



# Difference in biofilm development capability of vancomycin and ciprofloxacin resistant *Staphylococcus aureus* clinical isolates

Husain Saify, Rakesh Kumar Patidar, Mayuri Khare, K. N. Sahare and Vinod Singh\*

\*Correspondence: [vsingh3@rediffmail.com](mailto:vsingh3@rediffmail.com)

Department of Microbiology, Barkatullah University, Bhopal, Madhya Pradesh, India.

## Abstract

**Background:** *Staphylococcus aureus* has long been recognized as a major pathogen of urinary tract and hospital acquired infections. Over the last decade antibiotic resistance and biofilm formation potential by pathogens is a major challenge. In this study we screened *S. aureus* clinical isolates by antimicrobial susceptibility and biofilm assays to determine biofilm capability of vancomycin and ciprofloxacin resistant isolates.

**Methods:** Six clinical isolates of *S. aureus* were characterized by biochemical tests and further antibiotic susceptibility of vancomycin and ciprofloxacin were tested against *S. aureus* clinical isolates by disc diffusion method. Biofilm formation capability of these isolates were performed by microtiter plate, coverslip congoed agar and tube assays.

**Results and discussion:** In this study we found that two isolates were resistant to ciprofloxacin and three isolates were resistant to vancomycin, on the basis of CLSI guidelines. Ciprofloxacin resistant isolates showed moderate biofilm formation while vancomycin resistant showed strong biofilm formation.

**Conclusion:** We have concluded that ciprofloxacin and vancomycin resistant clinical isolates were showed differences in biofilm formation. Staphylococcal isolates having biofilm propensity exhibit more resistance to antibiotics, hence are difficult to treat.

**Keywords:** Antibiotic, biofilm, ciprofloxacin, resistance, vancomycin

## Introduction

*Staphylococcus aureus* is a gram positive cocci normally localize in the skin and mucous membranes in the nasal area of healthy humans and about 30% of the normal healthy population are transiently colonized by the organism [1]. These bacteria are associated with the several syndromes such as skin infections, osteomyelitis, bacteraemia, septicaemia, diarrhea, pneumonia and urinary tract infections [2-8]. Biofilm formation is the potential and major virulence trait [9] and has been described as a possible attribute to the resistance and major contributor to several infections. Biofilm impairs the action of both host immune system and antimicrobial activities. The formation of biofilms is an excellent example of a phenotypic change in *S. aureus* to adapt to its surroundings in the presence of environmental challenges and is a recognized method of some microorganism's capability to cause certain infections, and a way by which they increases its levels of antimicrobial resistance [10]. It is now well documented that biofilms are difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of abiotic surfaces becomes necessary [11]. The differentiation of Staphylococci with respect to its biofilm phenotype might help in diagnosis of infections related to hospital devices and prevention of device related infections [12].

The pathogenesis of Staphylococcal infection begins with primary attachment and colonization of the host tissues. Once a biofilm has been formed, it is a major concern for clinicians in the treatment of infectious disease because of multi drug resistance [13]. Therefore, a better understanding of bacterial

biofilms is urgently needed, and this may ultimately result in the development of novel therapeutics for the control of infections [14].

In this study we have screened *S. aureus* clinical isolates by antimicrobial susceptibility and biofilm assays to determine biofilm capability of vancomycin and ciprofloxacin resistant isolates.

## Materials and methods

### Bacterial isolates

In this study, urine samples were collected the patients of Hamidia hospital, Bhopal, India. Out of 32 isolates, 11 isolates were identified as Gram positive Staphylococci. Further identification of these isolates was done by catalase, coagulase, sugar fermentation tests and cultivation on blood agar, mannitol salt agar, CLED agar. Out of 11 Staphylococci isolates, 6 were *Staphylococcus aureus* and 5 were coagulase negative Staphylococci. *Staphylococcus aureus* MTCC 3160 strain was used as a control in the whole study.

### Antibiotic susceptibility testing

The antibiotic-resistance profile of *S. aureus* strains against ciprofloxacin (5 µg) and vancomycin (30 µg) was determined by the disc diffusion assay [15]. Mueller-Hinton agar plates were overlaid with the inoculum (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical strains. Antibiotic susceptibility was established and measured by the reference criteria of Clinical and Laboratory Standards

**Table 1. Distribution of antibiotic susceptibility and biofilm formation capability of *S. aureus* clinical isoates against ciprofloxacin.**

Isolates	Ciprofloxacin	Congo red agar assay	Tube assay	Microtiter assay	Coverslip assay
SA1	Sensitive	+	+	+	+
SA2	Resistant	++	++	++	++
SA3	Resistant	++	++	++	++
SA4	Sensitive	+	+	+	+
SA5	Sensitive	+	+	+	+
SA6	Sensitive	+	+	+	+
SA MTCC 1360	Sensitive	+	+	+	+

+biofilm forming, ++moderate biofilm forming.

Institute (CLSI) guidelines.

## Biofilm formation assay

### Congo red agar assay

Production of slime was studied by cultivation of all *S. aureus* strains on congo red agar media [16]. Briefly, the medium was composed of Brain Heart Infusion Agar (37 gms/L), sucrose (50 gms/L), and congo red stain (0.8 gms/L). Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the center of colonies was observed.

### Tube assay

A qualitative assessment of biofilm formation was determined by tube assay [17]. Briefly, overnight culture of *S. aureus* isolates were inoculated in tryptic soya broth with 1% sucrose and incubated for 24h at 37°C. The tubes were removed and washed with phosphate buffer saline and dried. Tubes were stained with 0.1% crystal violet for 10 min. Excess stain was removed and tubes were washed with distilled water. Tubes were then dried and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong. Experiment was performed in triplicate and repeated three times.

### Microtiter plate assay

Microtiter plate method is considered as a gold standard method for the quantification of biofilm formation. Briefly, overnight culture of *S. aureus* strains was grown in brain heart infusion broth (BHI) (Himedia Laboratories, India) for 18 h at 37°C. One ml of overnight culture was transferred to 10 ml of sterile BHI broth with the addition of 1% Sucrose for biofilm development. The suspensions were adjusted with the same BHI medium

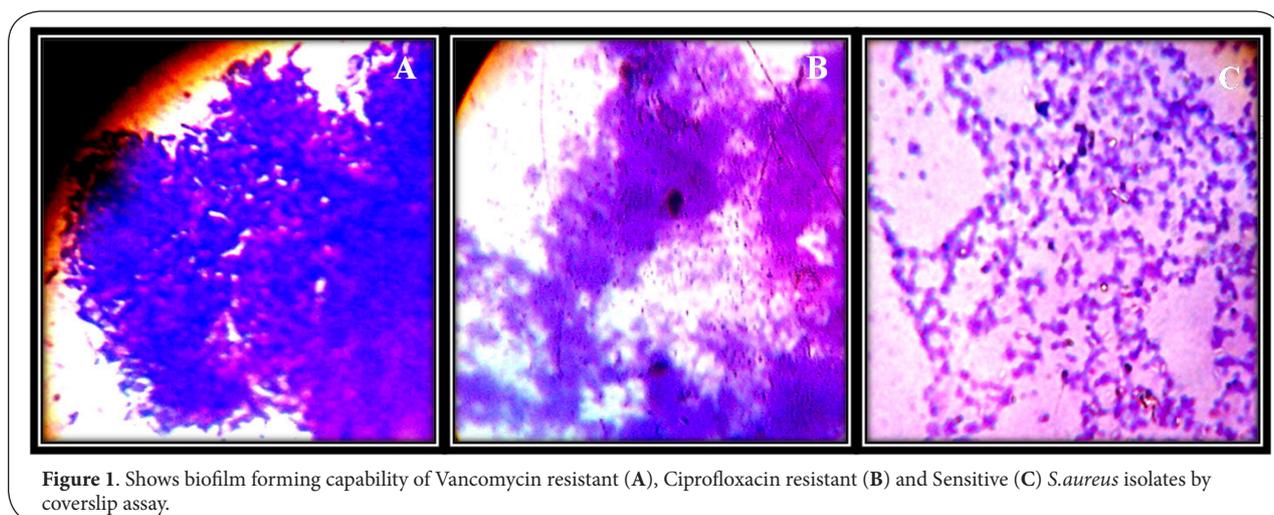
to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08-0.1 at 625 nm) in a spectrophotometer (Shimadzu, Australia), corresponding to approximately 102 CFU/ml. Then, from each culture, 250 µl volumes were transferred into wells of a microtiter plate (Himedia Laboratories, India). Blank wells contained broth, only. Plates were incubated for 24 hours at 37°C. At the end of 24 hours, the planktonic suspension and nutrient solution were removed and each well was washed three times with 300 µl of sterile physiological saline. The plates were vigorously shaken in order to remove planktonic cells. The remaining attached cells were fixed with 250 µl of 96% ethanol per well, and after 15 min, plates were made empty and left to dry. Each well was then stained with 200 µl of 0.1% crystal violet (CV Gram stain, Merck, Germany). The stain was rinsed off by placing the plates under tap water. After drying the stained plates, biofilms were visible as purple rings formed on the sides of each well. The quantitative analysis of biofilm formation was performed by adding 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Then the optical density (OD) of the stain was observed and measured at 570 nm by an ELISA reader (Lisa, Germany). The isolates were classified into three categories: nonadherent ( $A_{570} \leq 0.1$ ), weakly adherent ( $0.2 \geq A_{570} \geq 0.1$ ), strongly adherent, ( $0.2 < A_{570}$ ). Experiment was performed in triplicate and repeated three times.

### Coverslip assay

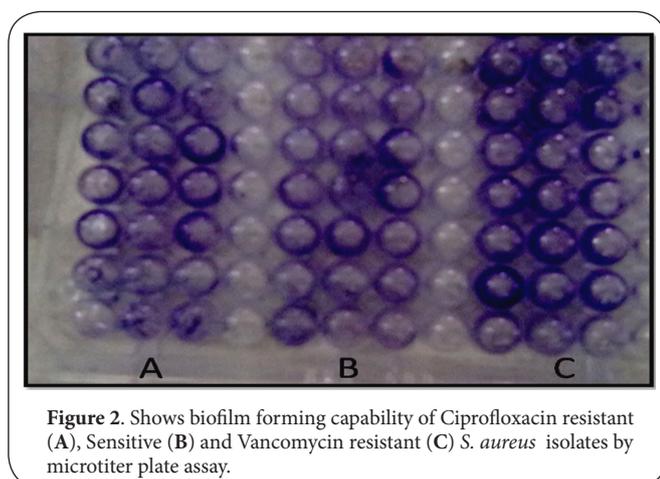
In coverslip assay [18], biofilms of *S. aureus* clinical isolates were grown as follows, individual sterile culture dishes were filled with 2.5 ml of BHI broth with 1% sucrose and sterile glass coverslip was added to each petriplate. Each sample was inoculated with defined volume of overnight culture. The dishes were incubated aerobically at 37°C for 72 h. Planktonic cells were rinsed off with phosphate buffer saline and sessile cells or biofilm were stained with 0.1% crystal violet for 5 min. Finally stained biofilms were observed microscopically.

## Results and discussion

*Staphylococcus aureus* is a gram positive coccal bacterium which is highly adaptive to human environment and are resistant to several antibiotics including vancomycin, which is the last effective antibiotic against *S. aureus*. There are many resistant species of *S. aureus* out of which the most infectious strains are vancomycin resistant and ciprofloxacin resistant *S. aureus*. In this study we found that two isolates were resistant to ciprofloxacin and three isolates were resistant to vancomycin (Tables 1 and 2), on the basis of CLSI guidelines. Over the last few decades the continuous increasing of bacterial resistance to antimicrobials has been a cause of worldwide concern and a great challenge. This situation is provoked by over the counter accessibility, indiscriminate and inappropriate use of antimicrobial agents. Ciprofloxacin and vancomycin are the most effective known drugs against urinary tract infections. In addition biofilm formation is known



**Figure 1.** Shows biofilm forming capability of Vancomycin resistant (A), Ciprofloxacin resistant (B) and Sensitive (C) *S.aureus* isolates by coverslip assay.



**Figure 2.** Shows biofilm forming capability of Ciprofloxacin resistant (A), Sensitive (B) and Vancomycin resistant (C) *S. aureus* isolates by microtiter plate assay.

**Table 2. Distribution of antibiotic susceptibility and biofilm formation capability of *S. aureus* clinical isoates against vancomycin.**

Isolates	Ciprofloxacin	Congo red agar assay	Tube assay	Microtiter assay	Coverslip assay
SA1	Sensitive	+	+	+	+
SA2	Resistant	+++	+++	+++	+++
SA3	Resistant	+++	+++	+++	+++
SA4	Sensitive	+	+	+	+
SA5	Resistant	+++	+++	+++	+++
SA6	Sensitive	+	+	+	+
SA MTCC 1360	Sensitive	+	+	+	+

+biofilm forming, ++moderate biofilm forming, +++ trrong biofilm forming.

major virulence determinant of *S. aureus*. In our study biofilm forming capability of ciprofloxacin and vancomycin resistant isolates showed differences in biofilm assays (**Tables 1 and 2**). Ciprofloxacin resistant isolates showed moderate biofilm

formation while vancomycin resistant showed strong biofilm formation (**Figures 1 and 2**). Staphylococci are bacterial pathogens that usually produce biofilms during different infectious processes, which are generally difficult to treat or control. It has been estimated that about 65 per cent of the nosocomial infections are associated with biofilm formation. These infections are 10 to 1000 times more difficult to eliminate with an otherwise successful treatment. The mechanism for enhanced antimicrobial resistance is believed to involve alteration or change in gene expression leading to a phenotypic difference between the planktonic and sessile condition. The sessile forms are more resistant as they produce exopolysaccharide, have different growth characteristics and take up nutrients and drugs differently from their planktonic counterparts [19].

### Conclusion

We have concluded that ciprofloxacin and vancomycin resistant clinical isolates were showed differences in biofilm formation. Vancomycin resistant *S. aureus* isolates showed strong biofilm formation while ciprofloxacin resistant isolates showed moderate biofilm formation.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

Authors' contributions	HS	RKP	MK	KNS	VS
Research concept and design	--	--	--	--	✓
Collection and/or assembly of data	✓	--	--	--	--
Data analysis and interpretation	--	✓	--	✓	--
Writing the article	✓	✓	--	--	--
Critical revision of the article	--	--	✓	--	--
Final approval of article	--	--	--	--	✓
Statistical analysis	--	--	--	✓	--

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