Susceptibility to Vancomycin of Biofilm Producing Staphylococci Isolated from Tertiary Care Hospital of Nepal

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ABSTRACT

Background: Methicillin resistance and biofilm-producing Staphylococci are emerging as multidrug-resistant strains narrowing the efficacy of antimicrobial therapy. Although vancomycin is used as the drug of choice to treat such isolates, different studies worldwide have documented the emergence of strains that are intermediately susceptible or resistant to this antibiotic. **Objective:** The study aimed to determine the minimum inhibitory concentration of vancomycin to methicillin-resistant and biofilm-producing staphylococci isolated from different clinical specimens. **Methods:** 375 staphylococci isolated from different clinical specimens over one year were included in the study. Biofilm formation was determined by the Tissue culture plate method (TCP), and *ica* genes were identified by Polymerase Chain Reaction (PCR). Antibiotic susceptibility and methicillin resistance were done following Clinical and Laboratory Standards Institute (CLSI) guidelines. The minimum inhibitory concentration (MIC) of vancomycin in all isolates was determined by the agar dilution method. **Results:** Among 375 Staphylococci studied, 43% and 57% represented *S. aureus* and Coagulase-Negative Staphylococci (CNS), respectively. The rate of Methicillin-Resistant *S. aureus* (MRSA) and Methicillin-Resistant Coagulase Negative Staphylococci (MRCNS) were 81.4% and 66.8% respectively and determined by the disc diffusion method. The most potential antibiotics were tetracycline and chloramphenicol showing sensitivity to more than 90% isolates. The Minimum Inhibitory Concentration (MIC) value of oxacillin for staphylococci ranged from 0.125-32 µg/ml. Oxacillin agar diffusion method showed 51.6% and 79.9% isolates as MRSA and MRCNS, respectively, revealing a very high percentage of *S. aureus* and CNS isolates as methicillin-resistant. All isolates had susceptible vancomycin MICs that ranged from 0.125-2 µg/ml. Two *S. aureus* isolated from Central Venous Catheter (CVC) and catheter specimens were detected with intermediate susceptibility to vancomycin. Similarly, three CNS isolated from blood, CVC, and wound/pus (w/p) were intermediately susceptible to vancomycin. Strong biofilm formation was observed in 22.1% of clinical isolates, and the *ica* gene was detected among 22.9% of isolates. Only one *S. aureus* detected as a biofilm producer by the TCP method was found to have intermediate susceptibility to vancomycin. **Conclusions:** The increment in vancomycin MIC among methicillin-resistant and biofilm-producing staphylococci is alarming. Strict control measures to prevent methicillin-resistant isolates spread and routine surveillance for vancomycin-resistant isolates must be incorporated in hospitals to prevent antimicrobial treatment failure.

Keywords: Staphylococci, Methicillin resistant *staphylococci*, Biofilm, *ica* genes, Vancomycin resistant staphylococci

RESUMEN

Antecedentes: Los estafilococos resistentes a la meticilina y productores de biopelículas están surgiendo como cepas multirresistentes que reducen la eficacia del tratamiento antimicrobiano. Aunque la vancomicina se utiliza como fármaco de elección para tratar dichos aislados, diferentes estudios realizados en todo el mundo han documentado la aparición de cepas intermedias susceptibles o resistentes a este antibiótico. **Objetivo:** El estudio tenía como objetivo determinar la concentración mínima inhibitoria de la vancomicina para los estafilococos resistentes a la meticilina y productores de biofilm aislados de diferentes muestras clínicas. **Métodos:** Se incluyeron en el estudio 375 estafilococos aislados de diferentes muestras clínicas durante un año. La formación de biopelículas se determinó mediante el método de la placa de cultivo de tejidos (TCP), y los genes *ica* se identificaron mediante la reacción en cadena de la polimerasa (PCR). La susceptibilidad a los antibióticos y la resistencia a la meticilina se realizaron siguiendo las directrices del Clinical and Laboratory Standards Institute (CLSI). La concentración inhibitoria mínima (MIC) de vancomicina en todos los aislados se determinó por el método de dilución en agar. **Resultados:** Entre los 375 estafilococos estudiados, el 43% y el 57% representaban *S. aureus* y estafilococos coagulasa-negativos (ECN), respectivamente. La tasa de *S. aureus* resistente a la meticilina (SARM) y de estafilococos coagulasa negativos resistentes a la meticilina (ECNM) fue del 81,4% y el 66,8%, respectivamente, y se determinó por el método de difusión de discos. Los antibióticos más potenciales fueron la tetraciclina y el cloranfenicol, que mostraron una sensibilidad superior al 90% de los aislados. El valor de la concentración inhibitoria mínima (CIM) de la oxacilina para los estafilococos osciló entre 0,125-32 µg/ml. El método de difusión en agar de la oxacilina mostró que el 51,6% y el 79,9% de los aislados eran SARM y MRCNS, respectivamente, lo que revela que un porcentaje muy elevado de los aislados de S. aureus y CNS son resistentes a la meticilina. Todos los aislados tenían MIC de vancomicina susceptibles que oscilaban entre 0,125-2 µg/ml. Se detectaron dos *S. aureus* aislados de muestras de catéteres venosos centrales (CVC) y catéteres con una susceptibilidad intermedia a la vancomicina. Del mismo modo, tres *S. aureus* aislados de sangre, CVC y herida/pus (w/p) fueron intermedianamente susceptibles a la vancomicina. Se observó una fuerte formación de biopelículas en el 22,1% de los aislados clínicos, y se detectó el gen *ica* en el 22,9% de los aislados. Sólo un *S. aureus* detectado como productor de biopelículas por el método TCP resultó tener una susceptibilidad intermedia a la vancomicina. **Conclusiones:** El incremento de la MIC de vancomicina entre los estafilococos resistentes a la meticilina y productores de biofilm es alarmante. Para evitar el fracaso del tratamiento antimicrobiano, deben incorporarse en los hospitales medidas de control estrictas para prevenir la propagación de los aislados resistentes a la meticilina y una vigilancia rutinaria de los aislados resistentes a la vancomicina.

Palabras clave: Estafilococos, estafilococos resistentes a la meticilina, biopelícula, genes *ica*, estafilococos resistentes a la vancomicina

INTRODUCTION

Staphylococci are present as normal flora on the skin and anterior nares of humans, and up to two-thirds of the population are estimated to be colonized by it. They are also responsible for causing skin and soft tissue infection to fatal endovascular infections and sepsis. In recent years, the importance of staphylococci has increased because of their causative role in nosocomial infections in both developed and developing countries (1-3). The widespread use of immunosuppressive drugs, indwelling intravascular catheters, artificial tools, and health care settings have been reported to be associated with the spread of these infections. The occurrence of bacterial infection had decreased with the discovery of penicillin in 1940 until *S. aureus* began producing β lactamase. The resistance to penicillin drove the development of methicillin drugs in 1960. But the first strain of methicillin-resistant *S. aureus* (MRSA) was reported in 1961, and since then, the infection caused by MRSA has been a major public health concern (4,5). The dissemination of MRSA has developed in parallel with increasing the prevalence of multi drug resistant strains (MDR).

One major virulence property of staphylococci is its ability to form biofilm. Biofilms are communities of microorganisms embedded in the extracellular polymeric substances (EPS) matrix. Bacterial biofilms provide considerable advantages like protection against antimicrobial agents, acquisition of new genetic traits, and resistance against phagocytosis and other components of the body defense system (6). Although vancomycin has been the most reliable therapeutic agent against infections caused by MRSA, an increasing number of vancomycin intermediates sensitive to *S. aureus* (VISA) are being reported. However, the vancomycin-resistant *S. aureus* (VRSA) is still limited worldwide (5,7). In addition, emerging data suggest that vancomycin may be less effective against severe MRSA infection with minimum inhibitory concentration (MIC) values at the higher end of the susceptibility range designated by Clinical and Laboratory Standards Institute (CLSI), resulting in treatment failure and increased mortality. Many researchers have reported that the higher MIC of vancomycin to MRSA, even in the susceptible range, correlates with the drug-resistant to different classes of antibiotics (7-9). Only limited data are available on the susceptibility of

MRSA to vancomycin in Nepal. Although VRSA has not been reported from Nepal, increasing trends of decreased susceptibility of MRSA to vancomycin is reported indicating the need for more research in this field (6,8,10). With the increasing occurrence of MRSA having decreased susceptibility to vancomycin and biofilm formation, the choice of medication remains one of the most challenging concerns in the management of infection caused by such isolates. Thus, this study was conducted to determine the changing susceptibility pattern of methicillin-resistant staphylococci isolates to vancomycin and biofilm producing capacity of *S. aureus* and coagulase negative staphylococci (CNS) isolated from different clinical specimens.

MATERIALS AND METHODS

Isolation and identification of staphylococci

The hospital-based cross-sectional descriptive study was conducted at the Department of Microbiology, B & B hospital, Gwarko, and KIST Medical College and Teaching Hospital, Imadol, Lalitpur, Nepal, from January 2018 to December 2018. 375 staphylococci were isolated from different clinical specimens processed during the study period. Specimens processed were wound/pus (w/s), blood, urine, body fluids, various tips (central venous catheter, catheter, endotracheal, transtracheal), etc. All clinical samples were collected and processed following standard microbiological procedures. The isolated colonies were identified by inoculating in Mannitol salt agar media, Blood agar media and performing Gram staining and specific biochemical tests. The slide and tube coagulase tests were performed to differentiate *S. aureus* and CNS (11). The species of CNS were identified based on a simplified scheme proposed by Cunha *et al.* using several biochemical tests (12). The biochemical tests performed were clumping factor, hemolysis, pyrrolidonyl arylamidase, urease, alkaline phosphatase, nitrate reduction, acetoin production, acid production from sucrose, mannose, trehalose, xylose, and susceptibility to novobiocin and desferrioxamine.

Antimicrobial susceptibility test

The modified Kirby-Bauer disk diffusion method was carried out to determine *in vitro* antibiotic susceptibility to nine antimicrobial agents selected based on different modes of action. Antibiotic discs (HiMedia, India) used was ciprofloxacin (5µg), clindamycin (2µg), erythromycin (15µg),

gentamicin (10µg), penicillin (10 units), cotrimoxazole (1.25/23/75µg), tetracycline (30µg) and chloramphenicol (30µg). The test used a bacterial suspension with turbidity adjusted equivalent to a 0.5 McFarland standard, and performed on Mueller Hinton agar plates (HiMedia, India) based on the Clinical & Laboratory Standards Institute (CLSI) guidelines (13). *S. aureus* ATCC 25923 strain was used as a positive control. Screening for Methicillin resistant staphylococci was carried out by cefoxitin disc diffusion method and interpreted according to CLSI guidelines. The growth of staphylococci with a zone of inhibition around cefoxitin disc (ZOI) ≥ 22mm was identified as methicillin sensitive *S. aureus* (MSSA), and that of ZOI ≤ 21 was identified as methicillin resistant *S. aureus* (MRSA). Similarly, for coagulase-negative staphylococci, ZOI ≥ 25 were identified as methicillin sensitive coagulase negative staphylococci (MSCNS) and ZOI ≤ 24 as methicillin resistant coagulase negative staphylococci (MRCNS), respectively.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) technique by agar dilution method was performed to determine the vancomycin-intermediate and resistant and oxacillin resistant strains of staphylococci isolate. MIC to vancomycin and oxacillin of all isolates was done using the agar dilution method following CLSI guidelines (14). Different concentrations of 0.125 to 32µg/ml of vancomycin and 0.125µg/ml to 128µg/ml of oxacillin incorporated plates were prepared. Positive growth controls were kept for each isolate, and *S. aureus* (ATCC 29213) of known MIC was included in each test to control antibiotic potency. All the experiments were performed in triplicate.

Detection of biofilm formation

The Tissue Culture Plate (TCP) method was used to determine the biofilm production of Staphylococcal isolates as described by Christensen *et al.* with some modifications (15,16). In this assay, a loopful of organisms was inoculated in 5mL Tryptone Soybroth (TSB) (HiMedia Laboratories Private Limited, India) supplemented with glucose and incubated at 37°C for 24 hours. The overnight culture was diluted 1:100 with fresh media, and 0.2mL of this diluted culture was then inoculated into individual wells of sterile polystyrene 96 well flat bottom tissue culture plates and incubated at 37°C for 24 hours. After incubation, the content of the tissue culture plate was removed by gentle tapping and washed with PBS (pH 7.2) 4

times to remove free-flowing planktonic bacteria. Biofilms formed by adherent sessile bacteria in the plate were fixed with sodium acetate (2%). It was then stained with crystal violet (0.1%) for 15 minutes at room temperature. Excess stain was rinsed off by washing with deionized water 4 times, and plates were dried. The optical density (OD) of stained adherent bacteria was measured with a micro ELISA auto reader at 630nm (OD $_{630nm}$). OD values from sterile medium, fixative, and dye were averaged and subtracted from all test values. Experiments were performed in triplicates and repeated three times. Bacterial adherence was classified based on OD values of the individual isolates: < 0.12, 0.12 - 0.24, and > 0.24 as weak, moderate, and strong biofilm formation, respectively.

Detection of *ica* gene by PCR

The biofilm of staphylococci is composed of the layer of extracellular polymeric substance called polysaccharide intercellular adhesion (PIA) encoded by *ica* operon (*icaADBC* genes). The genomic DNA was extracted from all 375 staphylococcal isolates as previously described (16) using the DNA extraction kit, following the manufacturer's instructions (Thermo Fisher Scientific Inc.). The sequences of *icaA* and *icaD* (accession number U43366) were taken from the GenBank sequence of the National Center for Biotechnology Information (NCBI) database. Primers (Solis Biodyne, Denmark) specific for *icaA* (forward 5'-TCTCTTGCAGGAGCAATCAA and reverse 5'-TCAGGCACTAACATCCAGCA) generating a product size of 188bp and *icaD* (forward 5'-ATGGTCAAGCCCAGACAGAG and reverse 5'-CGTGTTTTCAACATTTAATGCAA) with the product size of 198bp were designed by the Primer3 program. The PCR product was analyzed in 2% agarose gel stained with SYBR safe dye (Invitrogen).

Statistical Analysis

The data obtained were analyzed with the help of a statistical package for social sciences version 16.0. The Chi-square test was used to analyze the association between two variables, and *P* value less than 0.05 was considered statistically significant.

RESULTS

375 staphylococci were isolated from different clinical specimens collected at two tertiary care hospitals in Nepal, comprising 42.9 % *S. aureus* and 57.1 % coagulase-negative staphylococci (CNS). *S. epidermidis* (57.5 %) was the most commonly isolated CNS, followed by *S. saprophyticus* (18.7 %), *S. haemolyticus* (11.2 %), *S. hominis* (7.0 %), and *S. capitis* (5.6 %).

Distribution of methicillin resistant staphylococci

Out of 161 *S. aureus*, 81.4 % isolates were MRSA and 18.6% MSSA as detected by disc diffusion assay. Similarly, 66.8% represented MRCNS of 214 CNS isolates. The highest rate of resistance of MRSA isolates was against penicillin (96.2 %), followed by erythromycin (84 %). A similar resistant pattern was observed in MRCNS, with 96.5 % and 84.6 % isolates resistant to penicillin and erythromycin, respectively. All MSSA isolates were sensitive to clindamycin and chloramphenicol. Similarly, the highest resistance was seen towards penicillin for both MSSA (90 %) and MSCNS (81.7 %) (Table1).

Table 1. Antibiotic susceptibility pattern of methicillin resistant and methicillin sensitive staphylococci

Antibiotics	Potency (µg/disc)	MRSA (n=131)	MSSA (n=30)	p value	MRCNS (n=143)	MSCNS (n=71)	p value
Penicillin	10 units	126 (96.2%)	27 (90.0%)	0.160	138 (96.5%)	58 (81.7%)	0.000
Ciprofloxacin	5	102 (77.9%)	17 (56.7%)	0.017	69 (48.3%)	7 (9.9%)	0.000
Tetracycline	30	8 (6.1%)	1 (3.3%)	0.551	22 (15.4%)	5 (7.0%)	0.084
Clindamycin	2	16 (12.2%)	-	0.044	53 (37.1%)	10 (14.1%)	0.001
Chloramphenicol	30	4 (3.1%)	-	0.332	13 (9.1%)	6 (8.5%)	0.877
Erythromycin	15	110 (84.0%)	17 (56.7%)	0.001	121 (84.6%)	34 (47.9%)	0.000
Cotrimoxazole	1.25/23.75	72 (55.0%)	18 (60.0%)	0.616	65 (45.5%)	15 (21.1%)	0.001
Gentamicin	10	58 (44.3%)	9 (30.0%)	0.152	42 (29.4%)	3 (4.2%)	0.000

MRSA= Methicillin resistant *Staphylococcus aureus*, MSSA= Methicillin sensitive *Staphylococcus aureus*, MRCNS= Methicillin resistant coagulase negative staphylococci, MSCNS= Methicillin sensitive coagulase negative staphylococci

Distribution of oxacillin and vancomycin MICs in Staphylococcal isolates

The staphylococcal isolates had MIC values for oxacillin from 0.125 to >32 μg/ml. According to the observed MIC values, there were 83 MRSA (MIC ≥4μg/ml) isolates and 171 MRCNS (MIC ≥0.5 μg/ml) isolates as per CLSI. *S. aureus* ATCC 29213 recorded MIC of 0.5 μg/ml used as positive control strain. Sixty-three CNS isolates and 39 *S. aureus* had MIC >32 μg/ml (Figure 1).
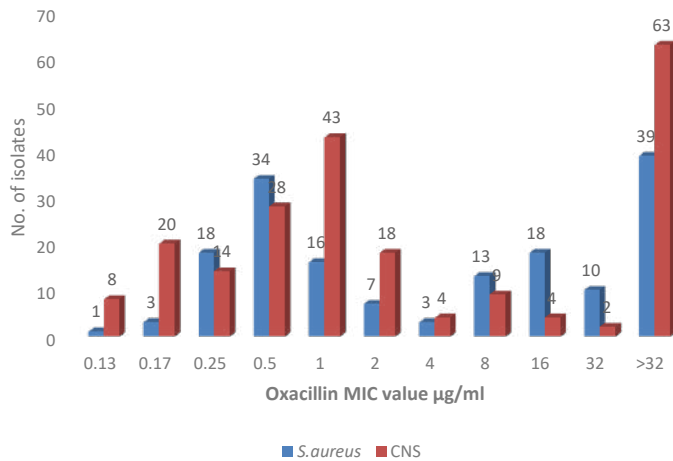

Figure 1. Oxacillin MIC values among Staphylococcal isolates

Most *S. aureus* strains were sensitive to vancomycin and the MIC value recorded was 0.125 μg/ml to ≤2 μg/ml, where 59 isolates had MIC value at a clinical breakpoint of 2 μg/ml. The two *S. aureus* isolates had a MIC value of 4 μg/ml; however, these are not resistant as per CLSI. These two isolates with intermediate susceptibility to vancomycin were obtained from central venous catheter (CVC) and catheter samples

of inpatients. Similarly, among CNS also, all isolates were sensitive with a MIC of ≤4 μg/ml, whereas 51 CNS had MIC at a clinical breakpoint of 4 μg/ml. Three isolates had MIC values >4 μg/ml representing intermediate susceptibility to vancomycin. These 3 isolates were methicillin-resistant and had been isolated from blood, CVC, and wound/pus (w/p) (Figure 2). *S. aureus* ATCC 29213 recorded a MIC of 1 μg/ml used as control strain.
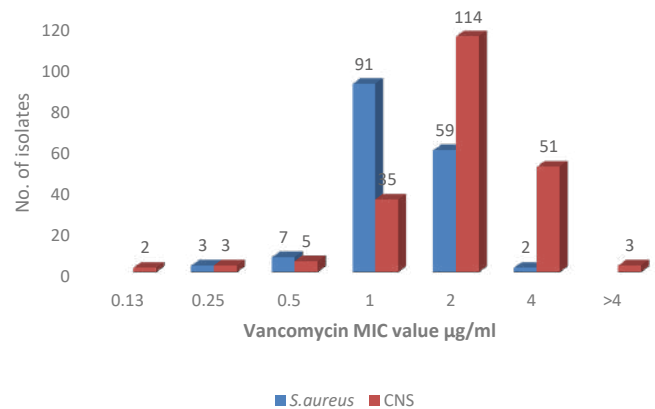

Figure 2. Vancomycin MIC values among Staphylococcal isolates

Distribution of oxacillin MIC in methicillin resistant isolates

Fifty-five isolates detected by cefoxitin disc diffusion assay as MRSA were susceptible to oxacillin by agar dilution method, whereas 7 MSSA isolates were resistant to oxacillin. Similarly, only 20 CNS isolates were detected as methicillin-resistant by disc diffusion assay and were susceptible to oxacillin, but 49 CNS isolates sensitive to methicillin were resistant to oxacillin (Table 2).

Table 2. MIC of oxacillin in methicillin resistant and methicillin sensitive staphylococci

MIC value of oxacillin (μg/ml)	MRSA (n,%)	MSSA (n,%)	MRCNS (n,%)	MSCNS (n,%)
0.125	3 (2.3%)	-	14 (9.8%)	15 (21.1%)
0.25	12 (9.2%)	7 (23.3%)	6 (4.2%)	7 (9.9%)
0.5	24 (18.3%)	9 (30.0%)	22 (15.4%)	6 (8.5%)
1	11 (8.4%)	4 (13.3%)	32 (22.4%)	15 (21.1%)
2	5 (3.8%)	3 (10.0%)	11 (7.7%)	7 (9.9%)
4	3 (2.3%)	-	4 (2.8%)	1 (1.4%)
8	-	1 (3.3%)	6 (4.2%)	2 (2.8%)
16	10 (7.6%)	2 (6.7%)	4 (2.8%)	-
32	17 (13.0%)	1 (3.3%)	2 (1.4%)	-
>32	46 (35.1%)	3 (10.0%)	42 (29.4%)	(25.4%)

Distribution of vancomycin MIC in methicillin resistant isolates

All staphylococcal isolates were sensitive to vancomycin irrespective of methicillin resistance. The MRSA had vancomycin MIC values from 0.25 to 4 µg/ml. Forty-nine MRSA isolates and 8 MSSA isolates had MIC values at a clinical breakpoint of 2 µg/ml. Three MRSA were detected with intermediate susceptibility to vancomycin. All CNS were also sensitive to vancomycin, but 34 MRCNS and 20 MSCNS had a clinical breakpoint of 4 µg/ml (Table 3).

Table 3. MIC of vancomycin in methicillin resistant and methicillin sensitive staphylococci

MIC value of vancomycin (µg/ml)	MRSA (n,%)	MSSA (n,%)	MRCNS (n,%)	MSCNS (n,%)
0.125	-	-	1 (0.7%)	1 (1.4%)
0.25	2 (1.5%)	2 (6.7%)	-	2 (2.8%)
0.5	4 (3.1%)	3 (10.0%)	5 (3.5%)	-
1	73 (55.7%)	17 (56.7%)	27 (18.9%)	8 (11.3%)
2	49 (37.4%)	8 (26.7%)	76 (53.1%)	40 (56.3%)
4	3 (2.3%)	-	34 (23.8%)	20 (28.2%)

Biofilm formation in staphylococci isolates

Biofilm formation detected by the TCP method showed that 22.1 % of staphylococci isolates were strong biofilm producers with 24.1 % MRSA and 9.3 % MRCNS. Similarly, the *ica* genes were detected among 22.9 % of staphylococcal isolates, where 18 % and 13.1 % represented MRSA and MRCNS, respectively (Table 4).

MIC of oxacillin in biofilm producing staphylococci

Out of 174 staphylococci biofilm producers detected by the TCP method, 48.3 % were *S. aureus* and 51.7 % CNS. Among them, 53.6 % isolates were MRSA (4 µg/ml), and 84.4 % were MRCNS (0.5 µg/ml). The

ica genes were detected in 86 isolates with, 52.3 % and 47.7 % isolates being *S. aureus* and 41 CNS, respectively. 44.4 % and 73.2 % of isolates were detected as MRSA and MRCNS by the agar dilution method (Table 5).

Among all staphylococci positive for biofilm production by the TCP method, only one was intermediate susceptible to vancomycin. This isolate was *S. aureus* obtained from CVP, which was also resistant to oxacillin. The other two isolates that were intermediately susceptible to vancomycin were non-biofilm producers and were isolated from catheters and w/p. The *ica* genes were absent in these three isolates (Table 5).

Table 5. MIC of oxacillin and vancomycin in biofilm producing staphylococci

Aspect	No of biofilm producer S. aureus isolates determined by		No of biofilm producer CNS isolates determined by	
	TCP method	*ica* genes	TCP method	*ica* genes
Oxacillin MIC (µg/ml)				
0.125	1	1	8	6
0.25	13	9	6	5
0.5	15	7	14	6
1	6	3	16	5
2	4	5	14	4
4	3	2	3	3
8	6	5	3	1
16	10	6	2	-
32	4	5	1	2
64	22	2	23	9
Vancomycin MIC (µg/ml)				
0.125	-	-	-	1
0.25	2	1	1	1
0.5	2	3	3	1
1	47	26	19	5
2	32	15	48	24
4	1	-	19	9

Table 4. Detection of biofilm formation by different methods among methicillin resistant and methicillin sensitive staphylococci

Method	Biofilm formation	MRSA	MSSA	P value	MRCNS	MSCNS	P value	Total
TCP method	Strong	39 (24.2%)	9 (5.6%)	0.681	20 (9.3%)	15 (7%)	0.412	83 (22.1%)
	Moderate	31 (19.3%)	5 (3.1%)		38 (17.8%)	17 (7.9%)		91 (24.3%)
	Weak/Non	61 (37.9%)	16 (9.9%)		85 (39.7%)	39 (18.2%)		201 (53.6%)
ica gene	Presence	29 (18%)	16 (9.9%)	0.001	28 (13.1%)	13 (6.1%)	0.824	86 (22.9%)
	Absence	102 (63.4%)	14 (8.7%)		115 (53.7%)	58 (27.1%)		289 (77.1%)

Distribution of MIC of oxacillin and vancomycin in different species of CNS

The agar dilution method detected 41.6 % *S. epidermidis*, 16.4 % *S. saprophyticus*, 11.2 % *S. haemolyticus*, 4.7 % *S. capitis*, and 6.5 % *S. hominis* resistant to methicillin.

All CNS species were susceptible to vancomycin. The MIC ranged from 0.125 µg/ml to 4 µg/ml for *S. epidermidis* and *S. saprophytics*, while for *S. haemolyticus*, the range was 0.5 µg/ml to 4 µg/ml, and for *S. capitis* and *S. hominis*, the MIC range was 1 to 4 µg/ml (Table 6).

Table 6 MIC of oxacillin and vancomycin in different species of CNS

Species of CNS	MIC of oxacillin (µg/ml)										
	0.125	0.25	0.5	1	2	4	8	16	32	>32	Total
S. epidermidis	22	12	17	28	9	1	5	1	-	28	123
S. saprophyticus	4	1	7	6	3	1	1	2	-	15	40
S. haemolyticus	-	-	2	10	2	-	1	1	1	7	24
S. capitis	2	-	1	1	1	1	1	-	-	5	12
S. hominis	1	-	1	2	3	2	-	-	1	5	15
	MIC of vancomycin										
S. epidermidis	1	2	3	20	67	30	-	-	-	-	123
S. saprophyticus	1	-	1	6	21	11	-	-	-	-	40
S. haemolyticus	-	-	1	5	11	7	-	-	-	-	24
S. capitis	-	-	-	2	8	2	-	-	-	-	12
S. hominis	-	-	-	2	9	4	-	-	-	-	15

DISCUSSION

In recent years, staphylococcal isolates have become one of the most dangerous pathogens due to their increased resistance to various commonly used antibiotics and biofilm formation. Studies showed that MRSA and MRCNS are the causative agent of hospital-acquired infection and incipient community pathogens in many geographical regions (6,8).

In our study, 81.4 % and 66.8 % of isolates were MRSA and MRCNS by cefoxitin disc diffusion assay, higher than in other studies from Nepal, where MRSA representation ranged from 19 % to 45.9 % (17-22). Higher isolation rates reported in our study can be attributed to several factors. Some of these include indiscriminate use of antibiotics, population and area studied, lack of awareness, and failure to observe simple yet effective infection control precautions like strict patient isolation and frequent hand washing by health care personnel (23).

Most MRSA and MRCNS isolates were sensitive to tetracycline and chloramphenicol, indicating exposure to newer drugs and susceptibility to older drugs. Most of these isolates were observed to be resistant to penicillin and erythromycin. This discrepancy suggests a change in antibiotic susceptibility pattern with a difference in study settings, geographical location, and population studied. This resistance pattern was in concordance with other studies (23). The notoriety of staphylococci in developing resistance to therapeutic agents has been known since the advent of penicillin resistance, particularly in response to selective antibiotic pressure.

The MIC value of *S. aureus* for oxacillin showed that 55 MRSA determined by the cefoxitin disc diffusion method were, in reality, susceptible to oxacillin. The MIC of MSSA isolates agreed with the methicillin status except for 7 MSSA isolates with MICs ≥ 4 µg/ml. For CNS, 20 methicillin-resistant isolates determined by the cefoxitin disc diffusion method were susceptible to oxacillin, but 49 methicillin-sensitive CNS were characterized as oxacillin resistant. Such elevated MICs are obtained due to mutation in one of their Penicillin Binding Protein (PBPs) (24) or hyperproduction of staphylococcal β lactamase (25) that could explain the observed aberration. This result in phenotypes expressing oxacillin MICs between the clinical breakpoint of susceptibility and resistance that are often termed Borderline oxacillin resistant *S. aureus* (BORSA) that lack the *mecA* gene (25). Oxacillin MIC determination

is limited to research purposes only. In contrast, for diagnostic purposes, the cefoxitin screening result is extrapolated for penicillinase-resistant penicillin's susceptibility to decide if they can be prescribed to patients (13). Due to limited treatment options for infections caused by methicillin-resistant staphylococci, the treatment of such infections is often difficult leading to a prolonged hospital stay and sometimes leading to treatment failure resulting in fatal outcomes (20).

Vancomycin remains the first choice parenteral agent for both empiric and definitive therapy of methicillin-resistant staphylococcal infections. There is limited literature regarding the MIC of vancomycin for staphylococci isolated from clinical samples in Nepal. All staphylococci isolates in the present study were sensitive to vancomycin, which was in concordance with the observations in clinical practices since resistance to the last resort drug is still rare. This result is corroborated by other studies conducted on different samples (6,26-28). Until now, staphylococcal isolates resistant to vancomycin has not been reported from Nepal (28). In the present study, two *S. aureus* and three CNS were detected with intermediate susceptible to vancomycin. Many other authors have reported VISA and VRSA from different countries (26). This reduced susceptibility to vancomycin is attributed to exposure of the staphylococci to this antibiotic.

In our study, all CNS isolates were found sensitive to vancomycin regardless of oxacillin sensitivity, whereas three *S. aureus* resistant to oxacillin had intermediate sensitivity to vancomycin. Pahadi *et al.* (2014) have also reported four VISA isolates with MICs of vancomycin to MRSA ranging from 0.5 μg/ml to 4 μg/ml. In a study carried out by Amatya *et al.* (2014), none of the MRSA isolates were reported to be VISA or VRSA but showed higher MIC (0.5 μg/ml to 2 μg/ml) of vancomycin. The haphazard use of this antibiotic causes selective pressure resulting in the emergence of VISA/VRSA. The use of antibiotics like vancomycin has a major impact on the genetic evolution of staphylococci during the development of drug resistance (9). As vancomycin is a reserve drug, it will be challenging to treat the infections caused by VRSA, and it is emerging as a serious public health problem worldwide (6).

In the present study, reduced susceptibility to vancomycin was associated with methicillin resistance detected by phenotypic methods. Several studies indicated that vancomycin heteroresistant staphylococci were significantly related to the isolation of methicillin-resistant staphylococci among patients with bacteremia (29,30). Inadequate and inaccurate vancomycin therapy for treating infections caused by methicillin-resistant staphylococci increased the selective antibiotic pressure resulting in the selection of non-susceptible strains like VISA or heterogenous vancomycin intermediate *S. aureus* (hVISA) or those with elevated MICs levels. The mechanism underlying the association between methicillin resistance and reduced vancomycin susceptibility remains unclear.

In our study, the resistance of CNS to oxacillin was 80.4 % which is in accordance with other studies (30,31). Irrespective of oxacillin susceptibility, all CNS isolates in the present study were sensitive to vancomycin. It agreed with other studies conducted on different samples that demonstrated all isolates of CNS being sensitive to vancomycin (32,33). Vancomycin resistance is still infrequent in CNS. However, heterogeneous resistance was reported among CNS and was associated with vancomycin therapy failure, which may be a precursor for developing vancomycin-resistant strains (5,6). These heterogeneous resistant strains have a susceptible vancomycin MIC, but they can grow in the presence of 4 μg/mL vancomycin, which is greater than their MIC (33).

Only 83 (22.1 %) isolates were detected to be strong biofilm producers by the TCP method, and among them, 48 (57.8 %) *S. aureus* and 35 (42.2 %) CNS comprised biofilm producers. The biofilm producers were observed more in methicillin-resistant isolates of *S. aureus* and CNS. The polysaccharide layer of biofilm formation was attributed to the *ica* gene. The *ica* genes were observed among 86 (22.9 %) staphylococcal isolates distributed among 45 (28 %) *S. aureus* and 41 (19.2 %) CNS. Most *ica* gene-positive isolates were also found to be methicillin-resistant. A study conducted by Mirani *et al.* (2013) reported that 57 % of MRSA isolates as biofilm producers. The regulation of the *ica* operon is complex, and the *ica* genes are expressed in a stressful environment, and many factors such as high osmolarity, anaerobic condition, increased temperature, and subinhibitory presence of some antibiotics contribute to its expression (34).

About 50% (45/86) of biofilm-producing *S. aureus* and more than half of the biofilm-producing CNS isolates (76/90) were resistant to oxacillin. Furthermore, several studies have demonstrated that methicillin susceptibility influences biofilm production and thereby the presence of *ica* genes

(35-37). In a biofilm, the frequency of horizontal plasmid transfer is much higher than between planktonic cells. Studies on staphylococcal biofilm showed that biofilm promotes the spread of plasmid-borne antibiotic resistance genes by conjugation/mobilization (38). This explains the acquisition of resistant genes to *ica* gene-positive strains (39,40).

The selective pressure generated due to the frequent use of vancomycin to treat methicillin-resistant staphylococcal infections has resulted in the emergence of resistant isolates or reduced susceptibility. In microbial communities growing in biofilm, vancomycin has a reduced effect due to the slow growth rate of bacteria and structured heterogeneity with biofilm (41). In the present study, one biofilm-producing *S. aureus* was detected as VISA, and 32 isolates with a clinical breakpoint of susceptibility, whereas 15 *ica* gene positive-isolates were detected with a clinical breakpoint of susceptibility. In the case of CNS, 19 biofilm producers and 9 *ica* positive isolates were found to have a clinical breakpoint of susceptibility. The emergence of many isolates with a clinical breakpoint of susceptibility to vancomycin can lead to treatment failure (39).

In our study, 89 (41.6 %) *S. epidermidis* were found resistant to oxacillin, followed by *S. saprophyticus* (35, 16.4 %). All CNS species were susceptible to vancomycin irrespective of oxacillin resistance, according to other studies (32, 33, 42). The study showed that although all CNS isolates were sensitive to vancomycin, isolates had a MIC of vancomycin with a clinical breakpoint of susceptibility. The presence of subpopulations with non-susceptible strains like VISA/hVISA or those with elevated MIC demonstrates the heterogeneity of CNS strains in terms of susceptibility to this antibiotic. Such resistance is endogenous (i.e., chromosomal mutation), and the mechanism is highly complex, often linking to a thickening of the bacterial cell wall, with hyperproduction of glycopeptide binding targets and altered autolysis. Additional tests are required to detect the main genes responsible for vancomycin resistance (*vanA* and *vanB*) (9). However, further investigations are required to elucidate the underlying mechanism of how these events occur in staphylococci.

Most of the oxacillin-resistant *S. aureus* and CNS were isolated from tips (11/18) and (45/52), respectively, indicating significant oxacillin resistance with the specimen. Staphylococci being commensal to the skin, easily gain access to the site of skin puncture and deep cuts, which cause uncomplicated infections but at times may develop into complicated infections to systemic failure. Device-related infections by staphylococci are a major problem in health care settings due to the propensity of biofilm formation by organisms that are difficult to eradicate and often lead to systemic, persistent infection and mortality (9,43,44).

The fact that the use of antimicrobial agents has a substantial impact on the genetic evolution of staphylococci during the development of drug resistance, like in the case of vancomycin, should not be ignored (44). The major drawback of this study was the lack of molecular characterization of the isolates and detection of virulent genes, which could be investigated in future research. Such a study may help physicians generate new treatment policies and develop new drugs against the resistant properties of isolates.

CONCLUSION

A very high number of methicillin-resistant isolates were observed among *S. aureus* and CNS. Determination of MIC showed vancomycin can still be used as the drug of choice to treat infections caused by methicillin-resistant staphylococci despite the detection of very few isolates with intermediate susceptibility. The present study demonstrated an association between the presence of the *icaADBC* operon, biofilm formation, and antimicrobial resistance in staphylococci isolated from various specimens. Furthermore, the detection of staphylococci subpopulations with intermediate vancomycin resistance associated with methicillin resistance highlights the role of this organism as an important multidrug-resistant microorganism and the consequent need to implement measures for the control of antibiotic use.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' 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.

REFERENCES

1. Papa R, Artini M, Cellini A, Tilotta M, Galano E, Pucci P, et al. A new anti-infective strategy to reduce the spreading of antibiotic resistance by the action on adhesion-mediated virulence factors in *Staphylococcus aureus*. Microbial Pathogenesis. 2013;63:44–53. DOI: https://doi.org/10.1016/j.micpath.2013.05.003

2. Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman CA, Stobberingh EF. The molecular evolution of methicillin-resistant *Staphylococcus aureus*. Clinical Microbiology and Infection. 2007;13:222-35. DOI: https://doi.org/10.1111/j.1469-0691.2006.01573.x

3. Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. Clinical Microbiology Reviews. 2014;27:870-926. DOI: https://doi.org/10.1128/CMR.00109-13

4. Berger-Bachi B, Rohrer S. Factors influencing methicillin resistance in staphylococci. Archives of Microbiology. 2002;178:165-171. DOI: https://doi.org/10.1007/s00203-002-0436-0

5. LaPlante KL, Rybak MJ, Amjad M, Kaatz GW. Antimicrobial susceptibility and staphylococcal chromosomal cassette *mec* type in community and hospital-associated methicillin-resistant *Staphylococcus aureus*. Pharmacotherapy. 2007;27:3-10. DOI: https://doi.org/10.1592/phco.27.1.3

6. Kshetry AO, Pant ND, Bhandari R, Khatri S, Shrestha KL, Upadhaya SK. Minimum inhibitory concentration of vancomycin to methicillin resistant *Staphylococcus aureus* isolated from different clinical samples at a tertiary care hospital in Nepal. Antimicrobial Resistance and Infection Control. 2016;5:27. DOI: https://doi.org/10.1186/s13756-016-0126-3

7. Sakoulas G, Moellering Jr RC, Eliopoulos GM. Adaptation of methicillin-resistant *Staphylococcus aureus* in the face of vancomycin therapy. Clinical Infectious Diseases. 2006;42 (Suppl 1):S40-50. DOI: https://doi.org/10.1086/491713

8. Gonzalez BE, Rueda AM, Shelburne III SA, Musher DM, Hamill RJ, Hulten KG. Community-associated strains of methicillin-resistant *Staphylococcus aureus* as the cause of healthcare-associated infection. Infection Control and Hospital Epidemiology. 2006;27:1051–1056. DOI: https://doi.org/10.1086/507923

9. Chen SY, Liao CH, Wang JL, Chianh WC, Lai MS, Chie WC. Method-specific performance of vancomycin MIC susceptibility tests in predicting mortality of patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. Journal of Antimicrobial Chemotherapy. 2014;69:211–218. DOI: https://doi.org/10.1093/jac/dkt340

10. Amatya R, Devkota P, Gautam A. Reduced susceptibility to vancomycin in methicillin resistant *Staphylococcus aureus*: a time for action. Nepal Medical College Journal. 2014;16:42–44. PMID: 25799810

11. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Gram positive cocci. In: Hensyl WR (ed). Bergey's manual of determinative microbiology, 9th ed., Williams and Wilkins, Baltimore; 1994: 527-558.

12. Cunha MDLRS, Sinzato YK, Silveira LV. Comparison of methods for the identification of coagulase-negative staphylococci. Memorias do Instituto Oswaldo Cruz. 2004;99:855–860. DOI: https://doi.org/10.1590/s0074-02762004000800012

13. Clinical Laboratory Standards Institute (CLSI). CLSI supplement M100. Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. Wayne, PA: CLSI; 2018.

14. Clinical Laboratory Standards Institute (CLSI). CLSI standard M0. Methods for Dilution Antimicrobial susceptibility test for Bacteria that Grow Aerobically. 11th ed. Wayne, PA: CLSI; 2018.

15. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology.1985;22:996–1006. DOI: https://doi.org/10.1128/jcm.22.6.996-1006.1985

16. Manandhar S, Singh A, Varma A, Pandey A, Shrivastava N. Biofilm Producing Clinical *Staphylococcus aureus* Isolates Augmented Prevalence of Antibiotic Resistant Cases in Tertiary Care Hospitals of Nepal. Frontiers in Microbiology. 2018;9:2749. DOI: https://doi.org/10.3389/fmicb.2018.02749

17. Sanjana R, Shah R, Chaudhary N, Singh Y. Prevalence and antimicrobial susceptibility pattern of methicillin-resistant *Staphylococcus aureus* (MRSA) in CMS-teaching hospi tal: a preliminary report. Journal of College of Medical Sciences Nepal. 2010;l6:1–6. DOI: https://doi.org/10.3126/jcmsn.v6i1.3595

18. Pandey S, Raza MS, Bhatta CP. Prevalence and antibiotic sensitivity pattern of Methicillin-Resistant *Staphylococcus aureus* in Kathmandu Medical College Teaching Hospital. Journal of Institute of Medicine. 2012;34:13–17. DOI: https://doi.org/10.3126/jiom.v34i1.9117

19. Mukhiya RK, Shrestha A, Rai SK, Panta K, Rai G, Prajapati A. Prevalence of Methicillin-Resistant *Staphylococcus aureus* in Hospitals of Kathmandu Valley. Nepal Journal of Science and Technology. 2012;12:185-190. DOI: https://doi.org/10.3126/njst.v13i2.7734

20. Ansari S, Nepal HP, Gautam R, Rayamajhi N, Shrestha S, Upadhyay G, et al. Threat of drug resistant *Staphylococcus aureus* to health in Nepal. BMC Infectious diseases. 2014;14:157. DOI: https://doi.org/10.1186/1471-2334-14-157

21. Bhatta DR, Cavaco LM, Nath G, Gaur A, Gokhale S, Bhatta DR. Threat of multidrug resistant *Staphylococcus aureus* in Western Nepal. Asian Pacific Journal of Tropical Diseases. 2015;5:617–621. DOI: https://doi.org/10.1186/s12879-016-1531-1

22. Pant ND, Sharma M. Carriage of methicillin resistant *Staphylococcus aureus* and awareness of infection control among health care workers working in intensive care unit of a hospital in Nepal. Brazilian Journal of Infectious Disease. 2016;20:218–219. DOI: https://doi.org/10.1016/j.bjid.2015.11.009

23. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug resistant, extensively drug-resistant and pandrug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection.2012;18:268–281. DOI: https://doi.org/10.1111/j.1469-0691.2011.03570.x

24. Ba X, Harrison EM, Edwards GF, Holden MTG, Larsen AR, Petersen A, et al. Novel mutations in penicillin-binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the mec gene. Journal of Antimicrobial Chemotherapy. 2014;69:594–597. DOI: https://doi.org/10.1093/jac/dkt418

25. Maalej SM, Rhimi FM, Fines M, Mnif B, Leclercq R, Hammami A. Analysis of borderline oxacillin resistant *Staphylococcus aureus* (BORSA) strains isolated in Tunisia. Journal of Clinical

Microbiology. 2012;50:3345-3348. DOI: https://doi.org/10.1128/jcm.01354-12

26. Nunes APF, Schuenck RP, Bastos CCR, Magnanini MMR, Long JB, Iorio NLP, et al. Heterogeneous resistance to vancomycin and teicoplanin among *Staphylococcus* spp. isolated from bacteremia. Brazilian Journal of Infectious Disease. 2007;11:345–350.

27. Pahadi PC, Shrestha UT, Adhikari N, Shah PK, Amatya R. Growing resistance to vancomycin among methicillin resistant *Staphylococcus aureus* isolates from different clinical samples. Journal of Nepal Medical Association. 2014;52:977–981.

28. Adhikari R, Pant ND, Neupane S, Neupane M, Bhattari R, Chaudhary R, et al. Detection of Methicillin Resistant *Staphylococcus aureus* and Determination of Minimum Inhibitory Concentration of Vancomycin for *Staphylococcus aureus* isolated from Pus/Wound Swab Samples of the Patients Attending a Tertiary Care Hospital in Kathmandu, Nepal. Canadian Journal of Infectious Diseases and Medical Microbiology. 2017;Article ID 2191532:6 pages. DOI: https://doi.org/10.1155/2017/2191532

29. Mashaly GE, ElMahdy RH. Vancomycin heteroresistance in coagulase negative Staphylococcus blood stream infections from patients of intensive care units in Mansoura University Hospitals, Egypt. Annals of Clinical Microbiology and Antimicrobials. 2017;16:63. DOI: https://doi.org/10.1186/s12941-017-0238-5

30. Ahlstrand E, Svensson K, Persson L, Tidefelt U, Soderquist B. Glycopeptide resistance in coagulase-negative staphylococci isolated in blood cultures from patients with hematological malignancies during three decades. European Journal of Clinical Microbiology and Infectious Diseases. 2011;30:1349–1354. DOI: https://doi.org/10.1007/s10096-011-1228-8

31. Mirani ZA, Aziz M, Khan MN, Lal I, Hassan N ul, Khan SI. Biofilm formation and dispersal of *Staphylococcus aureus* under the influence of oxacillin. Microbial Pathogenesis. 2013;61-62:66–72. DOI: https://doi.org/10.1016/j.micpath.2013.05.002

32. Al Tayyar IA, Al-Zoubi MS, Hussein E, Khudairat S, Sarosiekf K. Prevalence and antimicrobial susceptibility pattern of coagulase-negative staphylococci (CoNS) isolated from clinical specimens in northern of Jordan. Iranian Journal of Microbiology. 2015;7:294–301.

33. May L, Klein EY, Rothman RE, Laxminarayan R. Trends in antibiotic resistance in coagulase-negative staphylococci in the United States, 1999 to 2012. Antimicrobial Agents and Chemotherapy. 2014;58:1404–1409. DOI: https://doi.org/10.1128/AAC.01908-13

34. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews. 2002;15:167–193. DOI: https://doi.org/10.1128/CMR.15.2.167-193.2002

35. Abassi MS, Bouchami O, Touati A, Achour W, Hassen AB. Clonality and occurrence of genes encoding antibiotic resistance and biofilm in methicillin-resistant *Staphylococcus epidermidis* strains isolated from catheters and bacteremia in neutropenic patients. Current Microbiology. 2008;57:442-448. DOI: https://doi.org/10.1007/s00284-008-9227-4

36. Koksal F, Yasar H, Samasti M. Antibiotic resistance patterns of coagulase-negative staphylococcus strains isolated from blood cultures of septicemic patients in Turkey. Microbiological Research. 2009;164:404-410. DOI: https://doi.org/10.1016/j.micres.2007.03.004

37. Klingenberg C, Aarag E, Ronnestad A, Sollid JE, Abrahamsen TG, Kjeldsen G, et al. Coagulase-negative staphylococcal sepsis in neonates: association between antibiotic resistance, biofilm formation and the host inflammatory response. Pediatric Infectious Disease Journal. 2005;24:817-822. DOI: https://doi.org/10.1097/01.inf.0000176735.20008.cd

38. Savage VJ, Chopra I, O'Neill AJ. *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. Antimicrobial Agents and Chemotherapy. 2013;57:1968–1970. DOI: https://doi.org/10.1128/aac.02008-12

39. Loomba PS, Taneja J, Mishra B. Methicillin and vancomycin resistant *Staphylococcus aureus* in hospitalized patients. Journal of Global Infectious Disease. 2010;2:275-283. https://www.jgid.org/text.asp?2010/2/3/275/68535

40. Raad I, Hanna H, Jiang Y, Dvorak T, Reitzel R, Chaiban G. Comparative activities of daptomycin, linezolid and tigecycline against catheter-related methicillin resistant Staphylococcus bacteremic isolates embedded in biofilm. Antimicrobial Agents and Chemotherapy. 2007;51:1656-1660. DOI: https://doi.org/10.1128/AAC.00350-06.

41. Palazzo ICV, Araujo MLC, Darini ALC. First report of vancomycin-resistant staphylococci isolated from healthy carriers in Brazil. Journal of Clinical Microbiology. 2005;43:179-185. DOI: https://doi.org/10.1128/JCM.43.1.179-185.2005

42. Shrestha LB, Bhattarai NR, Khanal B. Antibiotic resistance and biofilm formation among coagulase-negative staphylococci isolated from clinical samples at a tertiary care hospital of eastern Nepal. Antimicrobial resistance and infection control. 2017;6:89. DOI: https://doi.org/10.1186/s13756-017-0251-7

43. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. *Staphylococcus aureus* biofilms. Virulence. 2011;2:445–459. DOI: https://doi.org/10.4161/viru.2.5.17724

44. Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials. 2012;33:5967–5982. DOI: https://doi.org/10.1016/j.biomaterials.2012.05.031