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Original Research Article

Methicillin resistance and biofilm formation in Staphylococcus Aureus isolated from clinical samples in a tertiary care hospital in Central India

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ABSTRACT:

Background: Staphylococcus aureus is a virulent organism resistant to most of the conventionally available antibiotics. Methicillin-resistant Staphylococcus aureus (MRSA) is the most common cause of nosocomial infections. One of the reasons is because of its capability to produce biofilms. The increasing drug resistance along with methicillin resistance and biofilm production among the strains of Staphylococcus aureus presents a serious problem to the treatment of the infections caused by S. aureus. **Objectives**- To detect methicillin resistance by antibiotic susceptibility test in Staphylococcus aureus and to detect biofilm formation in the isolates and assess its correlation with methicillin resistance **Material and methods**- In this prospective study conducted in Tertiary care Institute, Gwalior a total of 300 isolates of S. aureus were screened for Methicillin resistance using Cefoxitin disc by Kirby Bauer Disc Diffusion method on Mueller Hinton agar. For detection of Biofilm formation, two phenotypic tests were done - Tube method (TM) and Tissue culture plate method (TCP). **Results** - Out of 300 isolates, 78% were MRSA and 22% were MSSA. A total of 47% isolates were Biofilm producers and 53% were non biofilm producers. Among the biofilm producers, 89.5% were MRSA and only 10.5% were MSSA, thus depicting the higher prevalence of biofilm formation among MRSA. **Conclusion**- This study demonstrates the high prevalence of MRSA isolates producing biofilms in clinical staphylococcal samples. Since both are interdependent, detection of biofilm expression in clinical isolates would be beneficial in treatment decisions if done routinely.

Key words- MRSA (Methicillin resistant staphylococcus aureus), prevalence, Biofilms, Antibiotic susceptibility

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

1. INTRODUCTION:

Staphylococcus aureus is an opportunistic pathogen implicated as the most common agent of skin and soft tissue infections. It exists in the nasopharynx, skin, eye, intestine and urogenital tract as normal flora. However, it can breach the skin barriers through the wound or surgical incision and cause infection. [1] Staphylococcus aureus is a virulent organism that has developed resistance to most of the conventionally available antibiotics. The ability to acquire resistance to multiple antibiotics classes makes S. aureus a challenging pathogen to treat especially the methicillin-resistant S. aureus (MRSA). One of the main reasons for that is their capability of forming biofilms consisting of multilayered cell clusters embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism. The interior of the bacterial biofilms presents greater resistance to the opsonisation by antibodies and phagocytosis which explains the chronic character of these infections such as endocarditis, osteomyelitis and especially those infections associated with implanted medical devices that are difficult to be treated. Significant increase in both mortality and morbidity in humans has been reported in patients infected with MRSA due to the development of biofilms.

The aim of our study was to isolate and identify Staphylococcus aureus from various clinical samples and detect methicillin resistance by antibiotic susceptibility test. Secondarily, we observed the biofilm formation by the S.aureus isolates by two methods and assessed the correlation between MRSA and biofilm formation in Staphylococcus aureus.

2. MATERIAL AND METHODS:

This study was conducted from November 2019 to August 2021in department of Tertiary care Hospital, Gwalior (Madhya Pradesh).This is a prospective and observational type of study. A total of 300 S. aureus were isolated from various clinical specimens like pus, wound swab, aspirates, blood, urine, CSF and other sterile fluids. All samples were inoculated on nutrient agar, blood agar and Mac-Conkey agar and incubated at 37°C. The isolates were identified by colony morphology, Gram stain, catalase test, slide and tube coagulase test and standard biochemical tests. Antimicrobial susceptibility testing was done by the Kirby-Bauer disc diffusion method which was performed and interpreted as per the recommendations of the Clinical and Laboratory Standards Institute (CLSI) 2020 Guidelines. All antibiotic discs used in the study were procured from Hi-media and laboratories, Mumbai, India.S. aureus ATCC 25923 was used as control strains. Ethical clearance from the institute was taken with IEC details registration number as follows: 49/IEC-GRMC/2019

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

Detection of MRSA: Methicillin-resistant S. aureus were identified phenotypically based on its resistance to cefoxitin (30μg) by the Kirby Bauer's disc diffusion method performed on Muller Hinton agar plates. Based on the CLSI, 2020 guideline, the zone of inhibition was interpreted and grouped into methicillin-sensitive and methicillin resistant S. aureus. Isolates with cefoxitin zone size ≥ 22mm were considered methicillin susceptible i.e. MSSA and the isolates with zone size ≤ 21mm were considered methicillin resistant.

Detection of Biofilm:

The in vitro biofilm production was measured using two phenotypic assays: **Tube Method** (TM) and **Tissue Culture Plate** (TCP) method.

1. Tube Method: a qualitative method described by Christensen et al., 1982 [2] for the detection of biofilm formation was performed. Biofilm Production was considered positive when a visible film lined the inner wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and amount of biofilm formation was scored as 0-absent, 1-weak, 2- moderate, 3-strong.

2. Tissue Culture Plate: a quantitative method was used as described by Christensen et al., 1985 with slight modification [3], using Brain Heart Infusion (BHI) broth with 2% sucrose. Optical densities (OD) of both the dry plates and eluted stain was measured using micro ELISA auto reader at OD 620 nm. Mean OD value < 0.120, 0.120–0.240 and > 0.240 were classified as non/weak, moderate and strong biofilm adherence respectively[4].

STATISTICAL ANALYSIS:

The statistical analysis was performed using statistical software SPSS (2.1). The data was represented as percentages and proportions. Two or more set of variables were compared by using Chi-square test and Z-test. If the p-value was <0.05, it was considered statistically significant.

3. OBSERVATION AND RESULTS:

Out of 300 S. aureus samples, 111 were blood samples, 80 samples of pus, 76 of urine and 25 of CSF and other sterile fluids, and rest 8 others. In our study, prevalence of MRSA was found to be 78% and the MSSA were 22%.

To detect biofilm formation by S. aureus, we performed 2 methods namely Tissue culture plate(TCP) and tube method(TM), and out of 300 S. aureus isolates a total of 141(47%) produced biofilms and 159 (53%) did not produce biofilms.

Biofilm formation was detected by TCP method in 86 isolates and by TM in 74 isolates. Among 141 biofilm producers, MRSA (89.4%) and MSSA (10.6%). Among 159 non- biofilm producers,

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

MRSA (67.9%) and MSSA (32.1%). Similarly, among 234 MRSA strains, 53.8% produced biofilms and out of 66 MSSA strains, 22.7% produced biofilms .

Out of 141 samples that produced biofilms, 88.6% were IPD samples and 11.4% were OPD. Out of 159 non-biofilm producers, 34% were OPD and 66% were IPD. These findings show that there is a higher incidence of biofilm formation in the isolates of IPD patients (having catheters, attached prosthetic/medical devices).

Tables:

Table 1: ANTIBIOTIC RESISTANCE AND SENSITIVITY

ANTIBIOTIC DISCS	Sensitivity	RESISTANCE	% (SENSITIVITY)
Amoxacillin – Clavulinic acid (30 µg)	10	290	3.3%
Erythromycin (15µg)	49	251	16.3%
Clindymycin (2 µg)	277	23	92.3%
Cefoxitin (30 µg)	66	234	22%
Cotrimoxazole	223	77	74.3
Doxycycline (30 µg)	272	28	90.7%
Vancomycin (30 µg)	300	0	100%
Chloramphenicol (30 µg)	248	52	82.7%
Ciprofloxacin (5 µg)	15/224	209/224	6.7%
Nitrofurantoin (300 µg)*	55/76	21/76	72.4%
Norfloxacin (10 µg)*	22/76	54/76	28.9%

Table 2: PREVALANCE OF METHICILLIN RESISTANCE

ANTIBIOTIC RESISTANCE	PATIENT SAMPLES	PERCENT (%)
MRSA	234	78
MSSA	66	22
TOTAL	300	100

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

Table 3: PREVELANCE OF METHICILLIN RESISTANCE IN RELATION TO BIOFILM PRODUCTION

	MRSA	MSSA	TOTAL
BIOFILM PRODUCERS	126	15	141
NON-BIOFILM PRODUCERS	108	51	159
TOTAL	234	66	300

Figure 1: BIOFILM FORMATION ON TISSUE CULTURE PLATE

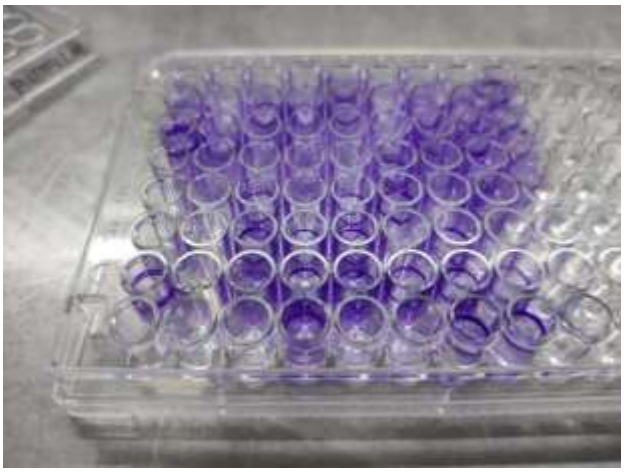

TABLE 1: ANTIBIOTIC RESISTANCE AND SENSITIVITY Antimicrobial susceptibility patterns of S. aureus isolated Among all the S. aureus, highest rates of susceptibility was seen toward Vancomycin (100%) followed by Clindamycin (92.3%), Doxycycline (90.7%) and Chloramphenicol (82.7%) and the least effective were Amoxy-clav (3.3%) and Ciprofloxacin (6.7%). TABLE 2: PREVALANCE OF METHICILLIN RESISTANCE Out of 300 samples, 234 samples were found to be of MRSA group i.e. 78% and 66 were sensitive to methicillin i.e. MSSA group (22%).

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

TABLE 3: PREVELANCE OF METHICILLIN RESISTANCE IN RELATION TO BIOFILM PRODUCTION

□

2 value = 20.013, P value < 0.001 (Significant)

Among 141 biofilm producers, 126 were MRSA (89.4%) and 15 were MSSA (10.6%).

Among 159 non-biofilm producers, 108 were MRSA (67.9%) and 51 were MSSA (32.1%).

Similarly, among 234 MRSA strains, 126 produced biofilms (53.8%) and out of 66

MSSA strains, 15 produced biofilms (22.7%).

Figiure 1: BIOFILM FORMATION ON TISSUE CULTURE PLATE DISCUSSION:

Staphylococcus aureus is one of the most common causes of nosocomial infections throughout the world and the epidemiology is quite rapidly changing worldwide. (Speller DC, et al., 1997).

Methicillin-resistant S. aureus (MRSA) poses a great risk to patients with wounds; significant increase in both mortality and morbidity in humans has been reported in patients infected with MRSA due to the development of biofilms. This has led to renewed interest in the usage of macrolide–lincosamide–streptogramin B (MLSB) antibiotics to treat S. aureus infections, with clindamycin being the preferable agent due to its excellent pharmacokinetic properties.[2] However, widespread use of MLSB antibiotics has led to an increase in the number of staphylococcal strains acquiring resistance to MLSB antibiotics.[5] In S. aureus, an active efflux mechanism encoded by msrA gene confer resistance to macrolides and streptogramin B antibiotics (so called MS phenotype), and modification of ribosomal target encoded by erm genes cause resistance to MLSB antibiotics; which is called as MLSB resistance. The latter mechanism can be constitutive MLSB (cMLSB), where the rRNA methylase is always produced; or can be inducible MLSB (iMLSB), where methylase is produced only in the presence of an inducing agent.

A total of 300 Staphylooccus aureus isolates were screened from various clinical samples in the hospital of which 234 (78%) were MRSA and 66 (22%) were MSSA. Out of 300 S. aureus isolates a total of 141(47%) produced biofilms and 159(53%) did not produce biofilms. Biofilm formation was detected by TCP method in 86 isolates and by TM in 74 isolates. Among 141 biofilm producers, MRSA were 126(89.4%) and MSSA were 15(10.6%). Among 159 non-biofilm producers, MRSA were 108(67.9%) and MSSA were 51(32.1%). Similarly, among 234 MRSA strains, 53.8% produced biofilms and out of 66 MSSA strains, 22.7% produced biofilms. Out of 141 samples that produced biofilms, 88.6% were IPD samples and 11.4% were OPD. Out of 159 non-biofilm producers, 34% were OPD and 66% were IPD. These findings show that

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

there is a higher incidence of biofilm formation in the isolates of IPD patients (having catheters, attached prosthetic/medical devices). Prevalence of MRSA in Staphylococcus aureus isolates-

Development of resistance against the therapeutic treatment options of infection caused by MRSA is an emerging problem. Therefore, this study was aimed to assess the prevalence of MRSA and antimicrobial susceptibility in Staphylococcus aureus. In study performed by **Mohammedaman mama et al**, in Ethiopia 2018 prevalence of MRSA was 82%. In a study by **Sarita Manadhar et al** in Nepal 2018 has found prevalence of MRSA was 81%. In a study by **Rania M.abdel Halim et al** in Egypt in 2017 has found 86% prevalence of MRSA . The MRSA prevalence statistics of our study was found in range to above studies. Our prevalence of MRSA was higher than the studies done by **Taruna Singh et al** in M.P. in 2019 of 38% MRSA prevalence, **Ankit Belbase et al** in Nepal 2017 has found 47% MRSA prevalence.

In our study, prevalence of MRSA was found to be 78% and the MSSA were 22%. In our study, higher percent of MRSA isolates were isolated from admitted patients. The colonized health care workers in the hospitals are the main sources of MRSA in hospitalized patients causing higher rates of infections among them [10]. The main source for the community acquired MRSA infections may be the colonized health care workers who transfer the MRSA to their household, spreading it to the community [10]. Further, the admitted patients who got colonized during hospital stay may also act as the alternative sources for the community acquired MRSA infections. The proportion of MRSA has increased worldwide since last two decades. Its prevalence varies markedly across different countries and among hospitals of the same country. Improper infection prevention practices in the hospital set up, indiscriminate use of antibiotics, intravascular catheterization, hospitalization in intensive care unit etc. contribute in the emergence of MRSA. The variations in the recent findings could be due to the differences in the circulating clones or due to the variations in infection prevention practices and trends of antibiotics prescription in different hospital set up. Prevalence of Biofilm Formation and relation with MRSA-

The biofilm-forming capacity of bacterial strains is a trait highly associated with bacterial persistence and virulence. Furthermore, chronic bacterial infections are linked to the formation of biofilms. Biofilm-producing capacity is closely related to clinical S. aureus strains, genetic lineages, multidrug-resistance profiles and highly virulent strains. For high disease burden of biofilm associated staphylococcalinfections, a reliable and prompt diagnostic method is essential in health care facilities. Therefore, in this study, we evaluated two phenotypic methods of in vitro biofilm detection**.**

In a study performed by **Fatima Khan et al** in Aligarh, India 2011, 64% isolates produced Biofilm by tube method and 65% by tissue culture plate method. **Abdolmajid Ghasemian et al**

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

in their study conducted in Iran 2014 found 36% MRSA and 29% MSSA isolates were strong biofilm producers.

In the study by **Sarita Manandhar et al** in Nepal 2018, total no. of biofilm producers were 61% out of the total isolates (45% biofilm producers were MRSA and 16% were MSSA**).** In a study by **Rania M. Abdel Halim et al** in Egypt in 2017, they biofilm producers were found to be 53.2% out of which 74% were observed by tissue culture plate method and 42% formed by tube method. The findings in this study were close to our study on biofilms as in our study, out of 300 samples, biofilm formation detected in 47% isolates and among them 89% were MRSA and 11% were MSSA which proves the association of Methicillin resistance with biofilm production. Also we found that by tissueculture method 60% isolates produced biofilms and by tube method 52.5% showed biofilm formation.

In our study, higher rates of multidrug resistance and methicillin resistance were found among biofilm producing strains in comparison to biofilm non-producing strains. These findings were in favour of the results reported by **Ghasemian et al**. [11]. Due to protective nature of the biofilm, the bacteria growing in it are intrinsically resistant to many antibiotics [12]. The antibiotic resistance among the strains of the bacteria residing in biofilm may increase up to 1000 times [12]. The main reasons for this may be difficulty in penetration of biofilm by antibiotics, slow growth rate of the bacteria and presence of antibiotic degradation mechanisms [12]. Further, biofilm formation gives platform for horizontal gene transfer among bacteria, causing the spread of drug resistance markers and other virulence factors [13].

Increasing cases of antibiotic resistance in staphylococcal infections pose a serious threat to public health as well as pronounced socio-economic burden across the world. S. aureus is a major human pathogen that causes acute to chronic systemic infections which are often refractory to antibiotics leading to treatment failures. Such recalcitrant infections are mainly associated with biofilms formed by S. aureus on indwelling devices such as catheters, CVC, prostheses etc. (**von Eiff et al., 2005; Murugan et al., 2010; Prasad et al., 2012**). Early detection of biofilm forming staphylococci therefore warrants one of the most essential steps for prevention, management, and cure of nosocomial infections.

LIMITATIONS:

In this study, due to lack of resources we could not use molecular methods to confirm our results but there are molecular methods like coagulase (coa) gene detection by polymerase chain reaction for identification of S. aureus and detection of mecA gene for identification of methicillin resistant S. aureus and we also did not perform the genotypic methods for detection of biofilm production by S. aureus.

ISSN: 0975-3583, 0976-2833 VOL14, ISSUE12, 2023

4. CONCLUSION

This study demonstrates the high prevalence of MRSA isolates producing biofilms in clinical staphylococcal samples. Since staphylococcal infections have a significant impact on morbidity and mortality, prevention and management of these infections should be a priority. This study, while bringing additional information about the status of biofilm producing clinical strains and their association with multiple antibiotic resistances, also highlights the importance of early detection strategies in routine diagnostics of S. aureus infections. Implementation of those will help to identify biofilm producing S. aureus cases to prevent occurrence of treatment failures of staphylococcal infections in Gwalior, M.P.

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