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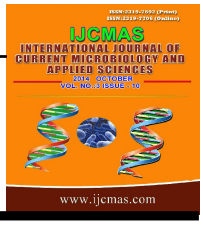


**Original Research Article**

# Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative *Staphylococcus* isolated from Non-clinical Isolates

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**A B S T R A C T**

Biofilms are a group of micro-organisms that attached to a surface and covered by an exopolysaccharide matrix. In recent years, Coagulase Negative Staphylococcus has become increasingly recognized as important agents of nosocomial infections. The Biofilm protects CONS against the action of antibiotics administered for treatment of these infections. There are various methods for detection of biofilm like Tissue Culture Plate Method (TCP), Tube Method (TM),Congo Red Agar Method(CRA). This study was conducted to compare these three methods for Detection of Biofilm produced by Coagulase negative staphylococcus. The study was carried out in Department of Microbiology, IGTAMSU, Arunachal Pradesh from January 2014 to June 2014.A total Number of 100 Non clinical isolates were subjected to Biofilm detection methods. Isolates were identified by standard microbiological methods. Biofilm detection was done by TCP, TM, CRA. The TCP method was considered as best method among the TM and CRA. From the total of 100 samples, TCP Method detected 83 (83%). From our study we can conclude that TCP is most reliable and easy method for detection of biofilm as compared to TM and CRA methods and it can be use as a general screening method for detection of Biofilm producing bacteria.

## Introduction

Coagulase-negative staphylococcus are among the most commonly isolated organism in the clinical microbiology laboratory but their important role as pathogens and their increasing incidence specimens. However, in recent years, CONS have become increasingly recognized as important agents of nosocomial infection. The frequent isolation of CONS from blood, other normally sterile body fluids,

intravenous catheters, peritoneal dialysates, and the various indwelling devices presents a recurring interpretive challenge to both clinical microbiologists and clinicians. If contaminant is mistakenly identified as a pathogen i.e. source of isolate was the skin and not an infective process, the patient will probably receive unneeded antimicrobial therapy. Their role as a significant pathogens following ophthalmologic, neurologic, cardiothoracic surgery, in immune compromised patients and in the patients with prosthetic devices has been established. Besides this we can see now-a-days, the infection of CONS are generally associated with the use of catheter and other medical devices. The capacity of adhere to polymer surfaces and consequent biofilm production are main virulence factor of CONS. The Biofilm protects CONS against the action of antibiotics administered for treatment of these infections and also the against the patient's immune system. In this respect it also becomes necessary to detect the biofilm production capacity along with speciation of CONS. There are various methods to detect biofilm production [5,7,8,9]. These include the Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA) etc.

## Materials and Methods

### Place and duration of the study

The study was conducted at the Department of Microbiology, IGTAMSU,Arunachal Pradesh from January 2014 to June 2014.

### Selection of the isolates:-

A total of 100 consecutive non-repeat clinical and non clinical CONS isolates were collected from January 2014 to June 2014 from students of various Departments of IGTAMSU and processed in Department of Microbiology, IGTAMSU.

### Processing of the Specimens

Isolates were identified by standard microbiological procedures (Gram staining, colonial morphology, slide and tube coagulase test, motility, biochemical tests). Reference strain of positive biofilm producer *Staphylococcus epidermidis* ATCC 35984, *Staphylococcus aureus* ATCC 35556, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus epidermidis* ATCC 12228 (non-slime producer) were used as control.

### Detection of biofilm production:

Early biofilm formation detection might help in treatment because long standing cases they may be very damaging and may produce immune complex squeal. There are three methods for detection of biofilm:

- Tissue Culture plate method
- Tube Method
- Congo Red method

### Tube method

10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopfull of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37$^{o}$c for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH7.3.The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation. Biofilm Production 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 amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong.

**Congo Red Agar Method (CRA)**

The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55°C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; weak producers usually remained pink, though occasional darkening at the centre of colonies was observed.

**Tissue Culture Plate Method**

Overnight culture of the isolate from nutrient agar plate is inoculated into Trypticase soy broth (TSB).

The primary inoculums are then inoculated in TSB with 1% glucose prepared in Different dilutions (1:20, 1:40, 1:80, and 1:100) and loaded into 96 wells flat bottom microtitre plate.

Plates are covered and incubated at 37°c for 24 hours in aerobic condition, the well are then decanted and washed three times with Phosphate buffer saline (PBS).

After washing, fixed with methanol for 15 minutes.

Then the wells are decanted and stained with crystal violet for 20 minutes.

The wells are again decanted and washed with distilled water.

Finally 33% glacial acetic acid is added to the wells to extract the stain and adherence of the stained cells to the wells.

Optical density of each well is measured at 490 nm using an automated ELISA plate reader.

**Calculation for optical density for detection of Biofilm**

Cut off OD=Negative control (3 standard deviation +mean)

Positive control= Average

Sample= Average

Weak biofilm= 0 cut off OD up to 2cut off OD value

Moderate =2 cut off OD up to 4 cut off OD value

Strong biofilm= more than 4 cut off OD value

## Results and Discussion

The total numbers of samples collected were 100 significant CONS isolates.

Out of 100 Samples, the following were:

Blood samples: 32
Urine Sample: 24
Others: 44

Among 100 isolates, TCP, the standard method, detected 36 as strong, 47 as moderate and 17as weak/non biofilm producers. The majority of the organisms associated with biofilm production were *S. epidermidis* (55%) followed by *S.heamolyticus* (21%), *S.sacharolyticus* (10%) *S.saprophyticus* and *S.lugdunensis* (5%). By TM, the number of strong biofilm producers were 21, moderate were 36 and weak or non-biofilm producers were 43. Very different results were observed by the CRA method, with which only five isolates showed black colonies with crystalline appearance.

**Statistical analysis of tissue culture plate, tube and Congo Red Agar methods**

The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods. In the

TCP method, the number of isolates showing biofilm formation was 83 (83%), and non or weak biofilm producers were 17 (17%).Tube method detected 57%isolates as biofilm producers and 43% as non-biofilm producers where CRA only detected 20% as biofilm producer and 80% as non or weak biofilm producer. On the other hand other parameters like sensitivity, specificity, false negative value, false positive value and accuracy were calculated. True positives were biofilm producers by TCP, TM and CRA method. False positive were biofilm producers by TM and CRA method and not by TCP method. False negative were the isolates which were non-biofilm producers by TM and CRA but were producing biofilm by TCP method. True negatives are those which were non biofilm producers by all the three methods.

The ability of Coagulase Negative Staphylococcus to form biofilm helps them to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections.
OLIVEIRA A and CUNHA MLRS [(1)] reported that the ability to produce biofilm is observed in a large group of bacteria, including coagulase-negative staphylococci (CNS) which are the predominant microorganisms of normal skin flora and have been implicated as the causative agents of hospital infections and *Staphylococcus epidermidis* shows mainly, here in our study we have see *S.epidermidis* is major biofilm producer which is 55% .

In the TCP method, the number of isolates showing biofilm formation was 83 (83%) and non or weak biofilm producers were17 (17%). Data from the study of Mathur. T etal**[(8)]** also showed that out of 152 isolates tested, the number of biofilm producers identified by TCP method was 53.9 %, and non-biofilm producers were 46%. As addition of sugar helps in biofilm formation so we have performed the TCP method by addition of 1% glucose in trypticase soy broth and found good result.

Tube method detected 57%isolates as biofilm producers and 43% as non-biofilm producers. If we correlate this method with TCP for identifying strong biofilm producers, but it was difficult to differentiate between moderate, weak and non-biofilm producers due to the change ability in the results detected by different observers. According to the previous studies, TM cannot be suggested as general screening test to identify biofilm producing isolates.[(3,8)]

In another study, Ruzicka et al.[(11)]that out of 147 isolates of S. epidermidis, TM detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates. They showed that TM is better for biofilm detection than CRA [(11).]Here in our study, we found Tube method detected 57%isolates as biofilm producers where CRA only detected 20% as biofilm producer. So we also recommend TM is better than CRA.

Knobloch et al.[(7)] did not recommend the CRA method for biofilm detection in their study. In their study, they found out of 128 isolates of *S. aureus*, CRA detected only 3.8% as biofilm producers as compared to TCP which detected 57.1% as biofilm producing bacteria.21 Here also if we compare the results of CRA method with TCP Method, we found that CRA detected only 20% as biofilm producer where TCP method detected 83% as biofilm producer.

**Table.1**

| No of Isolates | Biofilm Formation | TCP n (%) | TM n(%) | CRA n(%) |
|---|---|---|---|---|
| 100 | High | 36 | 21 | 5 |
| | Moderate | 47 | 36 | 15 |
| | Weak/none | 17 | 43 | 80 |

**Figure.1** Tissue culture plate method

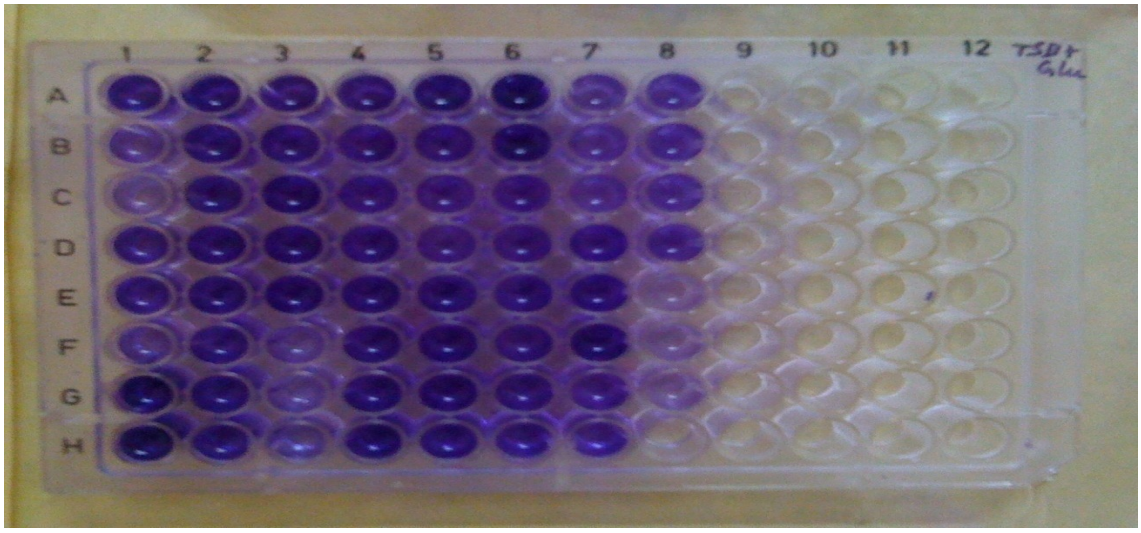
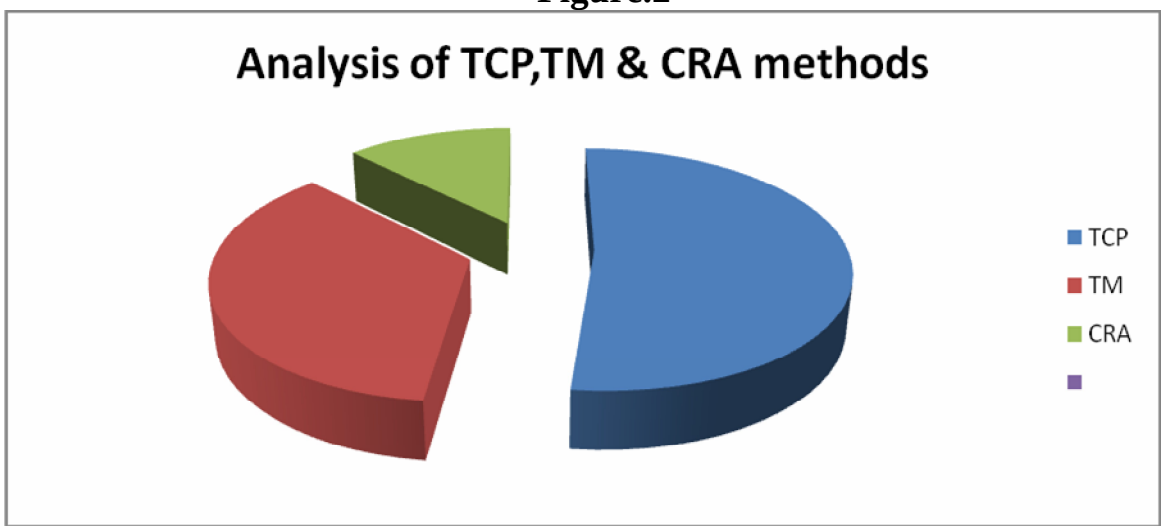



**Figure.2**



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