Multi Drug Resistance and Biofilm Production among *Staphylococcus* Isolates from Clinical Samples at a Tertiary Care Hospital in Kathmandu, Nepal

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ABSTRACT

Objectives: The purpose of the study was to determine multidrug resistance and biofilm production among *Staphylococcus* isolates from clinical samples at Nepal Armed Police Force Hospital, Kathmandu.

Methods: A hospital based cross sectional prospective study was conducted at Department of Microbiology, Nepal Armed Police Force Hospital, Balambu, Kathmandu from February to July 2022. A total of 1813 clinical samples were collected and processed in microbiology laboratory. Through cultural and biochemical analysis, *Staphylococcus spp*. were isolated and identified, and the Kirby-Bauer disc diffusion method was used to determine multidrug resistant among isolates. While the Tissue Culture Plate method was employed to detect *Staphylococcus spp.* that produce biofilm.

Results: Out of 1813 clinical samples, 304 (16.7%) exhibited bacterial growth; of these, 135 (44.4%) were *Staphylococcus spp*, among which 119 (88.1%) were *S. aureus*, 16 (11.8%) were coagulase negative Staphylococci (CONS) and 114 (84.4%) were MDR. Eighty-one (68.1%) of the 119 *S. aureus* isolates were methicillin resistant, and 11 (68.8%) of the 16 CONS were also resistant to the methicillin. Eighteen (13.3%) of the 135 *Staphylococcus spp*. were detected to be strong biofilm producers, 30 (22.2%) moderate, and 87 (64.4%) non or weak biofilm producers. Both biofilm producers and biofilm non-producers were found to be resistant against Ampicillin and Azithromycin.

Conclusion: The study reveals higher percentage of MDR among *Staphylococcus spp*, indicating the need to discourage self-medication, insufficient or over – medication. Moreover, the high rates of biofilm development in MDR *Staphylococcus spp* and MRSA underscores the need for monitoring of biofilm producers

Keywords: *Staphylococcus spp, Antimicrobial resistance, Multi Drug Resistance, Biofilm producers, Nepal*

INTRODUCTION

Staphylococci are a genus of extensively dispersed, gram-positive cocci that live in the mouth, colon, genito-urinary tract, upper respiratory tract, skin, skin glands, and mucous membranes, especially the nasal cavities. However, they are still capable of exhibiting pathogenic activity since they can enter many bodily areas through ruptured skin and mucus, which can lead to opportunistic infections (Bannerman 2004).

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The well-known human opportunistic pathogen *S. aureus* (Liu 2009), which is listed in the group of the "ESKAPE pathogens" (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species *specificall*) (Boucher et al 2009) responsible for a variety of infections (Otto 2013), ranges from toxinoses to systemic infections, including superficial lesions and wound infections (Bien et al 2011 and Aires et al 2004).

Similarly, despite of benign interaction of CONS with the host, they are now recognized to cause serious infections, especially in immune-compromised people, and they have a role in a variety of diseases that affect deep organs like the heart, joints, central nervous system, and bones (Heilmann et al 2019, Widerstorm et al 2012 and Casley et al 2007).

Biofilms are collections of microbial cells made up of a single bacterial species or a mix of bacterial species that are permanently attached to a surface and encased in extracellular polymeric substances (EPS). This enables the growth, survival, and slow rate of multiplication of the bacteria in hostile environments, as well as changes in genetic transcription and low antibiotic penetration, which leads to chronic infections (Tayal et al 2015, Niveditha et al 2012). Multidrug resistance (MDR) strain are resistant to at least one agent in three or more antimicrobial categories (Mahony et al 2020). It is challenging to treat infections brought on by organisms that exhibit MDR since so few medications offer effective therapy for MDR infections (CDC 2019).

S. aureus, K. pneumoniae, non-typhoidal *Salmonella,* and *Mycobacterium tuberculosis* were included as antibiotic-resistant organisms of global concern in the World Health Organization's (WHO) first global report on surveillance of antimicrobial resistance (AMR) (Prestinaci et al 2015), in which *S. aureus* methicillin resistance in several WHO regions, including Africa (19.1%), the Americas (31.9%), the Eastern Mediterranean (17.4%), Europe (67.9%), Southeast Asia (27.3%), and the Western Pacific (43.2%)was published (WHO 2014).

Additionally, even receiving excellent care, *S. aureus* is one of the main bacteria responsible for bloodstream infections, responsible for significant mortality rates of 20% to 50%, recurrent recurrences in 5-10% of patients, and long-term impairments in almost one third of survivors (Kwiecinski and Horswill 2020). *S. aureus* bacteremia is also known to cause more deaths than tuberculosis, viral hepatitis combined, and acquired immune deficiency syndrome (Gordon et al 2021).

The frequency of MRSA in community-acquired infections is still lower than that of hospital-acquired infections, which in South Asia ranges from 4.3% in India for community-acquired infections to 86.5% in Sri Lanka for hospital-acquired infections. According to which, when compared to other South Asian nations, Nepal has a higher prevalence of MRSA (Khanal et al 2021). Further, clinical isolates from Nepal have a high prevalence of *S. aureus* with 34.5%, a multi-drug resistance percentage of 57.1%, and an overall MRSA prevalence of 41.7% (Shrestha et al 2021).

Thereby, considering the clinical importance of various *Staphylococci* infection, prevention and management continue to be top priorities for the advancement of public health. This also necessitates the use of effective techniques to identify the production of biofilms in clinical samples during regular laboratory diagnosis, as biofilms hinder the penetration of antibiotics. In order to ascertain MDR and biofilm development among *Staphylococcus spp*, this investigation was carried out.

MATERIALS AND METHODS

Study site, duration and study population

This was hospital based cross-sectional prospective study carried out in Microbiology laboratory of Nepal Armed Police Force Hospital, Balambu, Kathmandu, Nepal from February to July 2022. A total of 1813 clinical samples including pus/wound swab, blood, urine, sputum and body fluids/tips from people visiting the hospital of all ages and both the sexes were included with proper requisition form filled for routine culture.

Isolation and identification of isolates

The obtained various clinical samples in the laboratory were inoculated on Blood agar (BA) and MacConkey agar (MA). Urine sample was inoculated on CLED agar media and incubated at 37°C for 24 hours of aerobic incubation. Pus/wound swab, sputum and body fluids were inoculated on MA and BA media and incubated at 37°C for 48 hours of aerobic incubation. For central venous catheter and

catheter tips, the tips were collected in sterile container and then mixed with 2 ml of nutrient broth (NB). After mixing by vortexing, loop-full of the suspension was streaked on MA and BA media and incubated at 37°C for 48 hours of aerobic incubation. For blood sample, the obtained volume of blood in 1:10 ratio was poured in Brain Heart Infusion (BHI) broth and subculture was performed after 24 hours of enrichment at 37°C at aerobic condition on MA and BA media for consecutive 7 days. The Gram positive bacterial isolates were sub cultured on nutrient agar (NA) and Mannitol Salt Agar (MSA) and identified on the basis of various biochemical tests – catalase test, O/F test, oxidase test, slide and tube coagulase test, VP test, DNase test and urease test.

Antibiotic susceptibility test and MDR analysis

The Different groups of antibiotics were used to test susceptibility of bacterial isolates according to the standard procedure of Kirby Bauer disk diffusion method recommended by CLSI 2020 using Mueller Hinton Agar (MHA). Antibiotics used in the study were Gentamicin (10µg), Azithromycin (30 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Norfloxacin (10 µg), Clindamycin (2 µg), Cotrimoxazole (25 µg), Chloramphenicol (30 µg), Ampicillin (10 µg), Linezolid (30 µg) and Ceftriaxone (30 µg). On the basis of sensitivity patterns of isolates, resistance to at least one antimicrobial agent in three or more classes of antibiotics were considered as multi drug resistant (Magiorakos et al 2012).

Detection of Methicillin Resistant *Staphylococcus spp*

MRSA and MRCONS were detected on the basis of an inhibition zone diameter shown by cefoxitin disc (30 µg) on MHA plate. An inhibition zone diameter of ≤ 21 mm for *S. aureus* was considered as cefoxitin resistant and reported as MRSA whereas an inhibition zone diameter of ≥ 22 mm was considered as cefoxitin sensitive indicating Methicillin Sensitive *Staphylococcus aureus* (MSSA). Similarly, an inhibition zone diameter of ≤ 24 mm for CONS was considered as cefoxitin resistant and reported as MRCONS whereas ≥ 25 mm was considered as cefoxitin sensitive indicating Methicillin Sensitive Coagulase Negative Staphylococci (MSCONS).

Screening of Biofilm Production by *Staphylococcus spp*

Tissue Culture Plate (TCP) method, a quantitative test described by Christensen et al, was employed to screen biofilm producers from weak/non-producers. Isolates were inoculated in 10 ml of trypticase soy broth with 1% glucose and incubated at 37°C for 24 hours. The cultures were then diluted 1:100 with fresh medium and individual wells of sterile TCPs were filled with 200 µl of the diluted culture including negative controls (sterile media) and positive control (*S. aureus* ATCC 25923). Plates were incubated at 37°C for 24 hours, after which contents of each well were removed by gentle tapping. The wells were washed with 0.2 ml of phosphate buffered saline (pH 7.2) for four times followed by fixing of wells by 200 µl of 2% sodium acetate for 10 minutes and discarded. 200 µl of 0.1% crystal violet was filled in each well to stain the biofilm formed for 30 minutes. Excess stain was removed by using deionized water and plates were dried. Optical density of stained adherent biofilms was read by micro ELISA auto reader (model 680, Biorad, UK) at a wavelength of 570 nm. The interpretation of biofilm production was done according to the criteria of Stepanovic et al. The test was performed in triplicate for each test organism in a microtitre plate and tests were repeated for 3 times.

Average OD value:	Biofilm Production:
OD ≤ ODc / ODc < OD ≤ 2*ODc:	Weak/ non- biofilm production
2*ODc < OD ≤ 4*ODc:	Moderate biofilm production
4*ODc < OD:	Strong biofilm production

Optical density cut-off value (ODc) = Average OD of negative control + 3* standard deviation (SD) of negative control.

Data Analysis

Microsoft Excel for Windows 10 was used to maintain the data gathered from the log entry and laboratory analysis. SPSS software (version 21) for Windows was used to organize and analyze the data kept in the computer sheets. Chi-square test was used to determine the significance of the data with various variables, and a result that had a $p < 0.05$ was considered significant.

RESULTS

Out of 1813 clinical samples, 304 (16.7%) showed bacterial growth among which 135 (44.4%) were *Staphylococcus spp* while remaining 169 (55.6%) were gram negative rods.

Among 135 *Staphylococcus spp*, 119 (88.1%) isolates were found to be *S. aureus* and remaining 16 (11.9%) were CONS.

The highest number 61 (45.2%) of *S. aureus* isolates were from pus/wound swab whereas CONS were isolated most from urine 7 (5.2%) (Figure 1).

Antibiotic Susceptibility Pattern of *S. aureus* and CONS

Among 119 isolates of S. aureus, 89 (74.8%) were sensitive to Chloramphenicol whereas 97 (81.5%) showed resistance against Azithromycin. Similarly, among 16 isolates of CONS, 14 (87.5%) were sensitive to Linezolid whereas 16 (100%) showed resistance against Ampicillin (Table 1).

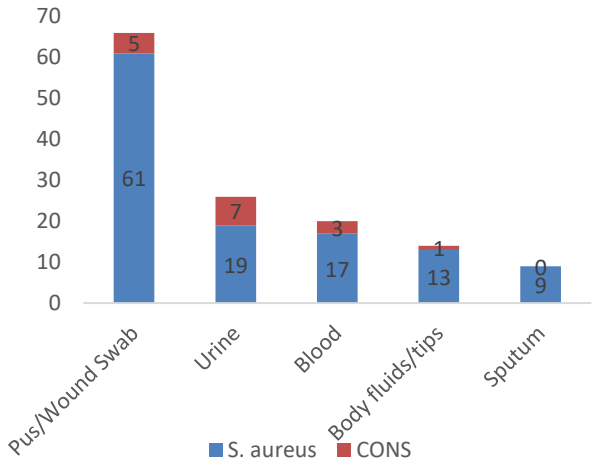

Figure 1: Distribution of *S. aureus* and CONS among different clinical samples

Prevalence of multidrug resistant isolates, methicillian resistant *S. aureus* and methicillin resistant CONS

Out of 135 isolates of *Staphylococcus spp*, 114 (84.4%) were multidrug resistant (MDR) isolates and 92 (68.2%) were methicillin resistant. Out of 119 isolates of *S. aureus* 100 (84%) were MDR and 81 (68.1%) were MRSA. Similarly out of 16 isolates of CONS, 14 (87.5%) were MDR isolates and 11 (68.8%) were MRCONS. The distribution of MDR and methicillin resistance among *S. aureus* was found to be statistically significant at $p< 0.05$. Also the distribution of MDR and methicillin resistance among CONS was found to be statistically significant at $p< 0.05$ (Table 2).

Similarly, out of 100 MDR S. aureus, 74 (74%) were found to be MRSA and among 19 non MDR *S. aureus*, 7 (36.8%) were found to be MRSA. Further out of 14 MDR CONS, 11 (78.6%) were found to be MRCONS and among 2 non MDR CONS, 2 (100%) were MSCONS (Figure 2).

Biofilm production among *Staphylococcus spp*

Among *S. aureus* 16 (11.9%) were strong biofilm producers, 27 (20%) as moderate and 76 (56.3%) as non/weak biofilm producers while, 2 (1.4%) were detected as strong biofilm producer, 3 (2.2%) as moderate and 11 (8.1%) as non/weak biofilm producer among CONS. The association between biofilm production by TCP method with *Staphylococcus spp* was statistically not significant at $p < 0.05$ (Table 3).

Table 1: Antibiotic susceptibility of *S. aureus* and CONS

Antibiotics	***S. aureus* (n=119)**		**CONS (n=16)**	
	Sensitive (%)	**Resistant (%)**	**Sensitive (%)**	**Resistant (%)**
Gentamicin	67 (56.3)	52(43.7)	12(75)	4(25)
Azithromycin	22(18.5)	97(81.5)	2(12.5)	14(87.5)
Ciprofloxacin	77(64.7)	42(35.3)	13(81.3)	3(18.8)
Levofloxacin	43(36.1)	76(63.9)	6(37.5)	10(62.5)
Norfloxacin	66(55.5)	53(44.5)	8(50)	8(50)
Clindamycin	61(51.3)	58(48.7)	9(56.3)	7(43.8)
Cotrimoxazole	43(36.1)	76(63.9)	8(50)	8(50)
Chloramphenicol	89(74.8)	30(25.2)	12(75)	4(25)
Ampicillin	7(5.9)	112(94.1)	0	16(100)
Linezolid	57(47.9)	62(52.1)	14(87.5)	2(12.5)
Ceftriaxone	84(70.6)	35(29.4)	12(75)	4(25)

Table 2: Prevalence of MDR, non-MDR, methicillin sensitive and methicillin resistant isolates

Isolates	MDR (%)	Non-MDR (%)	Total	Methicillin Resistant (%)	Methicillin Sensitive (%)	Total	p-value
S. aureus	100 (84)	19 (16)	119	81 (68.1)	38 (28.15)	119	0.006
CONS	14 (87.5)	2 (12.5)	16	11 (68.8)	5 (31.2)	16	0.025
Total	114 (84.44)	21 (15.6)		92 (68.2)	43 (31.8)		

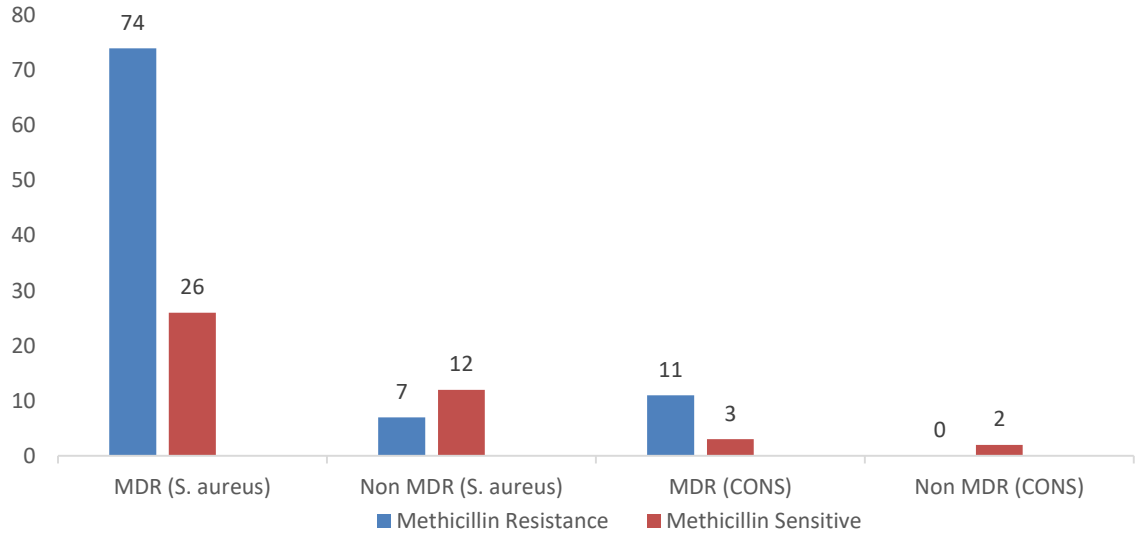

Figure 2: Distribution of methicillin resistant and methicillin sensitive isolates among MDR and non-MDR

Table3: Biofilm production by *Staphylococcus spp* by TCP method

Biofilm Formation	S.aureus	CONS	Total	p-value
Strong	16 (11.9%)	2 (1.4%)	18 (13.3%)	0.884
Moderate	27 (20%)	3 (2.2%)	30 (22.2%)	
Non/Weak	76 (56.3%)	11 (8.1%)	87 (64.4%)	

Table 4: Antibiotic resistance among biofilm producers and biofilm non-producers

	Producers(N:48)		Non Producers(N:87)	
Antibiotics	**Resistant**	**%**	**Resistant**	**%**
Gentamycin	24	50	32	36.8
Azithromycin	40	83.3	71	87.7
Ciprofloxacin	11	22.9	34	39.1
Levofloxacin	28	58.3	57	65.5
Norfloxacin	17	35.4	44	50.6
Clindamycin	21	43.8	45	51.7
Cotrimoxazole	29	60.4	54	62.1
Chloramphenicol	13	27.1	21	24.1
Ampicillin	45	93.8	83	95.4
Linezolid	15	31.3	31	35.6
Ceftriaxone	10	20.8	29	33.3

Table 5: Biofilm production among MDR, non-MDR, MRSA and MSSA

Isolates	**Strongbiofilm producer**	**Moderatebiofilm producer**	**Non/weakbiofilm producer**	**Total**	**p-value**
MDR	15 (15%)	21 (21%)	64 (64%)	100	0.523
Non-MDR	1 (5.3%)	6 (31.6%)	12 (63.1%)	19	
MRSA	15 (18.5%)	20 (24.7%)	46 (56.8%)	81	0.103
MSSA	1 (2.6%)	7 (18.4%)	30 (79%)	38	

Table 6: Biofilm production among MDR, non-MDR, MRCONS and MSCONS

Isolates	**Strong biofilm producer (%)**	**Moderatebiofilm Producer (%)**	**Non/weakbiofilm Producer (%)**	**Total**	**p-value**
MDR	2 (14.3)	2 (14.3)	10 (71.4)	14	0.451
Non-MDR	0	1 (50)	1 (50)	2	
MRCONS	2 (18.2)	1 (19.1)	8 (72.7)	11	0.254
MSCONS	0	2 (40)	3 (60)	5	

Antibiotic resistance among biofilm producers and biofilm non-producers

Among 48 biofilm producers, 45 (93.8%) were resistant to Ampicillin followed by 40 (83.3%) resistant to Azithromycin whereas among 87 non/weak biofilm producers, 83 (95.4%) were resistant to Ampicillin followed by 71 (87.7%) resistant to Azithromycin (Table 4).

Biofilm production among MDR and methicillin resistant isolates of *S. aureus*

Among 100 MDR isolates of *S. aureus*, 15 (15%) were found to be strong, 21 (21%) were moderate and 64 (64%) were non/weak biofilm producers. The relationship between biofilm production by TCP method with MDR and non MDR isolates was statistically not significant at $p < 0.05$. Similarly among MRSA isolates of *S. aureus*, 15 (18.5%) were found to be strong, 20 (24.7%) were moderate and 46 (56.8%) were non/weak biofilm producers. The relationship between biofilm production by TCP method with MRSA and MSSA isolates was statistically not significant at $p < 0.05$ (Table 5).

Biofilm production among MDR and methicillin resistant isolates of CONS

Among 14 MDR isolates of CONS, 2 (14.3%) were strong and moderate each and 10 (71.4%) were non/weak biofilm producers. The relationship between biofilm production by TCP method with MDR and non MDR isolates of CONS was statistically not significant at $p < 0.05$. Similarly, among 11 MRCONS isolates, 2 (18.2%) were strong, 1(9.1%) was moderate and 8 (72.7%) were non/weak biofilm producers. The relationship between biofilm production by TCP method with MRCONS and MSCONS isolates was statistically not significant at $p < 0.05$ (Table 6).

DISCUSSION

Staphylococcus aureus is an opportunistic pathogen which can breach the skin barriers through the wound or surgical incision and cause infection (Ziebuhr et al 2001) whereas CONS are the normal flora of human body that over past few decades has emerged as major cause of nosocomial infections (Seng et al 2017).

In this study, 135 (44.40%) were *Staphylococcus spp* comprising *S. aureus*: 119 (88.1%) and CONS: 16 (11.9%). The results were similar with findings of Pandey et al (2020), Upreti et al (2018) and Kumari et al (2008). The increasing isolation of *Staphylococcus spp* can be due to distribution of the organism along with the series of virulence factor possessed by the organism. Immune compromised individuals or patients under treatment with indwelling devices are more vulnerable to get CONS infections (Arciola et al 2005, Eiff et al 2006 and Otto M 2009). The highest number of *S. aureus* were isolated from pus/wound swab among various clinical specimens, which correlated with the mini review article by Shrestha et al (2021) where they mentioned highest isolation of *S. aureus* from pus sample in Nepal. *S. aureus*, meantime, is responsible for a wide range of skin and soft tissue infections, from the benign (such as impetigo and uncomplicated cellulitis) to the severe (such as necrotizing fasciitis and severe cellulitis), some of which can be immediately fatal. It is the most prevalent pathogen that has been found in cutaneous abscesses, purulent cellulitis, and infections at surgical sites. (Tong et al 2015). The majority of CONS are commensals of skin and mucosa, and further isolation of CONS from clinical samples including blood, urine, and pus/wound swabs reveals that they do not exhibit any tropism for specific niches but can be easily transferred from person to person through contact or skin sloughing. CONS are now often isolated bacteria in hospital acquired infections, especially in patients with surgical incisions and/or receiving foreign implants (catheters as well as any sort of prosthetic device) (Fey and Olson 2010, Otto 2010, Longaureova 2006).

The key to developing effective treatment strategies to combat bacterial illnesses is determining the susceptibility and resistance to antibiotics (Khan et al 2014). The findings regarding antibiotic resistance pattern hold in consistence with Shrestha et al (2021) and Adhikari et al (2017). Linezolid was found to be sensitive against *Staphylococcus spp* whereas most isolates were resistant against Ampicillin. The higher resistance to Ampicillin is due to the ability of organisms to produce β-lactamase (Ansari et al 2014). Similarly, the increasing resistance to every class of antibiotics, regardless of their natural or synthetic origins and the extensive exploitation of therapeutic agents, have led CONS to lose their susceptibility to most of the available antibiotics (Deurenberg and Stobberingh 2008). Moreover, higher proportion of MDR and MRSA isolates is reported in the study, than Belbase et al (2017) 59.2% MDR and 47.4% MRSA and Neopane et al (2018) 60.5% MDR and 30.2%

MRSA. These differences may be brought on by variations in the circulating clones, infection control procedures, trends in the prescribing of antibiotics in various medical settings, hospitalization in intensive care units, etc. (Adhikari et al 2017).

In the study 16 (11.9%) isolates of *S. aureus* were detected strong biofilm producer by employing TCP technique. However, Pandey et al (2020) and Tuladhar (2018) reported slightly less values of 7.7% and 8.8% strong biofilm producers respectively. Likewise, in case of CONS 2 (1.4%) isolates were detected as strong biofilm producers. Pandey et al (2020) reported 1.9% strong biofilm producers among CONS which was in consistent to the present findings. With regards to biofilm producers, 45 (93.8%) were resistant to Ampicillin followed by 40 (83.3%) resistant to Azithromycin. Pandey et al (2020) and Neopane et al (2018) had also reported highest resistance to antibiotics under β lactam and macrolides group by biofilm producers. Bacteria growing within biofilms are innately resistant to many antibiotics because of the protective structure of the biofilm. The presence of antibiotic degradation mechanisms, the horizontal gene transfer of drug resistance markers within biofilms, the slow growth of bacteria, among many other factors, may make it difficult for antibiotics to penetrate biofilms (Belbase et al 2017).

In this study 36 (36%) MDR isolates of *S. aureus* were biofilm producers which was less than that reported by Belbase et al (2017). Similarly, among MRSA isolates, 15 (18.5%) were found to be strong, 20 (24.7%) were moderate and 46 (56.8%) were non/weak biofilm producers. Azmi et al (2019) reported 21% strong, 46.4% moderate and 32.7% non/weak biofilm producers among 248 MRSA isolates and Piechota et al (2018) reported strong biofilm production by 39.7% of MRSA and 36.8% of MSSA strains. Both the mentioned findings were similar to this study. The expression of intercellular adhesions (IcaABCD) is necessary for the formation of biofilm whose expression is found to be more in MRSA than MSSA and expression of ica operon is found to vary with the strain of *S. aureus* distributed around the globe (Azmi et al 2019, Piechota et al 2018). Similarly, smaller proportion of strong biofilm producers among MRCONS was reported than by Sitthisak et al (2019) 38.1%.

CONCLUSION

A larger percentage of *S. aureus* than CONS was identified among isolated *Staphylococcus spp*. The isolates were found to be highly resistant to most of the first line drugs used in the treatment. *S. aureus* was found to be sensitive against Chloramphenicol and Gentamycin whereas CONS was found to be sensitive against Linezolid and Chloramphenicol. Additionally, the high rates of biofilm development in MDR and MRSA point to the need for monitoring of biofilm producers through techniques like TCP.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ETHICAL APPROVAL

Ethical approval for this study was taken from Institutional Review Committee, Institute of Science and Technology, Kirtipur, Kathmandu, Nepal (Ref. No. 965/078/079).

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