

RESEARCH ARTICLE

## High Prevalence of Multiple Drug Resistant and Biofilm Forming *Staphylococcus aureus* among HIV-Infected Patients with Suspected Pneumonia

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### Abstract

*Staphylococcus aureus* is one of the major causes of life threatening pneumonia, especially in immunocompromised population. In HIV positive patients, *S. aureus* associated pneumonia can be either health care associated or community acquired and responsible for high rate of mortality. In this study total 102 throat swab samples of HIV-Infected Patients with suspected pneumonia were collected during 2014-2016, out of them 46 samples (45.1%) were found positive for *S. aureus* by biochemical tests. 38 (82.6%) isolates were found multiple drug resistant while 9 (19.6%) strains showed resistance to cefoxitin antibiotic, were considered as methicillin resistant *Staphylococcus aureus* (MRSA). Only one strain (2%) was found vancomycin intermediate (VISA), remaining 98% isolates were sensitive to vancomycin antibiotic. In PCR test, all cefoxitin resistant strains were found positive for the presence of *MecA* gene. Biofilm former *S. aureus* were screened by tissue culture plate (TCP) methods. In TCP assay, 21 (45.6%) isolates were confirmed as high biofilm formers (OD value > 0.250), 16 (34.8%) were moderate biofilm formers (OD values- between 0.150 to 0.250), while 9 (19.6%) were low biofilm formers (OD value < 0.150). A significant association was found among multiple drug resistance and high biofilm formation (p value < 0.05). High prevalence of biofilm forming MDR isolates in airways of pneumonia suspected HIV patients is matter of great concern as poor antibiotic response may cause more severe diseases with increasing cost and duration of treatment. The *MecA* gene might be a cause of methicillin resistance among MRSA isolates.

**Keywords:** Multiple Drug Resistance, Biofilm, MRSA, Pneumonia, Immunocompromise, HIV.

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## INTRODUCTION

Opportunistic pneumonias are the major cause of HIV-associated severe respiratory illness with high rate of mortality<sup>1</sup>. The opportunistic pneumonias among HIV patients may include mycobacterial, viral, bacterial, parasitic and fungal pneumonias, among them bacterial pneumonia is the most common type of opportunistic pneumonia<sup>2-4</sup>. In HIV positive persons, the rate of bacterial pneumonia is higher than persons without HIV<sup>3,5</sup>. The clinical characteristics of pneumonia are fever, chills, chest pain, shortness of breath etc. Although bacterial pneumonia can occur at any stage of HIV infection but the incidence of bacterial pneumonia increases with declines in CD4 cell count<sup>1</sup>. Most common bacterial pathogens associated with community acquired pneumonia during HIV infection are *Streptococcus pneumoniae*, *Haemophilus* species and *Staphylococcus aureus*<sup>6</sup>.

In contrast, *S. aureus* normally colonize in skin and mucous membranes in the upper respiratory tract of healthy individuals without any complications, but in immunocompromised people, *S. aureus* has been associated with many syndromes such as pneumonia, bacteraemia, septicaemia, urinary tract infections, diarrhea and skin infections with increased severity<sup>7</sup>. The detection and characterization of *S. aureus* among clinical samples is generally done by biochemical tests such as mannitol fermentation, coagulase and DNase. Few studies suggested the polymerized chain reaction (PCR) as molecular technique using amplification of *nuc* gene for early diagnosis of *S. aureus*<sup>8</sup>.

The prevalence of multiple antibiotic resistances in *S. aureus* is increasing globally, which causes poor antibiotic response and economical burden especially in developing countries<sup>7</sup>. In the 1960s, first case associated with methicillin resistant *S. aureus* (MRSA) was reported and in the late 1990s It became a well known cause of community and hospital associated infections<sup>9</sup>. Previous studies suggested that resistance to methicillin among *S. aureus* is associated with presence of *mecA* gene which encodes penicillin binding protein 2a (PBP 2a). Staphylococcal chromosome cassette mec (SCCmec), a mobile genetic element of 21 to 60 kb contains *mecA* gene. SCCmec may also carry genes such as

pT181, Tn554 and pUB110 which may associated with resistance for non-beta lactam class of antibiotics<sup>10</sup>. Expression of PBPs provides capacity to bacterial cells to continue cell wall synthesis in presence of higher concentration of cell wall synthesis inhibitors<sup>11</sup>. Molecular detection of the highly conserved *mecA* gene using PCR amplification has been proved as a benchmark for the early diagnosis of MRSA in the clinical samples<sup>12</sup>. As an additional virulence factor many *S. aureus* strains have capacity to form biofilms on biotic and abiotic surfaces<sup>13</sup>. Biofilms are multilayered arrangement of bacterial cells enclosed in exopolysaccharide matrix, which helps bacterial cells in survival, providing protection against extreme environmental conditions, antimicrobial agents and host immunity<sup>14,15</sup>. Because of higher level of resistance against host immune responses and antibiotics, biofilm forming *S. aureus* may lead to severe and chronic disease in individuals with weak immunity such as HIV positive patients. Although in HAART era, a significant fall in opportunistic infections has been noticed, but HIV positive people are still at high risk of opportunistic bacterial respiratory infections<sup>16</sup>.

HIV patients are under high burden of medications, wrong selection of antibiotics for the treatment not only enhances duration of treatment and financial burden but also make disease more complicated and life threatening. Early detection of *S. aureus* and evaluation of their virulence such as antibiotic resistance, presence of *mecA* gene and biofilm formation ability is essential for selection of proper antibiotic for the treatment of pneumonia in HIV patients. The involvement of biofilm forming, MRSA in HIV patients suffering from pneumonia, is not well studied. The aim of this study is to detect the prevalence of multiple drug resistant and biofilm forming *Staphylococcus aureus* among pneumonia suspected HIV patients and evaluate the presence of *mecA* gene among isolates.

## MATERIALS AND METHODS

### Isolation and characterization of *S. aureus* from clinical samples

The isolation and characterization of *S. aureus* from clinical samples (throat swab) was done by direct throat swab inoculation on mannitol salt agar (MSA) (HiMedia laboratories private

limited, India) plates, followed by incubation at 37 °C for 24 h. Gram staining was performed followed by biochemical confirmation tests such as DNase, mannitol fermentation and coagulase tests. All strains which were positive for mannitol fermentation, coagulase and DNase tests were confirmed as *Staphylococcus aureus*<sup>17</sup>.

#### Antibiotic sensitivity and detection of MRSA by disc diffusion method

The antibiotic sensitivity test was performed by modified Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia laboratories private limited, India) for the antibiotic discs- azithromycin (25 µg), chloramphenicol (30 µg), cefoxitin (30 µg), clindamycin (2 µg), sulfamethoxazole/trimethoprim (25 µg), gentamycin (10 µg), levofloxacin ( 5 µg), linzolid (30 µg), vancomycin (30 µg) following Clinical and Laboratory Standards Institute (CLSI, 2013) guidelines<sup>18</sup>. All cefoxitin resistant strains were confirmed as MRSA.

#### Bacterial DNA Isolation

Bacterial log culture was prepared in tripticase soy broth (HiMedia laboratories private limited, India). 250 µL of broth culture was centrifuged at 5000 rpm for 10 min to settle bacterial cells. Pellet was resuspended in 50 µL of dd water, followed by addition of 5 µL lysostaphin solution (Sigma-Aldrich Corp.) and incubated at 37 °C for 30 min, followed by DNA extraction processes using PureLink™ genomic DNA mini Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Extracted bacterial DNA was stored at - 20 °C.

#### Detection of *nuc* and *mecA* genes by PCR amplification

A multiplex PCR was standardized for screening of *nuc* and *mecA* genes among isolates, using specific primers (Table-1). The thermal cycling conditions: an initial denaturation at 95 °C

for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 Sec, annealing at 51 °C for 30 Sec and extension at 72 °C for 90 Sec, final extension at 72 °C for 10 min., followed by agarose gel electrophoresis. DNA bands were visualized under gel imaging system (Bio-Rad Laboratories).

#### Detection of biofilm forming *S. aureus*

Screening of biofilm forming *S. aureus* strains was done by tissue culture plate (TCP) method<sup>19</sup> with some modification<sup>20</sup>. TSB media (HiMedia laboratories private limited, India) enriched with 1% glucose was inoculated with isolates and incubated for 24 h at 37 °C. Log cultures were diluted (1:100) with fresh TSB medium, each well of 96 well TCP was filled with inoculated media (200 µL) followed by incubation for 24 h at 37 °C. After incubation the content of plate was removed and washed three times with 200 µL of phosphate buffer saline (PBS). 0.25% crystal violet solution (200 µL) was added to each well and incubated for 20 min and rinse thrice with PBS. 200 µL of 95% ethyl alcohol was added. Optical density (OD) of stained biofilm in each well was determined by ELISA Reader (Thermo Fisher Scientific, India) at wavelength 630 nm. TCP assay was repeated three times for each strain.

#### Statistical analysis

Statistical analysis was performed using SPSS software (IBM SPSS statistics 20). The chi square test was performed to compare MDR and non MDR isolates for the presence of virulence factors such as biofilm formation and *mecA* gene.

## RESULTS

In this study total 102 throat swab samples of pneumonia suspected HIV patients were collected during 2014-2016. 46 samples (45.1%) were found positive for *S. aureus* by biochemical tests. In antibiotic sensitivity test, highest 65% of isolates were found resistant

**Table 1.** Primers used for the molecular detection of *nuc* and *mecA* genes

Primers	Sequences of primers	Size of amplified products (bp)
<i>Nuc-F</i>	5 -GCGATTGATGGTGATACGGTT-3	
<i>Nuc-R</i>	5 -AGCCAAGCCTTGACGAACTAAAGC-3	367 bp
<i>Mec-F</i>	5'-GTT GTA GTT GTC GGG TTT GG-3'	
<i>Mec-R</i>	5'CTT CCA CAT ACC ATC TTC TTT AAC-3'	533 bp

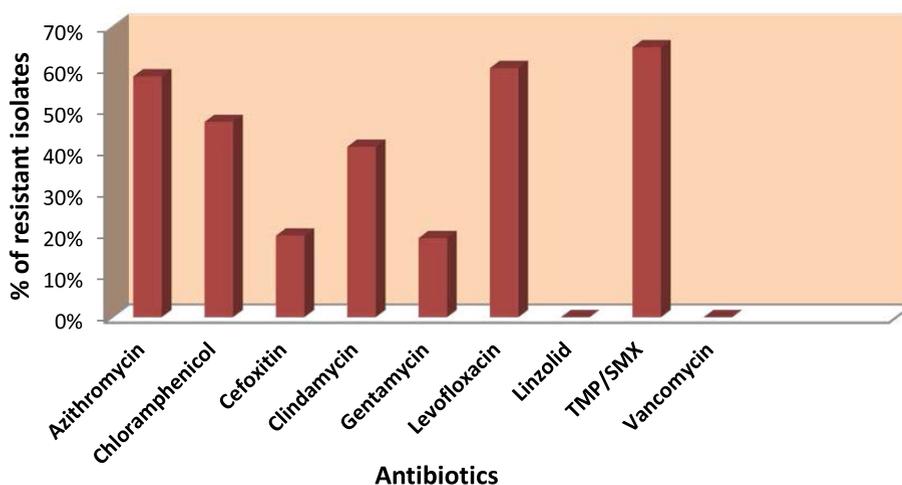
for antibiotic sulfamethoxazole/trimethoprim followed by levofloxacin 60%, azithromycin 58%, chloramphenicol 47%, clindamycin 41%, cefoxitin 20%, gentamycin 19%. Resistance for vancomycin and linzolid antibiotics was not found among isolates (fig. 1), while only one isolate (2%) was found vancomycin intermediate *S. aureus* (VISA). Out of total confirmed *S. aureus*, 38 (82.6%) isolates showed resistance for three or more antibiotics in antibiotic sensitivity test, considered as MDR, while 9 (19.6%) isolates showed resistance for cefoxitine antibiotic were confirmed as MRSA. All MRSA were found MDR. In PCR test, all confirmed

*S. aureus* showed amplification of *nuc* gene while all MRSA strains were found positive for presence of *mecA* gene (fig. 2). Biofilm forming *S. aureus* strains were screened by tissue culture plate (TCP) method (fig. 3). In TCP assay, 21 (45.6 %) isolates were confirmed as high biofilm forming isolates (OD value > 0.250), 16 (34.8 %) were moderate biofilm forming (OD values- between 0.150 to 0.250) while 9 (19.6 %) were low biofilm formers (OD value < 0.150) (fig. 4). A significant association was found among multiple drug resistance and high biofilm formation (p value < 0.05) (Table 2).

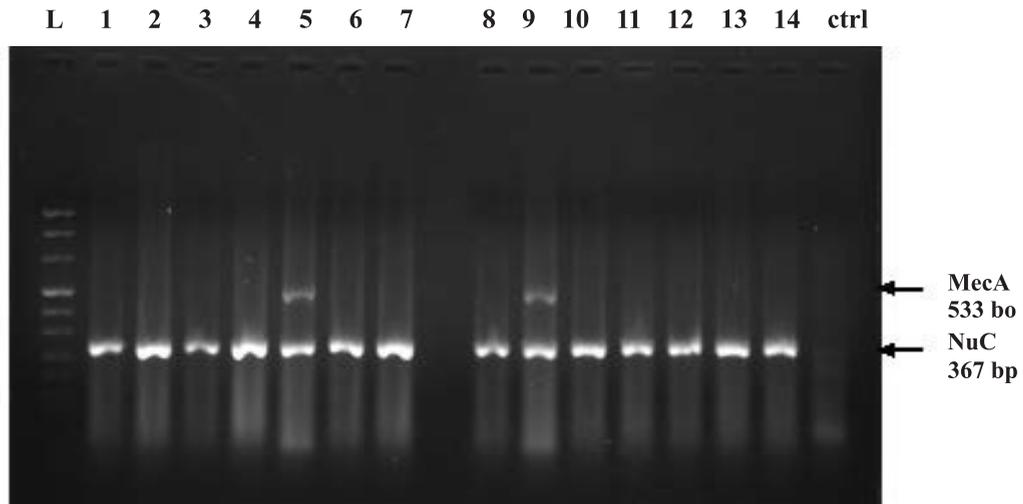
**Table 2.** Association of bacterial virulence factors (biofilm formation and presence of *mecA* gene) with antibiotic sensitivity

S.No.	Bacterial virulence Factors	<i>Staphylococcus aureus</i> Isolates (N=46)		p values*
		MDR isolates (N=38)	Non MDR isolates (N=8)	
1.	Biofilm formation			
	HBF	20 (95%)	1 (5%)	<b>0.03*</b>
	MBF	13 (81%)	3 (19%)	0.9
	LBF	5 (55%)	4 (45%)	<b>0.017*</b>
2.	Presence of <i>MecA</i> Gene	9 (100%)	0 (0%)	0.125

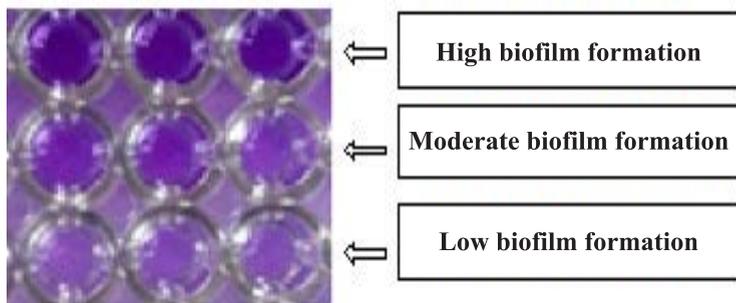
\*Chi-square test was performed to compare the bacterial virulence factors ((Biofilm formation and Presence of *mecA* gene) between MDR and non MDR *S. aureus* (p-value <0.05 was considered significant).



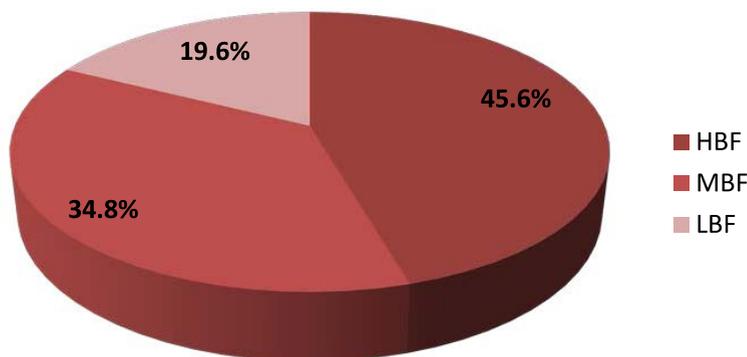
**Fig. 1.** Percentage of resistant isolates (*S. aureus*) against antibiotics



**Fig. 2.** Agarose gel images; Showing PCR amplification of specific *Nuc* (367 bp) and *MecA* (533 bp) genes of *Staphylococcus aureus* isolates: Lane- L is DNA Ladder (1000bp), Lane 1 to 14 – PCR amplification of *Nuc* gene, Lane 5, 9 – PCR amplification of *MecA* gene. Lane- ctrl is negative control.



**Fig. 3.** Screening of biofilm producing ability of isolates by tissue culture plate method



**Fig. 4.** Percentage of biofilm forming *Staphylococcus aureus* isolates; OD values less than 0.150 were recorded as low biofilm forming isolates, those with OD values between 0.150 to 0.250 were considered as moderate biofilm formers and those with OD values above 0.250, were considered as high biofilm forming isolates.

## DISCUSSION

Acquired immunodeficiency syndrome (AIDS) is a major health issue specially in developing world<sup>7</sup>. Weak immunity in HIV positive people place them at increased risk of opportunistic infections. Opportunistic associated respiratory illness is more common in immunocompromised people than healthy individuals<sup>3,5</sup>. Although most of the respiratory infection are self limiting and can be treated with medications but in HIV patients these infection may cause severe complications and life threatening conditions<sup>1</sup>. In HAART era the immunity status of HIV patients has significantly improved due to improvement in the level of CD4 cell count but the threat of opportunistic infections among HIV positive individuals cannot be denied<sup>16</sup>.

*S. aureus* is a common opportunistic pathogen associated with community acquired as well as nosocomial infections<sup>7</sup>. The emergence of antibiotic resistance and rise in level of virulence among *S. aureus* is a burning health issue both within the community and hospital settings. Occurrence of MRSA in respiratory infections has increased over time among HIV patients<sup>21,22</sup>. MRSA associated pneumonia among HIV patients can be community acquired or health-care associated and may lead to higher rate of morbidity and mortality. The rate of colonization by *S. aureus* is higher in HIV positive patients. As a colonizer MRSA may associated with increased risk of infections<sup>23, 24</sup>. In our study, out of 102 throat swab samples of HIV patients, 46 samples (45%) were positive for *S. aureus*. Out of the confirmed *S. aureus*, 82.6% were MDR while 19.6% were found MRSA. All MRSA were MDR. In a previous study, *S. aureus* was found associated with 25% of pneumonia cases in HIV-positive patients, among them 65% were MRSA<sup>25</sup>. Another study showed 85.7% prevalence of MRSA in cutaneous abscesses of HIV patients<sup>26</sup>.

In antibiotic sensitivity test, the highest resistance was noticed for sulfamethoxazole/trimethoprim antibiotic, while no resistance was found for vancomycin and linezolid antibiotic. A previous study supports our results, showing no resistance in MRSA for teicoplanin, vancomycin, and linezolid antibiotics<sup>27</sup>. In some other reports, MRSA were completely found sensitive for vancomycin<sup>28</sup> and linezolid antibiotics<sup>29, 30</sup>. While in another

study high resistance for vancomycin was observed and sulfamethoxazole/trimethoprim (TMP/SMX) antibiotic found effective against pathogenic *S. aureus* strains<sup>26</sup>, showing dissimilarity to our data.

The biofilm formation among *S. aureus* is a known virulence factor<sup>13</sup>. It is estimated that bacterial biofilms are associated with 65% of all bacterial infections. The involvement of biofilms found in both, device and non-device associated infections<sup>31</sup>. In our study 45.6 % isolates were found high biofilm formers, 34.8 % moderate biofilm former while 19.6 % were low biofilm formers. In a study 48 % biofilm forming *S. aureus* isolates were reported in different clinical samples of HIV positive patients. In our study, a significant association among high biofilm formation with multiple antibiotic resistance was observed. Similarly, Samie et al., correlated biofilm formation with  $\beta$ -lactamase production, out of the 14 strong biofilm formers, 9 (64.3%) were found  $\beta$ -lactamase positive<sup>7</sup>. However, the association of biofilm forming and multiple drug resistant *S. aureus* in pneumonia among HIV positive patients is not well studied. Higher level of drug resistance and biofilm formation among isolates, may lead to sever pneumonia, especially in immunocompromised patients. Early detection of *S. aureus* and evaluation of their virulence such as antibiotic resistance, presence of *mecA* gene and biofilm formation is essential for selection of proper antibiotic for the treatment of pneumonia in HIV patients.

## CONCLUSION

High prevalence of biofilm forming MDR isolates in airways of HIV-Infected Patients with suspected pneumonia is matter of great concern as poor antibiotic response may cause more severe diseases with increasing cost and duration of treatment. The methicillin resistance among MRSA isolates showed positive association with the presence of *mecA* gene. Biofilm formation among *S. aureus* isolates might be a cause of increasing multiple antibiotic resistance.

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