

Comparison of Standard Tube (ST) method and Congo Red Agar (CRA) method for detection of biofilm production among the isolated species of Coagulase Negative Staphylococci (CONS) in a rural tertiary care hospital

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Abstract

Introduction: CONS has the ability to colonize the surfaces of biomaterials by adhering in biofilm-structured communities of cells encased in a self-produced polymeric matrix known as biofilm which is responsible for resistance to host defense mechanisms and to the antibiotics. In pathogenesis of infections and drug resistance in CONS infections, biofilms play an important role.

Aims and Objectives: To compare Standard Tube (ST) method with Congo Red Agar (CRA) method for detection of biofilm production considering Tissue Culture Method (TCP) as the gold standard method among the isolated species of CONS from clinically significant samples.

Materials and Methods: 120 CONS strains isolated from clinically significant samples were identified by different conventional methods and biofilm production was detected by ST and CRA method considering TCP method, the gold standard method. **Results:** Among 120 CONS isolates, predominant isolated species were *S. epidermidis* 49(40.83%), *S. haemolyticus* 30 (25%). 74(61.67%), 64(53.33%) and 40(33.33%) CONS isolates were positive for biofilm production by TCP, ST and CRA method respectively. Comparison of ST method with TCP method by Pearson chi square test showed strong association between TCP and ST and this distribution is statistically significant with P value of 0.006 but CRA method showed poor association between TCP and CRA method and this distribution is statistically significant with P value of <0.01.

Conclusion: ST method can be used in routine hospital laboratories for detection of biofilm production as it shows reliable results with good sensitivity and specificity as compared to CRA method.

Keywords: Coagulase negative staphylococci (CONS), Congo Red Agar (CRA) method.

Introduction:

CONS are part of the normal skin flora increasingly recognized as significant nosocomial pathogens, particularly bloodstream infections and infections related to the prosthesis [1-2].

They are considered as non-pathogenic [1-2]. Healthy human skin or mucous membrane normally support from 10^1 to 10^6 colony forming units (CFU)/cm² of CONS, depending on the anatomical site. There are around more than 40 recognized species and subspecies of CONS, making them the most prominent microbes inhabiting on the normal skin and mucous membranes [3]. Infrequently, CONS causes primary invasive disease but they are considered as contaminants. Their increasing incidence and clinical importance has been studied in recent years. Increased importance of CONS may be attributed to elderly, morbid patients, increased use of implants and devices and increased number of immunocompromised patients [4].

The important characteristic of CONS is ability to colonize the surfaces of biomaterials by adhering in biofilm-structured communities of cells encased in a self-produced polymeric matrix known as biofilm which is responsible for resistance to host defense mechanisms and to the antibiotics [5]. In pathogenesis of infections and drug resistance in CONS infections, biofilms play an important role [5].

Antibiotic resistance is a global problem and intravenous treatment of systemic infections is required because CONS became increasingly immune to multiple antibiotics. Biofilms play a very important role in the pathogenesis and drug resistance of CONS infections [5].

Because of increasing clinical significance of CONS, accurate species identification of CONS and biofilm production is highly desirable.

Hence this study was undertaken with the subsequent aims and objectives.

Aims and Objectives:

1. To isolate species of CONS strains from clinically significant samples by conventional methods.
2. To study biofilm production by the isolated species of CONS by two different methods i.e Standard Tube method (ST) and Congo Red Agar (CRA) method and to compare these methods for biofilm production.
3. To study comparison of Standard Tube method (ST) and Congo Red Agar (CRA) method with Tissue Culture Plate (TCP) method for detection of biofilm production.

Materials and Methods:

Ethics Committee Approval: After obtaining approval from Institutional Ethics Committee, the study was conducted.

Locus of study: Study was carried out in department of Microbiology of Jawaharlal Nehru Medical College and Acharya Vinoba Bhave Rural Hospital, Sawangi (Meghe), Wardha which is a tertiary care hospital.

Study design: Cross sectional study.

Study duration: The study was conducted from October, 2016 to May, 2018.

Sample size and source of sample: After receiving in department of Microbiology, 120 CONS strains were isolated from clinically significant samples like blood, urine, indwelling catheter, pus (infected bone and joint prosthetic implants, surgical site infections etc.) and body fluids and processed according to conventional methods. All the samples were inoculated on blood agar, MacConkey agar and incubated overnight at 37°C.

The isolates were considered clinically significant when isolated in pure culture from infected sites or body fluids or if the same strain was isolated from repeat samples [6-7].

Various isolates were initially identified by colony morphology, gram staining, catalase and coagulase test (slide and tube method)⁸. Bacitracin (0.04 u) and Furazolidone (100ug) sensitivity were done to exclude Micrococcus and Stomatococcus [8].

Speciation of CONS was done by various conventional methods according to standard procedure [9].

Biofilm production was detected by standard tube (ST) method and congo red agar method (CRA) considering tissue culture plate method (TCP), the gold standard method for biofilm detection [10].

Biofilm production in test strains will be compared with reference strains [10].

- *S.epidermidis* ATCC 35983 (strong slime producer)
- *S. hominis* ATCC 35982 (Non slime producer)

Standard Tube (ST) method [11]:

Standard Tube method is a qualitative method for detection of biofilm production.

Procedure:

In this method, test strains of CONS along with positive and negative control strains were inoculated into 5 ml of Trypticase Soy Broth medium taken in the sterile test tubes and incubated at 37 °C for 48 h. At the end of this period, the tubes were emptied without shaking and by using phosphate buffer saline (pH 7.3) the tubes were washed and then allowed to dry.

The tubes were stained by using 5 – 6 drops of 0.25% saphranine and deionized water was used to remove excess stain. Tubes were kept in inverted position and allowed to dry.

Interpretation:

Biofilm production was determined by the development of a stained coating on the walls and the bottom of the tubes. Strains that developed stains in the form of rings at the air-liquid boundary were excluded.

Tubes were then examined and the amount of biofilm was scored as

- strong (+++)
- moderate (++)
- weak (+)
- absent (0)

Congo red agar (CRA) Method [11]:

Congo red agar method is a qualitative method for detection of biofilm production and medium used was Congo red agar (CRA) medium.

Procedure:

The CRA medium was prepared with brain heart infusion broth 37 g/l, sucrose 50 g/l, agar No 1 10 g/l and Congo red 0.8 g/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was added when the agar had cooled to 55°C. Plates of the medium were inoculated with test strains along with positive and negative control strains and incubated aerobically for 24 hours at 37°C.

Interpretation:

A positive result was indicated by black colonies with dry crystalline consistency.

Strong: Isolates producing black colonies were considered as strong biofilm producers.

Moderate: Dark colonies without dry crystalline colony morphology indicated moderate biofilm production.

Weak: Weak biofilm producers produced dark pink colonies.

Non-slime producers: Non-slime producers mostly turned out as dry red colonies.

Results:

Table 1. Sample wise distribution of CONS (n=120)

Samples	No of CONS isolates (n=120)
Blood	56(46.67%)
Pus	29(24.16%)
Urine	26(21.67%)
Catheter tips	5(4.16%)
Body fluids*	4(3.33%)
Total	120

- **Body fluids include [CSF (n=1), Ascitic fluid (n=1), Pleural fluid (n=2)].**

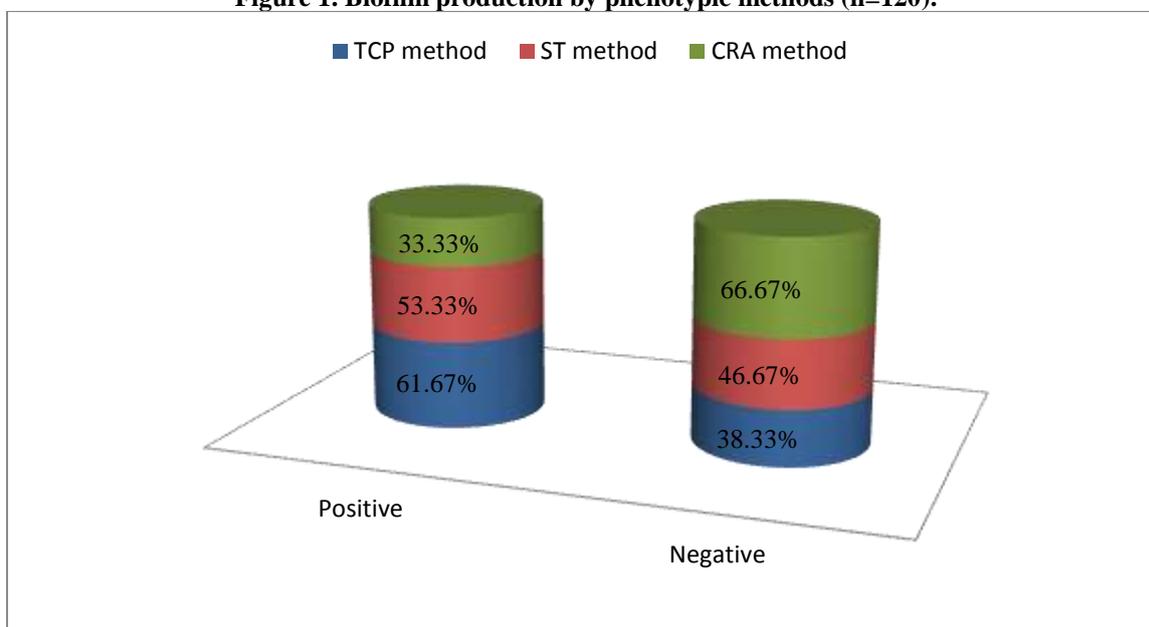
Among 120 CONS isolates, 56(46.67%) isolates were from blood samples, 29(24.16%) isolates from pus samples, 26(21.67%) isolates from urine samples, 5(4.16%) isolates from catheter tip samples and 4(3.33%) isolates from body fluids respectively.

Table 2. Species distribution of CONS isolates (n=120)

Species	No of CONS isolates (n=120)
S.epidermidis	49(40.83%)
S.haemolyticus	30(25%)
S. schleiferi	13(10.83%)
S.lugdunensis	12(10%)
S.saprophyticus	9(7.5%)
S.xylosus	3(2.5%)
S.intermedius	2(1.67%)
S.warneri	1(0.83%)
S. hominis	1(0.83%)

Among 120 CONS isolates from different clinical samples, predominant isolated species were S. epidermidis 49(40.83%), S.haemolyticus 30 (25%), S. schleiferi 13(10.83%) and S.lugdunensis 12(10%).

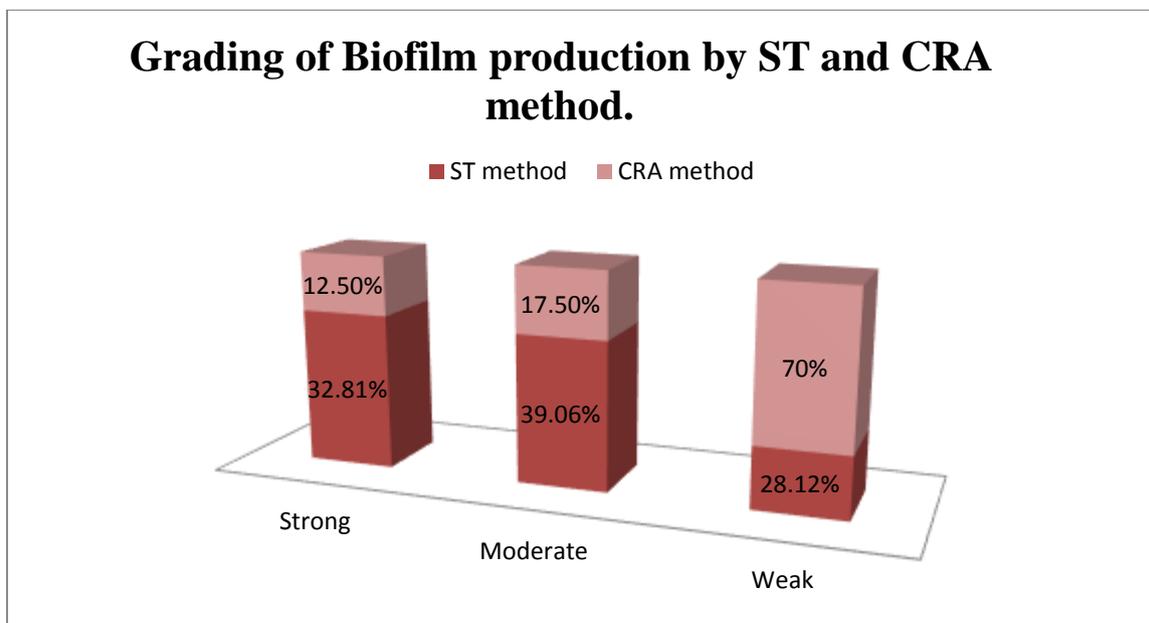
Figure 1. Biofilm production by phenotypic methods (n=120).



Biofilm production was detected by TCP method, ST method and CRA method. Among 120 CONS isolates, 74(61.67%), 64(53.33%) and 40(33.33%) CONS isolates were positive for biofilm production by TCP method, ST method and CRA method respectively.

So, from the table it was observed that TCP method showed higher biofilm production compared to the ST and CRA method. But, among ST and CRA method, ST method showed higher biofilm production compared to the CRA method.

Figure 2. Grading of positive biofilm production by ST and CRA method.



Amongst 64 biofilm producing CONS strains by ST method, 21(32.81%), 25(39.06%) and 18(28.12%) isolates showed strong, moderate and weak biofilm production respectively. Out of 40 biofilm producing CONS strains by CRA method, 5(12.5%), 7(17.5%) and 28(70%) isolates showed strong, moderate and weak biofilm production respectively.

Table 3. Comparison of ST method with TCP method.

ST method		TCP method	
		Positive	Negative
	Positive	63	1
Negative	11	45	

Considering TCP as the gold standard, out of 120 CONS isolates, 63 CONS isolates were positive by both TCP and ST method and 1 CONS isolate was negative by TCP but weak positive by ST method.

Comparison of ST method with TCP method by Pearson chi square test showed strong association between ST and TCP method and this distribution is statistically significant with P value of 0.006.

Considering TCP method as gold standard, sensitivity, specificity, PPV and NPV of ST method were found to be 85.13%, 97.82%, 98.43% and 80.35% respectively.

Analysis of Kappa agreement between TCP and ST method showed good agreement (Kappa=0.8) between TCP and ST method. So, it was observed that ST method is comparable to TCP method for detection of biofilm formation with respect to sensitivity, specificity, PPV and NPV with strong association and Kappa agreement was good agreement.

Table 4. Comparison of CRA method with TCP method.

CRA method		TCP method	
		Positive	Negative
	Positive	39	1
Negative	35	45	

Considering TCP as the gold standard, out of 120 CONS isolates, 39 CONS isolates were positive by both TCP and CRA method and 1 CONS isolate was negative by TCP but positive by CRA method.

Comparison of CRA method with TCP method by Pearson chi square test showed poor association between TCP and CRA method and this distribution is statistically significant with P value of <0.01.

Considering TCP method as gold standard, sensitivity, specificity, PPV and NPV of CRA method were found to be 52.70%, 97.82%, 97.50% and 56.25% respectively.

Analysis of Kappa agreement showed moderate agreement (Kappa=0.59) between the TCP and CRA method (Kappa value= 0.41-0.60=Moderate agreement).

So, it was observed that statistically association of CRA method with TCP method was found to be poor and kappa agreement was moderate agreement. So, CRA method alone cannot be recommended for detection of biofilm formation.

Discussion:

In the present study, 46.67% CONS isolates were from blood samples, 24.16% isolates were from pus samples, 21.67% isolates were from urine samples and 4.16% isolates were from catheter tip samples. This is in accordance to study done by Sadhvi

Parashar et al. [12] where 45.95% CONS isolates were from blood samples, 15.6% isolates were from pus samples and 19.46% isolates were from urine samples.

In the present study, predominant isolated species were *S. epidermidis* 49(40.83%), *S. haemolyticus* 30 (25%), *S. schleiferi* 13(10.83%) and *S. lugdunensis* 12(10%). This is in accordance to study done by Badampudi et al. [7] where predominant isolated species were *S. epidermidis* (40%), *S. haemolyticus* (26%) and *S. schleiferi* (13%). In the study, done by Roopa et al. [13] predominant isolated species were *S. epidermidis* (50.8%), *S. haemolyticus* (26.7%), *S. lugdunensis* (10.7%), *S. schleiferi* (7.1%) and *S. saprophyticus* (4.46%).

In present study, 53.33% and 33.33% CONS isolates were positive for biofilm production by ST method and CRA method respectively. This is in accordance with study done by Saumya Singh et al. [14] where 41.56% and 28.57% CONS isolates were positive for biofilm production by ST method and CRA method respectively.

In present study, 61.67% CONS isolates were positive for production of biofilm production by TCP method. This finding correlates with a study done by Devapriya F et al. [15] where 64.4 % CONS isolates were positive for production of biofilm production by TCP method.

In present study, 32.81% and 12.5% of CONS showed strong biofilm production by ST method and CRA method respectively. This finding correlates with a study done by Deka N et al. [16] where 21% and 5% of CONS showed strong biofilm production by ST method and CRA method respectively.

In present study, 39.06% and 17.5% of CONS showed moderate biofilm production by ST method and CRA method respectively. This is in accordance with study done by Deka N et al. [16] 36% and 15% of CONS showed moderate biofilm production by ST method and CRA method respectively.

Statistical analysis: Considering TCP method as the gold standard method for biofilm detection and when we compared ST method and CRA method with TCP method, ST method showed strong association with TCP method and kappa value was 0.8 (good agreement). Whereas, CRA method showed poor association with TCP method and kappa value was 0.59(moderate agreement). The sensitivity, specificity, PPV and NPV of ST and CRA method were found to be 86.06%, 98.71%, 99.05%, 81.91% and 54.09%, 98.71%, 98.50% and 57.89% respectively. So, ST method is comparable to TCP method but CRA method alone cannot be considered for biofilm detection by CONS. This observation correlates with study done by Mathur et al. [17] and Thilakavathy et al. [18] where they also recommended that CRA method alone cannot be used for detection of biofilm production.

Although, CRA method is a simple screening, rapid and easy to perform method for biofilm production but because of these variations of sensitivity, specificity, PPV and NPV in between ST and CRA method, CRA method alone cannot be used for detection of biofilm production. The reason behind these variations of sensitivity, specificity, PPV and NPV of ST and CRA method can be due to subjective errors during interpretation [19].

Conclusion:

This study suggests increasing pathogenic potential of CONS along with slime producing capacity that necessitates the need to adopt simple conventional laboratory methods to identify CONS species and to detect slime production which helps in understanding definitive therapy for CONS isolates from various clinical samples.

In present study, biofilm production was detected by 3 different phenotypic methods such as TCP method (61.67%), ST (53.33%) and CRA (33.33%) method and detection of biofilm production by TCP method was higher compared to the other phenotypic methods. For detection of biofilm production, TCP method is the gold standard method and when comparison of ST and CRA method with TCP method was done, it was found that ST method is comparable to TCP method but CRA method is not comparable to TCP method by CONS as ST method shows good sensitivity and specificity compared to the CRA method and ST method is also economic and easy to perform in hospital laboratories.

At last from this study, it can be inferred that ST method can be used in routine laboratories for detection of biofilm production where as CRA method alone cannot be used in routine laboratories for detection of biofilm production.

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