



E-ISSN: 2278-4136  
P-ISSN: 2349-8234  
JPP 2019; 8(3): 3508-3511  
Received: 16-03-2019  
Accepted: 18-04-2019

**Ankita A Singh**  
Ph. D. scholar, Department of  
Pharmaceutical Sciences,  
Kalaniketan Polytechnic College,  
Jabalpur, Madhya Pradesh,  
India

**OP Verma**  
Advisor, Department of  
Molecular and Cellular  
Engineering, JIBB, SHUATS,  
Prayagraj, Allahabad, Uttar  
Pradesh, India

**RR Mishra**  
Assistant Professor, Medical Lab  
Technology, DDU Kaushal  
Kendra Banaras Hindu  
University, Varanasi,  
Uttar Pradesh, India

#### Correspondence

**OP Verma**  
Advisor, Department of  
Molecular and Cellular  
Engineering, JIBB, SHUATS,  
Prayagraj, Allahabad, Uttar  
Pradesh, India

## Evaluation of biofilm formation on different nosocomial adherent materials like- pieces of catheters and micro titer plates

Ankita A Singh, OP Verma and RR Mishra

#### Abstract

A biofilm is a thin layer of microorganisms that adhere to the surface of an organic or inorganic structure, together with their secreted polymers. Formation of biofilm is a survival strategy for bacteria and fungi to adapt to their living environment, especially in the hostile environment. Under the protection of biofilm, microbial cells in biofilm become tolerant and resistant to antibiotics and the immune responses, which increases the difficulties for the clinical treatment of biofilm infections. As a result of DNA exchange, physiological changes, and the thick matrix in which these microorganisms embed themselves, they become highly resistant to conventional antibiotic treatments. Biofilms have demonstrated the ability to persist in 100 to 1000 times the concentrations of antibiotics and biocides that can inhibit planktonic cells. Similarly, granulocytes, macrophages, and other phagocytes are unable to engulf a biofilm as they would individual planktonic cells. The genotypic and phenotypic diversity of the biofilm allow adaptation to overcome multiple stresses and to survive most sequential therapies. Biofilm disease has been viewed as various diseases that affect a variety of tissues and structures, including ear, nose, throat, mouth, eye, lung, heart, kidney, gall bladder, pancreas, nervous system, skin, bone, as well as virtually every implanted medical device. Clinical observations and experimental studies indicated clearly that antibiotic treatment alone is in most cases insufficient to eradicate biofilm infections. Therefore, to effectively treat biofilm infections with currently available antibiotics and evaluate the outcomes become important and urgent for clinicians.

**Keywords:** Biofilm, bacteriophage, extracellular matrix

#### Introduction

*Staphylococcus aureus* is a major virulent human pathogen and colonizer in approximately 30–50 % of individuals on skin and the mucosal surfaces (Noble *et al.*, 1967) <sup>[1]</sup>. *S. aureus* causes a wide spectrum of disease including skin and soft tissue infections (SSTI), pneumonia, bacteremia, endocarditis, and osteomyelitis Lowy (1998) <sup>[2]</sup> and hospital care associated infections (HCAIs). Although *S. aureus* is often associated with antimicrobial drug resistance, large outbreaks of *S. aureus* predate the advent of widespread resistance. Methicillin resistance, conferred by a large transmissible staphylococcal cassette chromosome *mec* (SCC*mec*), first emerged in 1961 and for the first 30 years became endemic as hospital-associated (HA)-MRSA affecting patients with underlying co morbidities or exposure to the health-care setting Jevons MP *et al.* (1964) <sup>[3]</sup>. In 1961, Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in hospitals in the United Kingdom Jevons M (1961) <sup>[4]</sup>. Since the description of that initial case, MRSA has become increasingly prevalent and is now an increasing cause of infections worldwide. The proportion of healthcare-associated staphylococcal infections in United States' intensive care units due to MRSA has continued to rise, from 35.9% in 1992 to 64.4% in 2003 (Klevens *et al.*, 2006) <sup>[5]</sup>.

Phages are ubiquitous in nature and far outnumber their bacterial hosts. They have been reported to be present in soil and sediment at a titre of approximately 10<sup>9</sup> viral particles per gram and in aquatic systems at titres between 10<sup>4</sup> and 10<sup>8</sup> per ml Weinbauer (2004) <sup>[6]</sup>. Replication of these viral particles and release of the progeny generally leads to death of the host cell. Phages have been estimated to kill 20-40% of marine bacteria every day Suttle (2005) <sup>[7]</sup>. Phages can be readily isolated from environmental samples such as soil, sewage and water and their prominence in the environment means that humans are constantly exposed to phages without adverse effect. This fact, along with their specific bactericidal potential, are two important factors which justify utilising phages and phage based products for their therapeutic and *prophylactic* potential.

Bacteriophages (phages) are bacterial parasites. They have either DNA or RNA as their genetic material encapsulated in a protein coat. Phages are essentially found everywhere and

are known to infect >140 bacterial genera and can be regarded as the most abundant biological entities, with estimations of  $10^{31}$  phage particles in the world Bergh *et al.*, (1989) [8].

Following their discovery, it became evident that they possess two types of life cycle, lytic (used by both virulent and temperate phages) and lysogenic (used by temperate phages). Generally, phages bind to a receptor on the bacterial cell surface, insert their DNA and hijack the host cell machinery for subsequent replication of DNA and synthesis of phage proteins. Progeny phages then form intracellularly by a self-assembly process before being released following cell lysis Guttman *et al.*, (2005) [9]. In contrast, temperate phages can multiply via the lytic cycle or they can enter the lysogenic cycle by integrating their genome into the host chromosome. When the phage is residing in the chromosome, it is known as a prophage and is replicated along with the bacterial genome during cell replication. In some cases, prophage may encode virulence genes, which can be horizontally transferred from one bacterium to another by transduction Boyd and Brussow (2002) [10].

## Materials and Methods

### Collection of Bacterial strains from clinical sample

A total 25 *S.aureus* strains were identified and isolated from the 56 different clinical samples of Bacteriology section, HDRL Varanasi. The identified *S.aureus* strains were processed for confirmation and differentiation and antibiotic sensitivity.

### Morphological and Microscopic observation

Gram staining technique was used for microscopic observation. Smear of all strains were prepared and allowed it to dry. Initially it was stained with crystal violet as a primary stain. After washing the crystal violet stain, Gram's iodine was applied followed by decolorization with 95% alcohol solution. Finally, the smears were counterstained with safranin. The smears were allowed to air dry and the color, shape and arrangement of the bacterial cells were observed under the microscopic field and also observed, whether the bacteria is gram positive or not.

### Biochemical identification

Cagulase test was performed to confirm the presence of *S. aureus* in overnight culture media. Took a clean glass slide, add two drops of saline on it and then picked up the suspected colonies of *S. aureus* to be tested from agar culture and gently emulsify with drops of saline. Then added a drop of plasma to the bacterial suspension and mixed it properly. Rocked the slide, back and forth, and observed for the clumping of the bacterial suspension within 10-15 seconds.

### Antibiotic Disk Sensitivity test

Penicillin, Oxacillin, Ampicillin, Carbenicillin, Piperacillin+Tazobactam, Amoxicillin+Clavulanicacid, Cefazolin/Cephalexin, Cefuroxime, Cefotaxime/Ceftriazone, Ceftazidime, Gentamicin, Tobraycin, Amikacin, Netilmicin, H.S. Gentamicin, Nalidixic acid, Norfloxacin, Ciprofloxacin/Ofloxacin, Levofloxacin, Chloramphenicol, Erythromycin, Tetracycline, Trimethoprim+Sulfamethoxazole, Nitrofurantoin, Furazolidone, Vancomycin, Telcoplanin, Linezolid, Meropenem, Impenem, Polymyxin B were used for antibiotic sensitivity. Methicilin resistance *S. aureus* (MRSA)

was selected for phage generation and biofilm formation on different materials.

### In vitro biofilm formation on different materials

**Formation of *S. aureus* biofilm on microtiter plate:** Clean and sterilized 96 well microtiter plates were taken. 300µl of L.B. broth media was added in all the 96 wells with 10µl *S. aureus* culture of 10<sup>5</sup> CFU/ml. It was kept at shaker for 24hr. On the next day all the media was thrown in Lysol solution and all the wells were emptied. Then it was washed gently under running tap water. Above process was repeated for 7 days.

**Formation of *S. aureus* biofilm on pieces of catheters:** Two 50 ml clean and sterilized conical flasks was taken and 5ml of L.B. broth media was added in both the flasks. Added 10µl *S. aureus* culture of 10<sup>5</sup> CFU/ml to above flask and 5 pieces of catheters were kept and the flask was kept on the shaker for 24hr. On the next day media was thrown gently and fresh 5ml L.B. broth media was added and 10µl of pure cell suspension of 10<sup>7</sup> CFU/ml of staph culture was also added to the flask and kept at shaker for next 24hr. The above process was repeated for 7 days.

On the 8<sup>th</sup> day media was emptied from all the wells of microtiter plates. The pieces of catheters were transferred to sterilized petri-plates.

All the materials i.e. microtiter plates and catheters were washed gently under running tap water and then stained with 1% crystal violet solution and examined under compound microscope.

### Crystal violet assay

Total biomass attached to each well was measured by crystal violet assay. First, the wells were washed twice with a saline solution (0.9% NaCl (Merck) in distilled water) and then biofilms were fixed with 1 ml of methanol (Merck) for 15 min. After this time, methanol was removed and to each well was added 1 ml of crystal violet (1% v/v, Merck) for 5 min. The wells were then washed with water, and 1 ml of acetic acid (33% v/v, Merck) was added to dissolve the stain. The eluted stain was placed in a 96-well microtiter plate and its absorbance was read by an ELISA reader at 570 nm. Two independent experiments were performed in duplicate.

### XTT reduction assay

The determination of biofilm cellular activity was measured by the XTT reduction assay. Biofilms were washed twice with a saline solution (0.9% NaCl (Merck) in distilled water) and then 1 ml of XTT (200 mg /ml, Sigma) solution plus PMS (20 mg /ml, Sigma) was added to each well. The plates were incubated in the dark at 37 °C for 3 h. After this time, the solution was removed from each well and placed in a 96-well microtiter plate to determine its absorbance at 490 nm. Two independent experiments were performed in duplicate.

### CFU (Colony forming unit) counts

To determine the amounts of bacteria present in biofilms, CFU counts was performed using the micro drop technique. Briefly, wells of the microplates were washed once with saline solution (0.9% NaCl) to remove unattached bacteria and then 1 ml of fresh saline solution was added to each well and the biofilm scraped with a cell scraper prior to sonication (5 min). After this, the cellular suspension of each well was removed, centrifuged (5 min, 10000 rpm, 4°C) and the pellet re-suspended in 1 ml of saline solution (0.9%). The samples

were diluted in saline solution (0.9%) and one drop (10 ml) was placed in a petri plate containing Yeast extract peptone-dextrose (YPD) solid medium and allowed to run down the plate. Plates were incubated at 37°C for 16 to 18 h and after CFUs were counted. Three independent experiments were performed in duplicate.

**Results**

**Morphological observation of clinical sample**

The bacterial isolates, that were cultured on the Muller Hinton agar media, were identified as *S. aureus* on the basis of morphology, microscopic observation and biochemically such as coagulase test, from the bacterial growth appeared on the medium after overnight incubation (Figure 1). *S. aureus* produces opaque, circular colonies with butyrous consistency. Golden yellow pigment was demonstrated on MH agar media. On blood agar, *S. aureus* produces β-haemolytic golden yellow or white colonies. 25 possible clinical bacterial isolates of *S. aureus* were collected, of which 1 MRSA strain were used for the purpose of bacteriophage isolation.

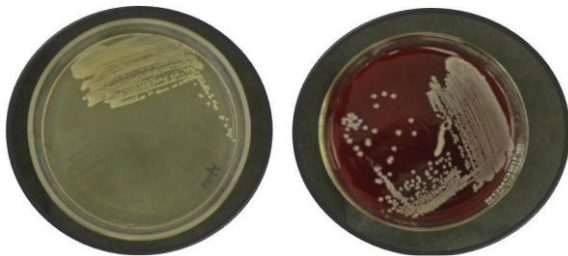


Fig 1: Growth of *S. aureus* on MH Agar medium and Blood Agar medium

**Microscopic and Biochemical observation**

In the microscopic observation the positive *S. aureus* strains appeared blue coloured cocci arranged in grape-like clusters (Figure 2). In slide test of coagulase, white precipitate and agglutination of the organism appeared within 10-15 seconds of mixing the plasma with the suspension of bacterial cells.

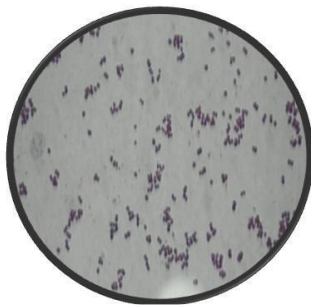


Fig 2: Microscopic observation of Gram's staining of loopful colonies.

**Antibiotic Disk Sensitivity test**



Fig 3: Antibiotic sensitivity of Methicillin resistant *S. aureus* strain.

**Biofilm formation on microtiter plate**

Microtiter plate was stained with 1% crystal violet solution after appropriate washing to confirm the formation of biofilm on it. Blue colour appeared on the walls of microtiter plates which confirms the presence of biofilm.

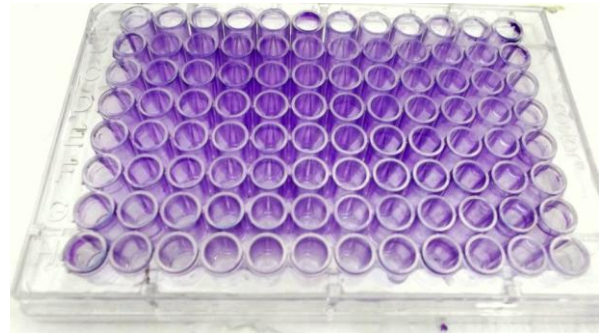


Fig 4: Formation of Methicillin resistant *S. aureus* biofilm on the walls of microtiter plate

**Biofilm formation on pieces of catheters**

One piece of catheter was taken and washed it properly under running tap water, then stain it with 1% crystal violet solution to confirm the presence of biofilm formation on it. Blue colour appeared on the walls of catheter which confirms the presence of biofilm on it.



Fig 6: Examination of pieces of catheter after biofilm formation.

**Confirmation Subculture:**

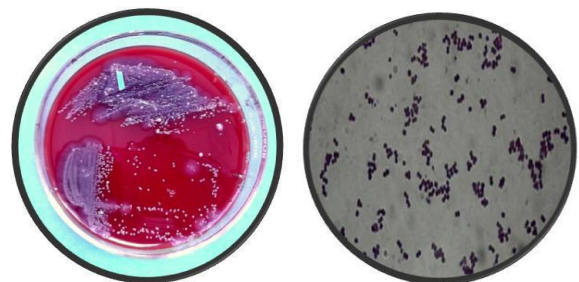


Fig 7: Gram's staining colonies. Gram positive cocci arranged in grapes-like clusters appeared which indicates the presence of *Staphalococcus aureus* species and it was confirmed to *Staphalococcus aureus* by positive coagulase test in which clumping of plasma appeared.

**Conclusion**

The use of bacteriophages to control bacterial infections shows therapeutic promise. The worldwide increase of pathogenic bacteria resistant to antibiotics makes it an imperative to exploit alternative strategies to combat this threat. Phages have increasingly become the subject of renewed interest as agents to treat infections in recent years.

In an era where antibiotic resistance is causing many problems particularly in nosocomial situations, phage and phage-based technologies may prove to be valuable antimicrobial alternatives for widespread applications in the future. So far, the positive growth of biofilm has been observed on different materials i.e on microtiter plates, pieces of glass cover slips and catheters of which 01 Methicillin resistance *S. aureus* (MRSA) strain will be used further for the purpose of bacteriophage isolation.

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