. The PVL gene was tested using a 25–766 bp DNA ladder.[11]

Statistical Analysis- The chi-square test was applied for categorical data after the initial data were entered into Microsoft Excel. The p-value<0.05 has been regarded as significant.

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RESULTS

A total of 97 isolates of MRSA were tested in this study. Among these isolates, none of them were found to be vancomycin-resistant. The highest resistance was seen for erythromycin 83.51% followed by ciprofloxacin 69.07%, cotrimoxazole 68.04% and least resistant against linezolid 7.22% as shown in Table 1.

Table 1: Antibiotic resistance pattern of MRSA isolated from pus samples.

Antibiotics	No. of resistant isolates out of n=97 (%)
Teicoplanin	11 (11.34%)
Gentamycin	54 (55.67%)
Amikacin	51 (52.58%)
Tobramycin	52 (53.61%)
Azithromycin	64 (65.98%)
Erythromycin	81 (83.51%)
Tetracycline	35 (36.08%)
Doxycycline	37 (38.14%)
Ciprofloxacin	67 (69.07%)
Clindamycin	39 (40.21%)
Linezolid	7 (7.22%)
Chloramphenicol	39 (40.21%)
Cotrimoxazole	66 (68.04%)

Out of the 97 MRSA isolates, 38 were biofilm producers while 59 did not produce biofilm (Table 2). The resistance for all antibiotics among biofilm producers was significantly higher as compared to non-biofilm producers. The most significant differences in antibiotic resistance due to biofilm production were found for aminoglycosides and clindamycin.

Table 2: Antibiotic resistance patterns of biofilm producer and non-biofilm producer MRSA isolates from wound infection.

Antibiotics	Biofilm Producer (n=38)		Non-Biofilm producer (n=59)		p-value
	R	%	R	%	
Teicoplanin	9	23.68	2	3.39	0.0020
Gentamycin	35	92.11	19	32.20	<0.00001
Amikacin	32	84.21	19	32.20	<0.00001
Tobramycin	27	71.05	25	42.37	0.0056
Azithromycin	30	78.95	34	57.63	0.030
Erythromycin	36	94.74	45	76.27	0.016
Tetracycline	21	55.26	14	23.73	0.0015
Doxycycline	20	52.63	17	28.81	0.018
Ciprofloxacin	32	84.21	35	59.32	0.0096
Clindamycin	28	73.68	11	18.64	<0.00001
Linezolid	6	15.79	1	1.69	0.0088
Chloramphenicol	24	63.16	15	25.42	0.00021
Cotrimoxazole	31	81.58	35	59.32	0.021

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A total of 46 isolates showed the presence of the PVL gene while 51 were negative for it. Antibiotic resistance was higher among isolates with the PVL gene than without it (Table 3). The significant differences in resistance pattern were shown against erythromycin (98.7% vs 70.59%, p=0.0003), ciprofloxacin (87.0% vs 52.94%, p=0.0029), cotrimoxazole (87.0% vs 50.98%, p=0.00014). All other antibiotics did not show significant differences in resistance patterns in the two categories.

Table 3: Resistance Patterns of PVL (Pantone Valentive leukocidin) positive and negative isolates (n=97).

Antibiotics	PVL positive (n =46)		PVL negative (n=51)		p-value
	R	%	R	%	
Teicoplanin	6	13.0	5	9.80	0.61
Gentamycin	34	73.9	30	58.8	0.117
Amikacin	30	65.2	21	41.18	0.017
Tobramycin	25	54.3	27	52.94	0.88
Azithromycin	40	87.0	24	47.06	0.00035
Erythromycin	45	97.8	36	70.59	0.0003
Tetracycline	20	43.5	15	29.41	0.14
Doxycycline	20	43.5	17	33.33	0.30
Ciprofloxacin	40	87.0	27	52.94	0.00029
Clindamycin	26	56.5	13	25.49	0.061
Linezolid	4	8.7	3	5.88	0.59
Chloramphenicol	25	54.3	24	47.06	0.48
Cotrimoxazole	40	87.0	26	50.98	0.00014

The demographic study of PVL positive and PVL negative strains has been shown in Table 4. The gender difference (p=0.53) and IPD vs OPD (p=0.70) distribution was not statistically significant. Age-wise distribution of PVL positive (mean=32.7, SD=15.1) and PVL negative isolates (mean=33.3, SD=13.2) were significantly similar. The presence of PVL gene significantly led the SSTIs to turn into invasive infections (p=<0.00001).

Table 4: Demographic data of MRSA isolated from skin and soft tissue infections in correlation with PVL detection

Demographic Data	PVL positive		PVL negative		p-value
	N	%	N	%	
Gender					
Male	26	44.82	32	55.17	0.53
Female	20	51.28	19	48.71	
IPD/OPD					
IPD	29	49.09	28	50.90	0.70
OPD	19	45.23	23	54.76	
Age (years)	Mean=32.7 SD=15.1±2		Mean=33.3 SD=13.2±2		—
Type of infection					
SSTIs	20	29.41	48	70.58	<0.00001
Invasive infection	26	89.66	3	10.34	

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DISCUSSION

SSTIs can be localized or turn into systemic inflammation depending on the severity and management strategies. Superbug infections, including MRSA, have long posed a serious risk to the hospital system and public health services. The treatment of bacterial infections of the wound is still difficult even with the wide range of antimicrobial treatments available. Due to the ability to produce many virulence factors, *S. aureus* is primarily responsible for skin and soft tissue infections in humans.[1,4] In this study, a total number of 97 MRSA isolates were included among them 38 were biofilm producers and 46 were positive for the PVL gene. The highest resistance was found against erythromycin at 83.51% and no resistance against vancomycin. Similar to our study, Sanchez et al. had shown high resistance for erythromycin 82% and gentamicin 96%.[12] In this study total of 38 (39.17%) isolates were biofilm producers while other studies show a higher number of biofilm producers like Neopane *et al.* 43.3% [13] and Gupta *et al.* 48%.[14] This phenomenon can be explained by the fact that the mecA gene produces the penicillin-binding protein (PBP2a) and deactivates the quorum-sensing regulator system auxiliary gene regulator (agr) augmenting the production of biofilms.[15],[16] Another factor that promotes MRSA biofilm development is a phenol-soluble modulin mec (PSMmec), which is encoded by PSM-MEC.[17] Biofilms can form on any wound when planktonic bacteria are not eliminated by the host's immune system or by exogenous antimicrobial agents.[15]

In the present study antibiotic resistance among biofilm producer isolates was significantly higher for all the antibiotics tested. These findings were in favor of the results reported by Ghasemian *et al*.[18], Belbase *et al*.[19], Golia *et al*.[20], Kulayta *et al*.[21] and Singh *et al*. [22] The bacteria that have a propensity to produce biofilm are resistant to many antibiotics because of their protective properties. The bacteria strains living in biofilm have the potential to become many times more resistant to antibiotics. The primary causes of this might include the bacterial slow growth rate, the inability of antibiotics to penetrate biofilm, and the existence of antibiotic degradation mechanisms.[19] High antibiotic concentrations could be required to get rid of the biofilm producers. The possibility of toxicity and associated side effects, however, may make this impractical in vivo. Thus, low-concentration combination therapies, such as those including MRSA, may be useful in eliminating staphylococcal infections associated with biofilms.[23] The selection of a suitable antimicrobial agent depends on the early identification and screening of biofilm producers, as well as the results of their antimicrobial susceptibility testing.

There has been a lot of deliberation on the role of PVL in the pathogenicity and virulence of *S. aureus*. PVL accelerates apoptosis and the death of polymorphonuclear and mononuclear cells, increasing the pathogenicity of *S. aureus* and thereby affecting the morbidity and mortality due to SSTIs.[24] Our study shows PVL gene was detected in 47.42% of MRSA isolates. In a study by Kaur *et al*., the PVL gene detection among MRSA was 85.1% which is higher than in our study.[25] While study by D'Souza *et al*. in Mumbai revealed that 64% of MRSA isolates with the PVL gene.[26] The study by Mohammad *et al.* showed combined resistance to co-trimoxazole and clindamycin in 29.1% of PVL-positive isolates.[27] The difference in drug resistance among PVL positive and negative isolates was not statistically significant for most of the antibiotics except erythromycin, ciprofloxacin andcotrimoxazole suggesting that PVL is not necessarily linked to drug resistance mechanisms.[28] This study shows no significance among demographic data such as gender, age and admission to the hospital among PVL positive and negative isolates. However, it was PVL was found to be responsible for SSTIs to turn into invasive infections.

Our study has a few limitations; firstly, it is a single center study with limited data. Secondly, minimum inhibitory concentration determination could have helped in a better understanding of the resistance levels. Overall, our study has a focused approach delineating the need to test for virulence markers of MRSA and de-escalating antimicrobials as soon as the AST reports are available to prevent the spread of resistant microorganisms.

CONCLUSIONS

This study focuses on the necessity of continual surveillance for antibiotic-resistant strains like MRSA. There is an urgent need to prepare local antibiograms based on the surveillance data, especially in tertiary care settings and hospitals situated in peripheral and rural areas as a lot of misinformation and inadvertent use of

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antibiotics is prevalent in these areas. We have highlighted the emerging threat of multidrug-resistant superbugs like MRSA strains possessing several virulence factors making them more formidable to treatment. However, for MRSA infection, vancomycin and linezolid continue to be the recommended treatments. A strong correlation has been established between antibiotic resistance and biofilm production whereas PVL positivity influences resistance pattern as well as an important indicator of severity of infection. Therefore, detection of biofilm production and PVL gene should be considered in difficult to treat MRSA infections. A robust infection control strategy, reliable diagnostic modalities and prompt antimicrobial stewardship activities can help us prevent and treat invasive infections.

CONTRIBUTION OF AUTHORS

Research concept- Bajarangi Lal Chaudhary, Sanjeev Sahai

Research design- Bajarangi Lal Chaudhary, Sanjeev Sahai, Shalini Upadhyay

Supervision- Bajarangi Lal Chaudhary, Sanjeev Sahai

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