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*Research Article*

# ANTIBIOTIC RESISTANCE PROFILE OF BIOFILM PRODUCING STAPHYLOCOCCI ISOLATED FROM DIFFERENT CLINICAL SAMPLES

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## ABSTRACT

Biofilm mediated infections by Staphylococci have a significant negative impact on patient health and necessitate reliable methods for detecting biofilm producers. The ability of isolates to produce biofilm makes them resistant to host immune response as well as available antibiotics. This study aims to detect biofilm producing ability among clinical staphylococci by phenotypic methods and presence of *icaAD* genes and determine their antibiotic profile. A total of 350 different clinical specimens received in the KIST Medical hospital, Gwarko, Nepal was examined from July 2018 to January 2019 and Staphylococci were identified following standard microbiological procedure. The antibiotic resistivity pattern was detected by Kirby Bauer disc diffusion method whereas biofilm formation was detected by three phenotypic methods viz. congo red agar (CRA), tube method (TM) and tissue culture plate (TCP) method. Furthermore, *icaAD* genes were detected by PCR method. Chi-square test and independent sample t-test were employed to calculate the significance. A total of 161 Staphylococci were isolated comprising *S. aureus* (63, 39.1%) and coagulase negative staphylococci (CNS) (98, 60.9%). Most isolates were found to be resistant to penicillin and erythromycin. Strong biofilm formation was detected among 6 (3.7%), 22 (13.7%) and 35 (21.7%) by CRA, TM and TCP method respectively. Similarly, *icaAD* genes were detected among 24 (14.9%) isolates. Staphylococci isolated from clinical sample showed biofilm forming ability by both phenotypic and genotypic method. The biofilm producing isolates were found to be more resistant to antibiotics than their planktonic counterparts with 56(89%) and 65 (66%) *S. aureus* and CNS observed as methicillin resistant respectively. Regular surveillance of biofilm formation by Staphylococci and their antimicrobial resistance profile may lead to early treatment of Staphylococcal infection.

**Keywords:** Antibiotic resistance, biofilm, *ica* gene, staphylococci

## INTRODUCTION

The emergence of antibiotic resistance poses an ever-growing challenge, significantly impacting the ability to combat infectious diseases effectively. Staphylococci, a diverse group of bacteria, have been at the forefront of this global concern as they are frequently implicated in a wide range of infections, both in health care and community settings (Becker *et al.*, 2014). Based on their ability to clot blood plasma with the production of enzyme coagulase, they are classified as coagulase positive Staphylococci (CPS) and coagulase negative Staphylococci (CNS). The most prevalent CPS in human infections are *Staphylococcus aureus* and CNS is *Staphylococcus epidermidis* (Zhang *et al.*, 2018).

*S. aureus* can cause a variety of diseases from uncomplicated skin infections like pyoderma to severe forms of bacteremia, osteomyelitis and endocarditis even leading to toxic shock syndrome (Moormeier *et al.*, 2014). CNS, being the normal commensal of skin and anterior nares, have long been considered as non-pathogenic and rarely reported to cause severe infection. However, as a result of the increased use of intravascular devices in the medical treatment as well as increase in hospitalized immune compromised patients, CNS have emerged as a major cause of nosocomial infection with *S. epidermidis* responsible for the majority of such infections (Zhang *et al.*, 2018).

The clinical importance of Staphylococci is attributed to its high virulence due to surface proteins, toxins and enzymes and its rapid development of drug resistance (Arvidson & Tegmark, 2001). In addition to antibiotic resistance, its ability to produce biofilm is another important complicating factor (O'Gara, 2007). Bacterial biofilms, which are micro-colonies encased in extracellular polysaccharide material, mediated by gene products of the *icaADBC* operon, are the sources of many bacterial

infections which hardly respond to routine treatments (Arciola *et al*., 2015). The *icaADBC* encodes four genes including *icaA, icaB, icaC*, and *icaD*, which collectively produce PIA, facilitate the cells binding together and forming into biofilms (Ghasemian *et al*., 2015).

The formation of biofilms not only facilitates bacterial colonization of a host, but also provides resistant to antibiotics and the host immune system. In fact, biofilms can resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free-floating bacteria (Piechota *et al*., 2018). Biofilm can also serve as foci of infection for metastatic spread of bacteria. Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in Staphylococci infections in clinical settings (Omidi *et al*., 2020).

Due to the emergence of resistant pathogen with the ability of biofilm formation, it makes a requisite to know the prevailing antibiotic susceptibility pattern of such isolates. However, the etiology of such infections is poorly characterized in Nepal, mainly due to limited laboratory resources, poor recording systems and an inadequate number of trained personnel. The aim of this study is to investigate the incidence of Staphylococcal infection from different clinical specimens and evaluate the antibiotic susceptibility pattern of the isolates.

## MATERIALS AND METHODS

### Collection and Identification of bacterial isolates

The hospital based cross sectional descriptive study was conducted at KIST Medical College and Teaching Hospital, Imadol, Lalitpur, Nepal from July 2018 to January 2019. Ethical approval was obtained from Institutional Review Committee (IRC) of KIST hospital (IRC No. 0069/2016/017). The sample size was 348 according to prevalence rate of 65.38% biofilm producing coagulase negative staphylococci (Shrestha *et al*., 2017). The sample size is determined by using Fisher's formula:

Sample size (N)= $Z^2$ X $pq/e^2$ where $p$= prevalence and $e$= allowable error
Thus, N=347.8 so we took 350 samples.

Different specimens such as catheter tips, central venous catheter (CVC), drain tip, suction tip, DJ stenting, endotracheal tube, urine, wound/pus, blood etc were processed by standard microbiological technique as described by Cheesebrough (2000). The isolates were identified as Staphylococci following Gram staining and different biochemical tests. Coagulase enzyme production by slide and tube method and DNase production were used to confirm the isolates as *S. aureus*. The species of CNS were identified based on simplified scheme proposed by Cunha *et al*. (2004) (Table 1).

**Table 1. Interpretative criteria for identification of coagulase negative Staphylococci.**

| Result with biochemical tests | | | | | | | | | | | Identification |
|---|---|---|---|---|---|---|---|---|---|---|---|
| URE | PYR | TCO | SCO | FOS | NOV | POL | TRE | MAN | XYL | ACE | |
| + | - | - | - | + | S | R | _ | + | - | + | *S. epidermidis* |
| + | - | - | - | - | R | S | + | - | - | + | *S. saprophyticus* |
| - | + | - | - | - | S | S | + | - | - | + | *S. haemolyticus* |
| + | - | - | - | - | S | S | - | - | - | - | *S. hominis* |
| - | - | - | - | - | S | S | - | + | - | - | *S. capitis* |

URE=urease, PYR=pyrrolidonyl arylamidase, TCO=tube coagulase, SCO=slide coagulase, FOS=alkaline phosphatase, NOV=novobiocin susceptibility, POL=polymixin B susceptibility, TRE=D trehalose, MAN=D-mannose, XYL=D-xylose, ACE=acetoin, +=positive, -=negative, R=resistant, S=susceptible

### Antibiotic susceptibility test

The antibiotic susceptibility test (AST) was performed towards various antibiotics by the modified Kirby Bauer disk diffusion method within the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2018). The isolates were tested against the clinically relevant antibiotics (HiMedia, India) as penicillin-G (10 units), cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg) and cotrimoxazole (25 µg). Cefoxitin disk was used to detect methicillin resistance. *S. aureus* ATCC 25923 was used as control strain in each AST assay along with test isolates.

### Assessment of biofilm formation

Three phenotypic methods i.e., Congo Red Agar method (CRA), Tube method (TM) and Tissue Culture Plate Method (TCP) and polymerase chain reaction (PCR) for the detection of *ica* genes were used for detection of biofilm formation ability of isolates. All tests were performed using *Staphylococcus epidermidis* ATCC 35984 as positive control and repeated three times.

CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10g/L and Congo Red indicator 8 g/L. CRA plates were inoculated with test organisms and incubated at 37ºC for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production (Freeman *et al*., 1989; Manandhar *et al*., 2018).

In TM method, a loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose. After incubation at 37ºC for 24 h, tubes were decanted, washed with phosphate buffer saline (pH 7.3) and stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position.

Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1 (weak/none), 2 (moderate) and 3 (high/strong) (Christensen *et al.*, 1985; Manandhar *et al.*, 2018).

In TCP Method, test organisms were inoculated in 10 mL of Brain Heart Infusion (BHI) broth supplemented with 2% sucrose and incubated at 37ºC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture plates were filled with 200 µL of the diluted cultures. After incubation at 37ºC for 24 h, contents of each well were removed by gentle tapping and washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water. Optical density (OD) of stained adherent biofilm was obtained by using micro-ELISA auto reader at wavelength 570 nm. The OD value of < 0.12, 0.12 - 0.24 and > 0.24 were considered as weak, moderate and strong biofilm producers respectively (Christensen *et al.*, 1985; Manandhar *et al.*, 2018).

The genomic DNA was extracted using the DNA extraction Kit following the manufacturer instructions (Thermo Fischer). The primer used for the detection of *icaA* was forward 5'-TCTCTTGCAGGAGCAATCAA and reverse 5'-TCAGGCACTAACATCCAGCA generating a product size of 188-bp. Similarly, for detection of *icaD*, forward and reverse primer used were 5'-ATGGTCAAGCCCAGACAGAG and 5'-CGTGTTTTCAACATTTAATGCAA, respectively, with the product size of 198 bp. The PCR product was analyzed in 2% agarose gel stained with SYBR safe (Invitrogen) dye (Manandhar *et al.*, 2018).

**Data analysis**

The statistical analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, United States) software. Chi-square test was used to compare between groups of clinical isolates and $p$ values < 0.05 were considered statistically significant.

## RESULTS

Among 4063 samples analyzed, 654 showed significant growth where 161 were identified as Staphylococci. Five species were identified among all CNS isolates including *S. epidermidis* (59.2%); the most frequently isolated species followed by *S. saprophyticus (*19.4%), *S. haemolyticus* (9.2%), *S. homonis* (8.2%) and *S. capitis* (4.1%). Among 161 Staphylococcal isolates, *S. aureus* was isolated in high number from W/P (47, 29.2%) whereas CNS from blood (54, 33.5%) (Fig. 1).

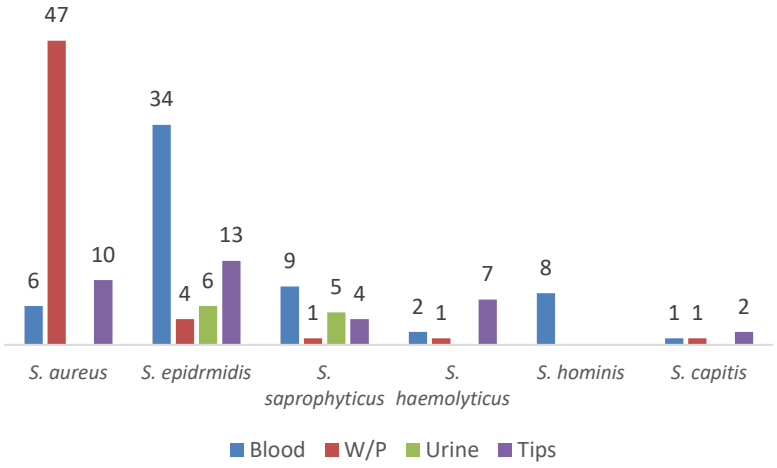


**Figure 1. Distribution of Staphylococci in different clinical specimen (w/p=wound/pus, tips=catheter tips, suction tips, drain tips, DJ stenting tips, transtracheal tips, central venous catheter).**

**Antibiotic susceptibility profile of isolates**

*S. aureus* was found to be sensitive towards commonly used antibiotics such as tetracycline (100%), chloramphenicol (98.4%) and clindamycin (87.3%) but resistant towards penicillin (95.2%) and erythromycin (93.6%). Similarly, CNS were also found to be resistant towards penicillin (93.9%) and erythromycin (75.5%) and sensitive towards chloramphenicol (92.9%), tetracycline (86.7%) and clindamycin (72.4%) As indicated by cefoxitin disc diffusion assay, 56 (89%) of *S. aureus* were methicillin resistant and 65 (66%) were methicillin resistant CNS (Table 2).

**Table 2. Antibiotic resistant pattern of Staphylococci.**

| Antibiotics | Antibiotic class | Potency (µg/disc) | Resistant cases | | Total (n=161) |
|---|---|---|---|---|---|
| | | | *S. aureus* (n=63) | CNS (n=98) | |
| Penicillin | β lactams | 10 units | 60 (95.2%) | 92 (93.9%) | 152 (94.4%) |
| Ciprofloxacin | Fluroquinolone | 5 | 41 (65.1%) | 31 (31.6%) | 72 (44.7%) |
| Tetracycline | Tetracycline | 30 | 0 | 13 (13.3%) | 13 (8.1%) |
| Clindamycin | Lincosamide | 2 | 8 (12.7%) | 27 (27.5%) | 35 (21.7%) |
| Chloramphenicol | Phenicols | 30 | 1 (1.6%) | 7 (7.1%) | 8 (5%) |
| Cefoxitin | β lactams | 30 | 56 (88.9%) | 65 (66.3%) | 121 (75.2%) |
| Erythromycin | Macrolides | 15 | 59 (93.6%) | 74 (75.5%) | 133 (82.6%) |
| Cotrimoxazole | Folic acid synthesis inhibitors | 1.25/23.75 | 34 (54.0%) | 37 (37.7%) | 71 (44.1%) |
| Gentamycin | Aminoglycosides | 10 | 14 (22.2%) | 13 (13.3%) | 27 (16.8%) |

**Detection of biofilm formation among Staphylococci**

Among all the Staphylococci isolates, black colonies were produced by 6 (3.7%) isolates in CRA while 16 (10%) isolates were moderate biofilm producers. The remaining 139 (86.3%) isolates were found to be biofilm non-producers whose colony color was pink to red. Strong biofilm production was observed only among CNS.

By TM method, the biofilm production was observed among 6 (3.7%) *S. aureus* and 9 (5.6%) CNS. The TCP method detected 5(3.1%) biofilm producers among *S. aureus* and 14 (8.7%) among CNS. In total of 161 isolates, 24 (14.9%) isolates were found to possess both *icaA* and *icaD* genes comprising 6 (3.7%) *S. aureus* and 18 (11.2%) CNS isolates (Table 3).

**Table 3. Detection of biofilm formation among Staphylococci by different phenotypic and genotypic methods.**

| Method | Biofilm formation | No. of isolates (n) | | Total (n=161) |
|---|---|---|---|---|
| | | *S. aureus* (n=63) | CNS (n=98) | |
| CRA method | Strong | 0 | 6 (6.1%) | 6 (3.7%) |
| | Moderate | 1 (1.6%) | 15 (15.3%) | 16 (9.9%) |
| | Weak/Non | 62 (98.4%) | 77 (78.6%) | 139 (86.3%) |
| TM method | Strong | 3 (4.8%) | 19 (19.4%) | 22 (13.7%) |
| | Moderate | 8 (12.7%) | 8 (8.2%) | 16 (9.9%) |
| | Weak/Non | 52 (82.5%) | 71 (72.4%) | 123 (76.4%) |
| TCP method | Strong | 21 (33.3%) | 14 (14.3%) | 35 (21.7%) |
| | Moderate | 14 (22.2%) | 28 (28.6%) | 42 (26.1%) |
| | Weak/Non | 28 (44.4%) | 56 (57.1%) | 84 (52.2%) |
| Detection of *ica* gene | Presence | 6 (9.5%) | 18 (18.4%) | 24 (14.9%) |
| | Absence | 57 (90.5%) | 80 (81.6%) | 137 (85.1%) |

**Methicillin resistivity among *ica* positive isolates**

In total of 161 isolates, 24 (14.9%) isolates were found to possess both *icaA* and *icaD* genes comprising 6 (3.7%) *S. aureus* and 18 (11.2%) CNS isolates. None of the genes were identified in 137 (85.1%) isolates. The *ica* genes were harbored by methicillin resistant than methicillin sensitive isolates of both *S. aureus* and CNS (Table 4).

**Table 4. Presence of *ica* gene among Staphylococci.**

| *ica* genes | MRSA | MSSA | *p* value | MRCNS | MSCNS | *p* value | Total |
|---|---|---|---|---|---|---|---|
| Presence | 4 (6.3%) | 2 (3.2%) | 0.069 | 14 (14.3%) | 4 (4.1%) | 0.255 | 24 (14.9%) |
| Absence | 52 (82.5%) | 5 (7.9%) | | 51 (52.0%) | 29 (29.6%) | | 137 (85.1%) |

**Evaluation of different methods for the detection of biofilm production**

When different methods for the detection of biofilm formation were analyzed, it was found that TM method is statistically significant when compared with presence of *ica* genes whereas other two phenotypic methods were statistically insignificant (Table 5).

**Table 5. Screening of biofilm formation by different methods.**

| Biofilm formation | CRA | TM | TCP | *ica* genes |
|---|---|---|---|---|
| High | 6 (3.7%) | 22 (13.7%) | 35 (21.7%) | 24 (14.9%) |
| Moderate | 16 (10.0%) | 16 (9.9%) | 42 (26.1%) | |
| Weak/non | 139 (86.3%) | 123 (76.4%) | 84 (52.2%) | 137 (85.1%) |
| *p* value | 0.268 | 0.000 | 0.272 | |

**Antibiotic resistant pattern among biofilm positive isolates**

The biofilm positive isolates as detected by TM and TCP method were found to be resistant to penicillin (90% & 94%) and erythromycin (71% & 82%) respectively. Similarly, those isolates which possess *icaAD* genes were also resistant to penicillin (100%) and erythromycin (83%) (Table 6).

**Table 6. Antibiotic resistivity and biofilm formation by different methods.**

| Antibiotics | Biofilm detection methods | | |
|---|---|---|---|
| | TM method (n=38) | TCP method (n=77) | *icaAD* genes (n=24) |
| Penicillin | 34 (89.5%) | 72 (93.5%) | 24 (100%) |
| Ciprofloxacin | 20 (52.6%) | 36(46.8%) | 10(41.7%) |
| Tetracycline | 4 (10.5%) | 4 (5.2%) | 1 (4.2%) |
| Clindamycin | 8 (21.1%) | 15 (19.5%) | 5 (20.8%) |
| Chloramphenicol | 1 (2.6%) | 3 (3.9%) | 1 (4.2%) |
| Cefoxitin | 25 (65.8%) | 58(75.3%) | 18(75%) |
| Erythromycin | 27(71.1%) | 63(81.8%) | 20 (83.3%) |
| Cotrimoxazole | 12 (31.6%) | 36(46.8%) | 10 (41.7%) |
| Gentamycin | 3 (7.9%) | 10 (13%) | 1 (4.2%) |

## DISCUSSION

A total of 161 clinically significant Staphylococci were studied. More than half of the isolates were CNS (98, 61%) as compared to *S. aureus* (63, 39%). The result is in harmony with the study done by Gad *et al.* (2009); Warren and Peter (2009).

Among the isolates, five different species of coagulase-negative staphylococci were encountered: *S. epidermidis* (58, 59%), *S. saprophyticus* (19, 19%), *S. haemolyticus* (9, 9%), *S. hominis* (8, 8%) and *S. capitis* (4, 4%). The findings of the present study are in agreement with the various studies which show *S. epidermidis* as the most common CNS (McCann *et al.*, 2008; Oliveira and Cunha 2010). Staphylococci are commensal of skin and commonly gain access to site of skin puncture and deep cuts which most time cause uncomplicated infections but at times may develop into complicated infections leading to systemic failure (Mendoza-Olazarán *et al.*, 2015).

Staphylococci are commensals as well as pathogens of human beings and because of their versatile nature they were isolated from different clinical samples. Out of 161 Staphylococci, the highest number of CNS were isolated from blood 54 (33.5%) and *S. aureus* from W/P 47(29.2%). Increased antibiotic resistance, in addition to the increased frequency of invasive surgery, use of intra vascular devices, and increased number of patients with immune compromised status because of HIV infection or immunosuppression after transplantation or cancer treatment, has led to sharp increases in the incidence of *S. aureus* bacteremia and *S. aureus* infective endocarditis and is associated with significant mortality and morbidity (Mahajan *et al.*, 2007). Bloodstream infection with *S. aureus* is associated with mortality rate of about 30% and the incidence is increasing (Khan *et al.*, 2014).

The emergence of antimicrobial resistance among Staphylococci isolates is one of the important factors in nosocomial infection. Both *S. aureus* and CNS were found to be resistant to penicillin 60 (95.2%) and 92 (93.9%) followed by erythromycin 59 (93.6%) and 74 (75.5%) respectively. Fortunately, the *S. aureus* and CNS were found to be susceptible to common antibiotics such as tetracycline (100%), 85 (86.7%) and chloramphenicol 62 (98.4%) and 91 (92.9%), respectively.

*S. aureus* infections are very common, and MRSA continues to be a serious and dreadful challenge as their prevalence is reported to be increasing exponentially. The present study reported MRSA 56(34.8%), MSSA 7(4.3%), MRCNS 65(40.4%) and MSCNS 33(20.5%) among 161 Staphylococci. The prevalence of MRSA is 47.05% (48) lower than the result reported from south India (John and Murugan, 2014). In studies carried out in similar settings in Nepal, 75.6%, 69.1% and 54.9% MRSA were reported, higher than present study (Rijal *et al.*, 2008; Sharma *et al.*, 2009). The difference in prevalence of MRSA may be because of the factors like healthcare facilities available in the particular hospital and rationale antibiotics usage which

varies among hospitals in different parts of the world. The important reservoirs of MRSA in hospitals/institutions are infected or colonized patients and transient hand carriage is the predominant mode of transmission from patient to patient. But the considerable increase in the prevalence of MRSA has been observed globally (Rijal *et al*., 2008). Likewise, prevalence of MRCNS is (12) 25% which was in accordance with other studies (Cabrera-Contreras *et al*., 2013; Shrestha *et al*., 2017) but opposed with the findings of others (Seng *et al*., 2017). Similarly, prevalence of MRCNS ranging from 48.2% to 60% has been reported in India (John and Murugan, 2014) which was comparatively higher than our study.

Both phenotypic and genotypic methods were used to analyze the ability of biofilm production in all isolates. Investigation of biofilm by CRA showed 22 (13.7%) staphylococcal isolates positive for the slime production. Among CRA positive, only 6 isolates formed black colonies representing the strong biofilm production. Variable results were obtained from various research (McCann *et al*., 2008; Gad *et al*., 2009; Oliveira and Cunha, 2010). Slime formation is not always indicative of biofilm formation *in vivo* as highlighted by Arciola *et al.* (2011) and Mathur *et al.* (2013). The consistency and color of the colony developed depends on strains of bacteria, nutrient composition, origin of specimen, physiology of isolates as well as incubation time.

Investigation of biofilm production by the tube method showed 24 (14.9%) isolates as strong biofilm producers, 16 (9.9%) moderate and 121 (75.2%) weak/non-biofilm producers. This result is comparable with Mathur *et al.* (2013) (11.8%) but the data is less than that observed by other researchers (Hassan *et al*., 2011; Cue *et al*., 2012). The result of tube method is based on visual observation of adherent on the wall of tube. So, it is difficult to discriminate between weak and biofilm negative isolates due to the variability in observed result by different observers.

The TCP method detected 35 (21.7%) strong and 84 (52.2%) weak biofilm producers. The TCP method is a convenient and quantitative technique that directly detects the polysaccharide production by measuring the adherent biofilm by spectrophotometer. TCP is the most widely used and was considered as standard test for the detection of biofilm formation (Oliveira and Cunha, 2010; Hassan *et al*., 2011). This method has been reported to be the most sensitive, accurate and reproducible screening method for the determination of biofilm production by clinical isolates of Staphylococci and has the advantage of being a quantitative tool for comparing the adherence of different strains (Christensen *et al*., 1985; Hassan *et al*., 2011).

Previous studies have shown the presence of *ica* locus in clinical isolates emphasizing their increased virulence as compared to the saprophytic strains (Los *et al*., 2010). Besides, plethora of studies has demonstrated the causal link between staphylococcal biofilm and the presence of *ica* operon (*icaADBC* genes) (Los *et al*., 2010; Mathur *et al*., 2013), which in turn are involved in the PIA production; the most extensively characterized staphylococcal biofilm component. In *ica* operon, mainly co-expression of *icaA* and *icaD* has been demonstrated to be necessary for phenotypic expression of biofilm production in clinical staphylococcal isolates (McCann *et al*., 2008; Los *et al*., 2010). Besides, being reliable yet efficient, PCR of *ica* genes has been extensively used for the detection of biofilm formation (Gad *et al*., 2009; Oliveira and Cunha, 2010; Los *et al*., 2010). In the present study, concomitant presence of *icaA* and *icaD* genes was detected in 24 (14.9%) staphylococcal isolates comprising of 6 (3.7%) *S. aureus* and 18 (11.2%) CNS isolates. Previous studies have also demonstrated the presence of *ica* genes in clinical staphylococcal isolates. Los *et al.* (2010) showed the prevalence of *ica* operon in 27.4% nasopharyngeal *S. epidermidis* isolates from hospitalized patients. Oliveira & Cunha (2010) detected *ica* genes in 40% CNS isolated from clinical specimen and nares of healthy individuals. Likewise, Cafiso *et al.* (2004) found 35% of the isolates positive for *icaA* and *icaD* genes, Silva *et al.* (2002) showed 40% staphylococcal isolates positive for *ica* genes respectively. Altogether, these results indicate the importance of *ica* genes in biofilm production in device associated infections.

This low rate of *ica* detection as compared to the previous studies (Silva *et al*., 2002; Cafiso *et al*., 2004; McCann *et al*., 2008; Mathur *et al*., 2013) may be due to difference in *in-vivo* and *in-vitro* conditions possibly contributing to the physiological changes of the pathogen modulating biofilm formation capabilities. For instance, *ica* genes are expressed in stressful environments such as high osmolarity, anaerobic condition, high temperature, and sub-inhibitory presence of some antibiotics (Mathur *et al*., 2013; Mirani *et al*., 2013). Studies have also demonstrated biofilm formation via PIA-independent mechanisms in *S. aureus* (Silva *et al*., 2002). Likewise, biofilm-associated protein (Bap), the first gene known to form biofilm via *icaADBC* independent in *S. aureus* from bovine mastitis isolates. Although initially, it appeared to be absent in human clinical *S. aureus* isolates, Bap protein has now emerged associated with more than 100 surface proteins that are involved in biofilm formation (Los *et al*., 2010). However, given the undeniable role of *icaADBC* in biofilm matrix formation and that PCR enables rapid diagnosis of slime producing virulent strains assays; implementation of genotypic measure is strongly suggested in routine diagnostic laboratory. We reason many factors as environment, nutrition, sub inhibitory concentration of certain antibiotics, and stress (temperature, osmolarity) might play a significant role in biofilm formation resulting

in varied frequency of biofilm producers among clinical isolates (Mahajan *et al*., 2007; Aricola *et al*., 2011).

In consistency with previous studies, CRA and TCP method correlated well in positive results (Silva *et al*., 2002; McCann *et al*., 2008; Hassan *et al*., 2011). However, evidence of false negative results in CRA method while comparing with TCP method suggest that CRA method alone cannot be solely depended upon for the precise detection of biofilm formation. Taken together, in this study, the modified TM method showed the best correlated result with genotypic assay suggesting its importance in routine diagnostic laboratories. Oliveira & Cunha (2010) also reported good sensitivity and specificity for the tube test and PCR when analyzing isolates obtained from infection. According to Cunha *et al.* (2006), the test provides reliable results for biofilm detection in CNS and is adequate for routine use.

Due to resource limitation, the molecular characterization of resistance determinants, clonal lineages or genetic determinants of biofilm-production was not performed which would improve our understanding of these processes.

## CONCLUSIONS

The study showed a significant association between phenotypic production of biofilm and presence of *ica* genes. Taken together, this study demonstrates the high prevalence of methicillin resistant isolates producing biofilms in clinical staphylococcal samples. Since staphylococcal infections have a significant impact on morbidity and mortality, prevention and management of these infections remain a priority. This study, while bringing additional information about the status of biofilm producing clinical isolates and their association with multiple antibiotic resistances, highlights the importance of early detection strategies in routine diagnostics. Therefore, we recommend regular surveillance of biofilm formation in clinical Staphylococcal isolates and their antimicrobial resistance profiles. Implementation of those will help to identify biofilm producing cases to prevent occurrence of treatment failures of staphylococcal infections in Nepal.

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## AUTHOR CONTRIBUTIONS

RST and SM conceived the study. RST carried out experiments. RJ, SL and SM analyzed data. RST, SM, SL and MC drafted the manuscript and all authors contributed to preparing the final version of the manuscript. All authors read and approved the final manuscript.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interests.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

## ETHICAL STATEMENT

The ethical clearance and consent to participate was approved by Institutional Review Committee (IRC) of the hospital. The consent to participate was not required as all the investigated isolates were taken as part of standard routine diagnostic purposes.

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