Vancomycin Resistant *Staphylococcus aureus* Reported from Tertiary Care Hospital in Nepal

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ABSTRACT

Objectives: The study was conducted to assess the rate of Methicillin-resistant *Staphylococcus aureus* (MRSA) among patients and healthcare personnel at Manmohan Memorial College and Teaching Hospital, Kathmandu, Nepal and to evaluate the minimum inhibitory concentration of Vancomycin to MRSA isolates.

Methods: A total of 1433 different clinical specimens from patients and 33 nasal swabs from healthcare personnel were subjected to bacteriological investigation following standard protocol. *S. aureus* were isolated and identified by using standard Microbiological tools. Those isolates were subjected to Antimicrobial susceptibility testingusing modified Kirby-Bauer's disc diffusion method following CLSI guidelines.

Results: The rate of *S. aureus* carriage was found to be 65 (18.9%) in the samples from clinical patients and 24 (72.7%) in the samples from healthcare personnel. The rate of MRSA was found to be 57(85.1%) in patients and 24 (100%) in healthcare personnel. The high distribution of MRSA was found in female of age group 21-30 years (patients: 10.4%; healthcare personnel: 70.8%). Amikacin was found to be most effective antimicrobial. All *S. aureus* isolates were found to be multidrug resistant (100%).On performing D-test, 10 (17.5%) and 22 (38.6%) of MRSA from clinical specimens showed inducible and constitutive Clindamycin resistance respectively. Whereas, 11 (45.8%) and 4 (16.7%) of MRSA from nasal swabs were found to be inducible and constitutive Clindamycin resistance respectively. Upon performing minimum inhibitory concentration (MIC) test for clinical isolates, 3.5% (2) of MRSA were found to be Vancomycin resistant (VRSA), 54.4% (31) were Vancomycin intermediate (VISA) and 42.1% (24) were found to be Vancomycin sensitive (VSSA). All of the nasal swab MRSA isolates were found sensitive to Vancomycin. Congo red agar method was done for biofilm production. For clinical isolates, 32 (47.8%) were found to be strong, 6 (8.9%) moderate and 29 (43.3%) were non biofilm producer. For nasal swab isolates, 66.7% (16) and 33.3% (8) were found as strong and non-biofilm producer respectively.

Conclusion: This study reported the case of VRSA which hasn't been reported in Nepal. Though present study showed that Vancomycin remains the main choice of treatment of MRSA infection. Therefore, to preserve its value, use of vancomycin should be limited only to those cases where there are clearly needed.

Key words: *S. aureus*, MRSA, D-test, Inducible Clindamycin resistance,VRSA

INTRODUCTION

Staphylococcus aureus is one of the common human pathogens capable of causing a wide range of infections. A great deal of virulence from the organism occurs through cross infection by patient to patient in hospitals and other institutional settings. In contrast, healthy individuals have a small risk of invasive infection caused by *S. aureus*, but they can

be carriers of the organism (Foster 2004). Infection due to Methicillin-Resistant *S. aureus* (MRSA) are an increasing problem worldwide in community as well as hospital environment (Boyce et al. 2005; Skoy et al. 2006). The incidence of community-acquired and hospital-acquired *S. aureus* infections has been rising with increasing emergence of drug-resistant strains called Methicillin resistant *S. aureus* (MRSA) (Steinberg et al. 1996).

The resistance of *S. aureus* to Methicillin is caused by the *mecA* gene which codes the low affinity 78-Kda penicillin-binding protein (PBP2a). Beta-lactam antibiotic normally binds to PBPs in the cell wall, resulting in the disruption of synthesis of the peptidoglycan layer and death of bacterium. Since the beta-lactam antibiotics cannot bind to low affinity PBP2a, synthesis of peptidoglycan layer and cell wall are able to continue (Duerenberg 2007). MRSA infections often require systematic antibiotic therapy. The spread of MRSA can also be potentially minimized by prevention of the risk factors such as previous antibiotic use, contact with the healthcare workers or nursing home resident, daycare attendance, hospitalization, admission to an intensive care unit, intravenous drug use, invasive indwelling devices, haemodialysis or peritoneal dialysis, immunosuppression, chronic illness, and previous isolation of MRSA (Cohen 2007).

Following the spread of MRSA, glycopeptides (usually Vancomycin and more recently Teicoplanin) have become the mainstay of treatment for MRSA infections (CDC 2013). Vancomycin is the choice of drug for MRSA isolates. Patients unable to tolerate vancomycin have been treated with fluoroquinolones, Trimethoprim-Sulfomethoxazole, Clindamycin or Minocycline (Shah 2008). As Vancomycin is commonly used for the treatment of MRSA infections, which has resulted into development of Vancomycin-Intermediate *S. aureus* (VISA) and Vancomycin-Resistant *S. aureus* (VRSA).

Clindamycin, a lincosamide drug, has been used to treat serious infections caused by susceptible *S. aureus* in children for more than 30 years (Woods 2009). It is also an alternative choice in case of Penicillin allergic patients. Clindamycin is recommended in some European countries for suppression of panton-valentine leukocidin (PVL) toxin, along with Linezolid and Rifampin (Adaleti et al. 2010). In vitro, *S. aureus* isolates with constitutive resistant are resistant to Erythromycin and Clindamycin while isolates with inducible resistant are resistant to Erythromycin but appear susceptible to Clindamycin (Steward et al. 2005). Inducible MLSB (Macrolide, Lincosamide and Streptogramin B) resistant can be detected by discapproximation test (D-test) by placing Erythromycin and Clindamycin discs in adjacent positions (Fiebelkorn et al. 2003).

Biofilms are communities of microorganisms embedded in extracellular polymeric substances (EPS) matrix. Bacteria in biofilms demonstrate distinct features from their free-living planktonic counterparts, such as different physiology and high resistance to immune system and antibiotics that render biofilm a source of chronic and persistent infections. Extracellular polymeric matrix plays various roles in structure and function of different biofilm communities. Adhesion to the surface provides considerable advantages such as protection against antimicrobial agent, acquisition of new genetic traits and the nutrients availability and metabolic co-operability. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defense system (Donlan and Costerton 2002).

The study was thus done to determine the rate of Methicillin resistant, emergence of Vancomycin resistant and inducible Clindamycin resistant *S. aureus* among biofilm producing and non-producing isolates of *S. aureus*.

MATERIALS AND METHODS

Study design: A hospital based cross sectional descriptive study was conducted.

Study period, site and population: The study was conducted at Manmohan Memorial Medical College and Teaching Hospital, Swoyambhu, Kathmandu, Nepal in collaboration with Kantipur College of Medical Science, Sitapaila, Kathmandu, Nepal from April 2014 to October 2014. All the clinical specimens obtained from individuals of all ages and sexes visiting hospital during the study period were included in the study.

Sample size: A total of 1344 clinical specimens including blood, urine, sputum, vaginal swab, eye swab, ear swab, throat swab, wound swab, clavical swab, body fluids like pus, synovial fluid, pleural fluid, asiatic fluid, peritoneal fluid and catheter swabs and urethral discharge; were processed in the study. For the study of hospital acquired MRSA, a total of 33 nasal swabs were collected from the hospital personnel.

Laboratory diagnosis

Sample collection: Sterilized sample collection container was used for the collection of all clinical specimens. Blood was collected with sterile syringe and then poured in leak proof, dry and sterilized container.

Sample processing and bacterial identification: All the clinical samples were inoculated onto blood agar, chocolate agar and mac-conkey agar plates. Blood samples were inoculated into brain heart infusion broth and incubated at 37°C for 7 days and then further inoculated into agar media. All the culture plates were then incubated at 37°C for overnight. The plates showing growth of bacterial were processed for identification of *S. aureus* using standard microbiological procedures by inoculating the organism on mannitol salt agar and performing specific biochemical tests catalase test, coagulase test and oxidative-fermentative test.

Antimicrobial susceptibility testing and screening of multi drug resistant (MDR) *S. aureus*: All the identified isolates of S. aureus were undertaken in-vitro antibiotic susceptibility test by using modified Kirby-Bauer's disc diffusion method (CLSI 2013). The antibiotics used were Cefoxitin (5mcg), Ciprofloxacin (5mcg), Cefixime (5mcg), Tetracycline (30mcg), Amikacin (30mcg), Azithromycin (30mcg), Vancomycin (30mcg), Cloxacillin (5mcg), Cefotaxime (30mcg), Clindamycin (10mcg), Ceftriaxone (30mcg), Erythromycin (15mcg), Gentamicin (10mcg), Penicillin (10mcg), Co-trimoxazole (25mcg), Mupirocin (5mcg), and Chloramphenicol (50mcg). The organism resistant to three or more antibiotics of different classes were classified as MDR isolates (Magiorakos et al. 2012). Intrinsic resistance to any of the employed antibiotics was not counted.

Screening of methicillin resistant S. aureus: Screening for Methicillin resistant*S. aureus* was carried out by Cefoxitin disc diffusion method and interpreted according to CLSI (2013) guidelines. The growth of *S. aureus* with zone of inhibition around Cefoxin disc (ZOI) ≥ 22mm were identified as Methicillin sensitive *S. aureus* and that of ZOI ≤ 21 were identified as Methicillin resistant *S. aureus*.

Detection of Inducible clindamycin resistance (ICR): In this assay, two discs namely Erythromycin and Clindamycin were placed 18mm away edge-to-edge on Muller Hinton agar plates that were previously inoculated with 0.5 McFarland bacterial suspensions. Plates were observed after 18 hours of incubation at 35±2°C. Flattening of the zone of inhibition adjacent to the Erythromycin disc (referred to as D-zone) or hazy growth within the zone of inhibition around Clindamycin (even if no D- zone is apparent) is regarded as positive test, i.e. Inducible Clindamycin resistance (CLSI 2013).

Determination of minimum inhibitory concentration of vancomycin: Minimum inhibitory concentration (MIC) technique was performed to determine the Vancomycin intermediate and resistant strains of *S. aureus* isolates MIC to Vancomycin in isolated MRSA was done by agar dilution method following CLSI guidelines (CLSI 2013). Different concentrations ranging from 0.06-32µg/ml of Vancomycin incorporated plates was prepared. Positive growth controls were kept for each isolates and *S. aureus* (ATCC 25923) of known MIC was also included in each test as control for antibiotic potency.

Biofilm production: Biofilm detection was carried out by Congo Red Agar method (CRA**):** CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose. CRA plates was inoculated with test organisms and incubate at 37°C for overnight aerobically. Black colonies were considered as biofilm producing isolates (Freeman et al. 1989).

RESULTS

Out of total 1433 clinical specimens, *S. aureus* was isolatesd from 67 specimens (4.7%), among them 57 (85.1%) were found to be MRSA. Whereas from 33 nasal swab specimens, 24 (72.7%) *S. aureus* were isolated and all of them were found to be MRSA (100%). All of the *S. aureus* isolates from clinical as well as nasal swab specimens were multi-drug resistant (MDR) (Figure 1).

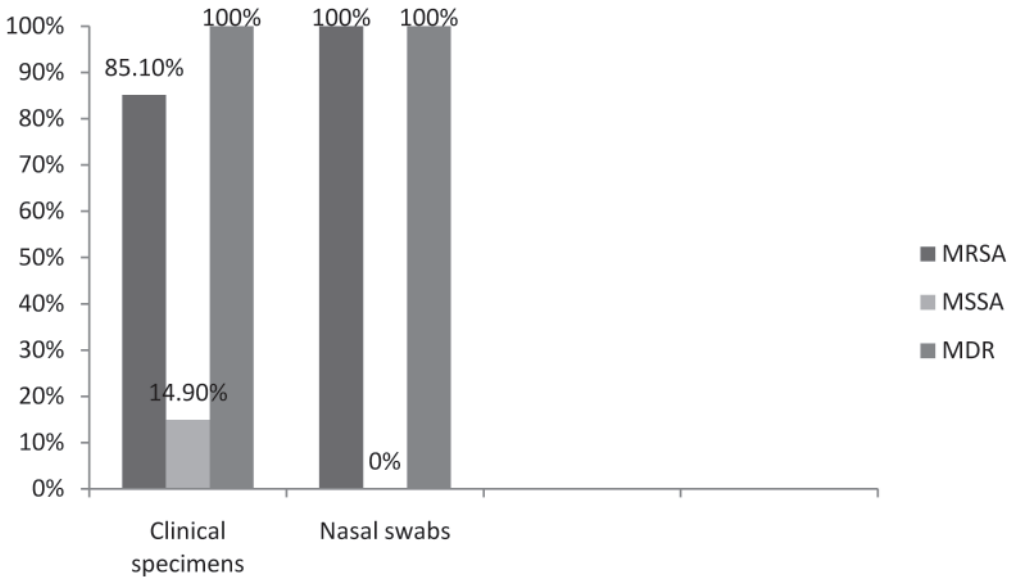

Figure 1: Distribution of MRSA, MSSA and MDR S. aureus

Among total of 67 *S. aureus* isolates, 27 (40.3%) from male and 30 (44.8%) from female. High rate of MRSA 12 (17.9%) was obtained from age group 21-30 year (Table 1).

Table 1: Age and sex wise distribution of MRSA from clinical specimens

Age group (year)	Total No. of samples	Male Number (%)			Female Number (%)			Total no. of MRSA (%)
		Samples	*S. aureus*	MRSA	Samples	*S. aureus*	MRSA	
<10	109	56	3(4.5)	2(2.9)	53	1(1.5)	1(1.5)	3(4.5)
11-20	204	99	7(10.4)	4(5.9)	105	2(2.9)	2(2.9)	6(9)
21-30	382	121	6(9)	5(7.5)	261	7(10.4)	7(10.4)	12(17.9)
31-40	202	86	3(4.5)	2(2.9)	116	4(5.9)	4(5.9)	6(9)
41-50	124	65	6(9)	6(9)	59	4(5.9)	4(5.9)	10(14.9)
51-60	128	40	1(1.5)	1(1.5)	88	6(9)	5(7.5)	6(9)
61-70	108	55	3(4.5)	3(4.5)	53	5(7.5)	3(4.5)	6(9)
71-80	87	37	2(2.9)	2(2.9)	50	3(4.5)	2(2.9)	4(5.9)
>80	89	51	2(2.9)	2(2.9)	38	2(2.9)	2(2.9)	4(5.9)
Total	**1433**	**610**	**33 (49.2)**	**27 (40.3)**	**823**	**34 (50.7)**	**30 (44.8)**	**57 (85.1)**

Among 24 nasal swab MRSA isolates, 2 (8.3%) were from male and 22 (91.7%) were from female and High rate of MRSA was 19 (79.2%) obtained from age group 21-30 year (Table 2).

Table 2: Age and sex wise distribution of MRSA from nasal swabs

Age group (year)	Total no. of samples	Male Number (%)			Female Number (%)			Total no. of MRSA (%)
		Samples	*S. aureus*	MRSA	Samples	*S. aureus*	MRSA	
<10	-	-	-	-	-	-	-	-
11-20	4	1	-	-	3	2(8.3)	2(8.3)	2(8.3)
21-30	22	3	2(8.3)	2(8.3)	21	17 (70.8)	17 (70.8)	19 (79.2)
31-40	7	-	-	-	4	3(12.5)	3(12.5)	3(12.5)
Total	**33**	**4**	**2 (8.3)**	**2 (8.3)**	**29**	**22 (91.7)**	**22 (91.7)**	**24 (100)**

MRSA from clinical specimens were 100% resistant to Penicillin G, Co-trimoxazole and Cefixime, followed by Cloxacillin (94.7%), Ceftriaxone (93%), Vancomycin (92.2%), and Cefotaxime (84.2%). Whereas MMSA isolates were 100% resistant to Cefixime, followed by Cefotaxime (90%), Ceftriaxone (90%), Penicillin G (80%) and Co-trimoxazole (80%). For nasal swab MRSA isolates, 100% showed resistance towards Cefixime, Cefotaxime, Penicillin G and Co-trimoxazole, followed by Cloxacillin (75%), Clindamycin (54.2%), and Tetracycline (50%). Whereas no MMSA isolates were obtained (Table 3).

Table 3: Antimicrobial resistance profile of MRSA and MSSA

Antibiotics	Clinical specimens		Nasal swab specimens	
	Antibiotic profile of MRSA (n=57)	Antibiotic profile of MSSA (n=10)	Antibiotic profile of MRSA (n=24)	profile of MSSA Antibiotic (n=0)
Cefoxitin	57(100%)	-	24(100%)	-
Ciprofloxacin	15(26.3%)	3(30%)	8(33.3%)	-
Cefixime	57(100%)	10(100%)	24(100%)	-
Tetracyclin	32(56.1%)	-	12(50%)	-
Amikacin	8(14%)	-	4(16.7%)	-
Azithromycin	25(43.9%)	1(10%)	6(25%)	-
Vancomycin	53(92.9%)	4(40%)	8(33.3%)	-
Cloxacillin	54(94.7%)	1(10%)	18(75%)	-
Cefotaxime	48(84.2%)	9(90%)	24(100%)	-
Clindamycin	42(73.7%)	4(40%)	13(54.2%)	-
Ceftriaxone	53(93%)	9(90%)	24(100%)	-
Erythromycin	32(56.1%)	-	8(33.3%)	-
Gentamicin	13(22.8%)	-	4(16.7%)	-
Penicillin G	57(100%)	8(80%)	24(100%)	-
Co-trimoxazole	57(100%)	8(80%)	24(100%)	-
Mupirocin	43(75.4%)	3(30%)	18(75%)	-
Cloramphenicol	11(19.3%)	-	3(12.5%)	-

Upon performing D-test, 10 (17.5%) and 22 (38.6%) of MRSA from clinical specimens showed inducible and constitutive Clindamycin resistance respectively. Whereas, 11 (45.8%) and 4 (16.7%) of MRSA from nasal swabs were found to be inducible and constitutive Clindamycin resistance respectively (Table 4).

Table 4: D-test of MRSA isolates

Phenotype	Erythromycin	Clindamycin	D-test	MRSA (%)	
				Clinical isolates	Nasal swab isolates
iMLSB	Resistance	Sensitive	Positive	10 (17.5)	11 (45.8)
cMLSB	Resistance	Resistance	Negative	22 (38.6)	4 (16.7%)

From clinical specimens, 47.8% (32), 8.9% (6) and 43.3% (29) of *S. aureus* isolates were found to be strong, moderate and non-biofilm producer respectively. Among nasal swab specimens, 66.7% (16) and 33.3% (8) of *S. aureus* isolates were found to be strong and non-biofilm producer respectively (Table 5).

Table 5: Biofilm production by *S. aureus*

Serial no.	Strong producer	Moderate producer	Non producer
1.clinical specimen (n=67)	32(47.8%)	6(8.9%)	29(43.3%)
2.Nasal swab (n=24)	16(66.7)	-	8(33.3%)

On performing MIC of MRSA from clinical specimens, 42.1% (24) of isolates were reported as VSSA (showed MIC value of 2µg/ml), 54.4% (31) as VISA (showed MIC value of 4-8µg/ml) and 3.5% (2) of MRSA isolates were reported as VRSA (showed MIC value of ≥16µg/ml) (Figure 2). Whereas all MRSA isolates from nasal swabs were found to be Vancomycin sensitive (VSSA).

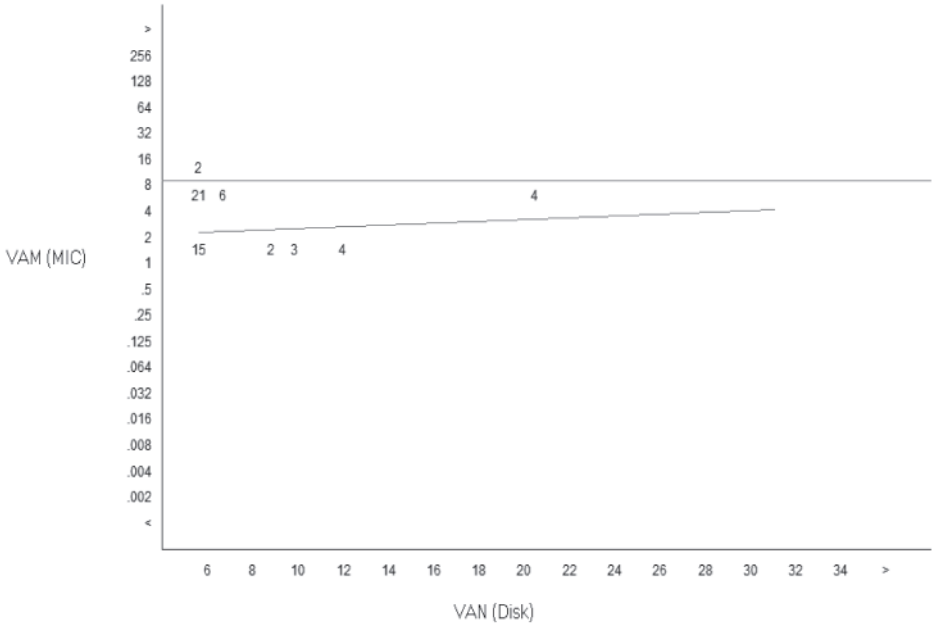

Figure 2: Scatter plot analysis of MIC of vancomycin for MRSA isolates from clinical specimens (WHONET 5.6)

DISCUSSION

S. aureus has remained a versatile and potent pathogen in humans, since it is one of the most common causes of nosocomial and community acquired infections (Rajbhandari et al. 2003). *S. aureus* is a major cause of infectious morbidity and mortality around the world, causing a wide variety of clinical manifestations ranging from localized infection to toxin mediated diseases and invasive blood stream infections(Vandecasteele et al. 2008).

In this study, the rate of MRSA isolation was found to be 85.1% from clinical specimens. This result is higher than the many other studies conducted by Kumari et al. (26.14%), Shakya et al. (12.5%) and Tiwari et al. (69.1%) (Tiwari et al. 2006; Kumari et al. 2008; Shakya et al. 2010). All of the *S. aureus* isolates from nasal swab specimens were found to be MRSA i.e. 100%. Nasal carriage rate of MRSA among health care workers in hospital setting ranges from 6-17.8% (Cesur and Cokca 2004; Pant and Rai 2007). Nasal carriage rate of 43.8% has been reported among the healthcare personnel of a Medical College Teaching Hospital in Kathmandu (Pant and Rai 2007). The nasal carriage rate of *S. aureus* in this study i.e. 72.7% was found to be greater than the study conducted by Shakya et al. i.e. 12.5% (Shakya et al. 2010). The result is also in agreement with the study by Gonsu et al. (Gonsu et al. 2013).

Regarding the sex wise distribution of MRSA clinical specimens, the study showed high incidence of MRSA from female patients (44.8%) than males (40.3%). The present study showed the opposite variation with the study conducted by Boucher and Corey (Boucher and Corey 2008) showing males (64.4%) were more predisposed than females (35.6%). The highest distribution of MRSA was found within the age group of 21-30 years (17.9%) and the lowest in the age group below 10 years (4.5%). However, the study conducted by Arch et al. (Arch et al. 2006) and Lucet et al. (Lucet et al. 2003) showed high rate of MRSA colonization among the population with age group 60 years and above.

The nasal carriage rate of MRSA reported in present study was found to be higher (72.7%) than the previous studies conducted in Nepal by Shakya et al. and Rijal et al. (Rijal et al. 2008; Shakya et al. 2010).

Penicillin was found resistant to all of MRSA isolates

i.e. 100%. This result is higher than that of Shrestha et al. who reported 91.94% (Shrestha et al. 2009). In present study, clinical MRSA isolates showed rate of resistance to antibiotics Co-trimoxazole (100%), followed by Cloxacillin (94.7%), Ceftriazone (93%), Vancomycin (92.9%), Cefotaxime (84.2%), Mupirocin (75.4%), Clindamycin (73.7%), Tetrcyclin (56.1%) and Erythromycin (56.1%). Rijal et al. reported the rate of resistance to Cloxacillin (68.8%), followed by Tetracycline (15.6%) and Erythromycin (9.4%) (Rijal et al. 2008). Resistance to Erythromycin is seen to be greater than the finding disseminated by study conducted by Mishra i.e. 14.29% (Mishra 2008)and lower than the finding disseminated by Tiwari et al. i.e. 68.7%(Tiwari et al. 2006).

All isolates were found to be multi drug resistant (MDR) in this study. The rate of MDR-MRSA (100%) is higher than that of the result reported in the studies conducted by Tiwari et al. i.e. 40.1% and Pandey et al. i.e. 75.86%. Though this study is in accordance with the previous studies from Nepal and other countries showing high percentage of MDR among MRSA; >65% by Kumari et al., 93% by Rahimi et al. and 63% by Salah et al. (Kumari et al. 2008; Salah et al. 2012; Rahimi et al. 2013).

In this study, 17.5% and 38.6% isolates were found to be inducible and constitutive Clindamycin resistance respectively. Among nasal swab specimens taken from hospital staffs, 45.5% and 16.7% were found to be inducible and constitutive Clindamycin resistance respectively. In the study conducted by Ujwol et al. (Bhomi et al. 2016), D-test positive isolates were found to be 18.03% and study also reported constitutive resistance in 36.06% of isolates.

Upon performing MIC, 3.5% (Skoy et al. 2006)) of clinical MRSA isolates were reported as VRSA, 54.4% (31) as VISA and 24.1% (Vandecasteele et al. 2008) as VSSA. Assadullah et al. (Assadullah et al. 2003); Sharma and Vishwanath (Sharma and Vishwanath 2012) reported 18.3% and 11.54% VISA among MRSA respectively. In the study carried out by Venubabu et al. (Venubabu et al. 2011), who reported 1.9% VRSA from India. Likewise Tiwari and Sen (Tiwari and Sen 2006) reported two strains of VRSA and six strains of VISA in the Northen part of India.

Biofilm production by *S. aureus* was found to be 47.8% strong, 8.955% moderate and 43.3% biofilm non producers. Whereas 66.7% and 33.3% of isolates were found to be strong and biofilm non-producer from nasal swab specimens respectively. A study, conducted by Mirani et al. (Mirami et al. 2013) reported 57% of MRSA isolates as biofilm producer. Rewatkar and Wadher (Rewatkar and Wadher, 2013) reported 90% of strong biofilm producer and remaining 10% of weak/ none producer.

CONCLUSION

Higher rate of MRSA was found in female in age group 21-30 years. Amikacin was found to be most effective drug, whereas penicillin G was found as the least effective drug. Upon performing MIC test of MRSA isolates from clinical specimens, 3.5% (2) of MRSA isolates were found as VRSA and 54.4% (31) and 42.1% (24) were as VISA and VSSA respectively. Whereas all nasal swab isolates were sensitive to Vancomycin. From this study, it could be concluded that all of the MRSA isolates i.e. 100% were multi drug resistant (MDR), which is the significant public health problem in context of Nepal, indicating the high risk of staphylococcal infections in our context. This high load of MDR organisms provokes the necessity of strictly performing susceptibility testing before starting antibiotic therapy, or there may be chance of clinical failure. Thus determination of MIC of Vancomycin is crucial. Inducible Clindamycin resistance test cannot be observed in routinely done antibiotic susceptibility testing by Kirby Bauer method hence specific D-test should be performed before treatment with Clindamycin. There are various methods for detection of biofilm production and both tube test and agar plate methods can be carried out for comparative study.

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