Molecular Detection of *mecA* and *aac(6′)-aph(2″)* Genes in *Staphylococcus aureus* Isolates from Clinical Specimens

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ABSTRACT

Objectives: The main aim of this study is to assess the prevalence of *Staphylococcus aureus* in clinical samples and its antimicrobial susceptibility pattern based on the detection of *mecA* and *aac(6′)-aph(2″)* genes conferring resistant ability to *S. aureus* isolates.

Methods: A prospective hospital based cross-sectional study was conducted among the patients visiting Kirtipur hospital for 6 months from May 2020 to November 2020. The clinical samples were collected and processed for culture and identified following standard microbiological procedures. Antibiotic sensitivity testing was performed by Kirby Bauer disc diffusion method and methicillin resistance was determined by using cefoxitin (30 µg) disc and interpreted as per CLSI guidelines. The chromosomal and plasmid DNA were extracted and *aac(6′)-aph(2″)* and *mecA* genes were detected by conventional polymerase chain reaction.

Results: Out of 1969 clinical samples, the overall prevalence of *S. aureus* was 9.82% and among the staphylococci, 50(75.8%) were MRSA. Male patients from 31-45 age group and those visiting OPD were more prone to MRSA infection, although only patient's status and age group were statistically significant. Most of the MRSA were recovered from wound swab (78.2%). A majority of the MRSA isolates were resistant to Ciprofloxacin (90%). Similarly, resistance to Gentamicin was observed in only 12(24%) isolates. Resistance to Gentamicin was statistically associated with types of samples but not with gender, age group and patient status. Among the 50 MRSA isolates, 10 were identified as co-harboring both the *aac(6′)-aph(2″)* and *mecA* genes. Notably, excluding only 2 MRSA isolates, the remaining 48 isolates demonstrated the presence of the *mecA* gene.

Conclusion: High prevalence of MRSA in this study underscores the need for more commitment towards infection control measures that meet the standard protocols aimed at reducing the spread of infection by MRSA among susceptible individuals.

Keywords: *Staphylococcus aureus*, MRSA, Gentamicin resistance, *aac(6′)-aph(2″)*

INTRODUCTION

Staphylococcus aureus is a facultative aerobic gram positive cocci discovered by Scottish surgeon Sir Alexander Ogston (Elward, McAndrews and Young, 2009) which causes a wide variety of infections such as vascular, catheter related infections, pleuro pulmonary infections, skin and soft tissue infections, infective endocarditis, osteoarticular infections (Tong et al. 2015). The skin, rectum, vagina, gastrointestinal system and axilla are all places where *Staphylococcus aureus* can be found with the anterior nares serving as the main reservoir colonizing up to 30% of the human population

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asymptomatically and permanently (Sakr et al. 2018). It is the most common cause of soft tissue infection followed by *Streptococcus* species and *Pseudomonas* species (Bouvet et al. 2017). *S. aureus* is responsible for a high extent of serious infections in hospitals-both admitted and outpatient (Fry and Barie, 2011). In clinical settings, a major issue associated with *S. aureus* has been the remarkable level of acquisition of resistance against multiple antibiotic classes, complicating treatment. Historically, *S. aureus* resistance emerged within 2 years of the introduction of penicillin (Kirby, 1944). Methicillin resistance *Staphylococcus aureus* (MRSA) is due to penicillin binding protein encoded by *mecA* gene (Tominaga and Ishii, 2020) which shows worse therapeutic outcomes than Methicillin Sensitive *Staphylococcus aureus* (MSSA) as resistance to oxacillin or cefoxitin implies non-susceptibility to all categories of β-lactam antimicrobials (i.e. penicillin, cephalosporins, β-lactamase inhibitors and carbapenems) and subsequently the isolates is termed as Multi Drug Resistant (MDR) (Kaur and Chate, 2015). Aminoglycoside such as gentamicin was found highly effective in the treatment of staphylococcal infection. Aminoglycoside such as gentamicin was highly effective against staphylococcal bacteraemia, infections of the skin, soft tissue, pneumonia and lung abscess, gastrointestinal and genitourinary system (Liu et al. 2011). Resistance to aminoglycosides is mostly by the inactivation of antibiotics by aminoglycoside-modifying enzymes (AMEs) whereas resistance to gentamicin in most of the *S. aureus* isolate is mediated by *aac(6')-aph(2")* gene (Udo and Dashti, 2000). These gene has also been detected in *Streptococcus agalactiae* (Doumith et al. 2017), group G *Streptococcus* (Galimand et al. 1999) and *Streptococcus mitis* (Kaufhold and Potgieter, 1993). The incidence of MRSA in Nepal in last two decades has been found to be increasing from 26.1% in 2004 to 26.14% in 2008 (Kumari, Mohapatra and Singh, 2008), 39.6% in 2010 (Sanjana et al. 2010), 42.4% in 2013 (Shrestha, 2013), 55.3% in 2018 (Ansari et al. 2014) reaching 70.6% in 2019 (Belbase et al. 2017). Even though the gentamicin resistance has been observed in MRSA isolates, no any scientific works targeting gentamicin resistant gene among MRSA isolates have been done so far in the context of Nepal. Therefore this study was aimed at isolating *Staphylococcus aureus* from various clinical samples and to detect methicillin resistance gene, *mecA* and gentamicin resistance gene, *aac(6')-aph(2")* in MRSA as well as MSSA in one of the tertiary care hospitals in Nepal. The results of the work also provide the information about prevalence of gentamicin resistance in methicillin resistant *Staphylococcus aureus* (MRSA) as well as gentamicin resistance in methicillin sensitive *Staphylococcus aureus* (MSSA).

MATERIALS AND METHODS

Study design and settings

A prospective hospital based cross-sectional study was conducted in patients visiting Kirtipur hospital during 6 months from May 2020 to November 2020. Data were collected using a semi-structured questionnaire. Chi-square test ($\chi 2$) was used to determine significant associations between various attributes including age and gender of the patients with the prevalence rate of MRSA.

Sample size, inclusion and exclusion criteria

A total of 1969 urine samples were collected from all suspected patients. Samples showing mixed growth and non-significant growth on the culture were rejected and only the samples showing significant growth were included in the study. Samples with inappropriate labeling and leaked samples were also excluded from the study.

Sample processing and identification

A loop full of urine and pus samples were streaked on Cysteine Lactose Electrolyte Deficient (CLED) agar and rest of the samples were cultured on Blood Agar and incubated at 37°C for 24 h aerobically (Kitara et al. 2011). Deep yellow colonies in CLED agar and beta-hemolytic colonies on blood agar were suspected colonies of Staphylococci which were further sub-cultured on MSA. Golden yellow colonies of *S. aureus* were further confirmed by performing coagulase and DNase tests. All the test were performed in duplicates in order to ensure that the growth was not due to contamination.

Antibiotic susceptibility test (AST) of the isolates

Antibiotic sensitivity testing was performed by Kirby Bauer disc diffusion method and methicillin resistance was determined by using cefoxitin (30 µg) disc and interpreted as per CLSI guidelines (Sapkota et al. 2019). Resistance to at least one drug from 3 different antibiotics of different structural classes was considered MDR as described elsewhere (Regmi et al. 2020) .

Extraction of chromosomal DNA

All *Staphylococcus aureus* isolates were analyzed for the presence of *mecA* and *aac(6')-aph(2")* gene. The isolates

were grown in Luria Bertani (LB) broth at 37°C for 24 hours in an orbital shaker at 120 revolutions per minute (rpm). Chromosomal DNA from *Staphylococcus aureus* was harvested by following chromosomal DNA extraction method as described by (Regan et al. 2012). Briefly, the bacterial cells were lysed with 3-5 mg/mL lysozyme in the presence of 1/10 volume of 10% sodium dodecyl sulfate (SDS) at high pH and the lysate was then neutralized with subsequent deproteinization with 1:1 phenol:chloroform. Chromosomal DNA was precipitated with ethanol by spinning at high speed and extracted DNA was stored at -70°C (Sahm et al. 2001).

Extraction of plasmid DNA

MRSA and MSSA isolates were grown in Luria Bertani (LB) broth at 37°C in an orbital shaker at 120 revolutions per minute for 24 hours. Plasmid DNA from *Staphylococcus aureus* was harvested by following alkaline lysine method (Green and Sambrook, 2012).

Detection of *mecA* gene and *aac(6′)-aph(2″)* gene by Polymerase Chain Reaction

The primer pair with forward primer 5′-ACTGCTATCCACCCTCAAAC- 3′and reverse primer 5′-CTGGTGAAGTTGTAATCTGG –3′ were used for the amplification of *mecA* gene (Shah et al. 2020). The reaction mixture for the *mecA* gene was 25 µL and consisted of 21 µL of 1X Qiagen master mix, 0.5 µL of 10 picomolar primer (forward and reverse) and 3 µL of extracted DNA template. Amplification was performed following the conditions which were initial denaturation at 95°C for 15 minutes, denaturation at 94°C for one minute, annealing at 57°C for one minute 30 seconds, extension at 72°C for one minute of 35 cycles and final extension at 72°C for seven minutes. The primer pair with forward primer 5′-CCAAGAGCAATAAGGGCATACC- 3′and reverse primer 5′- CACACTATCATAACCACTACCG –3′ were used for the amplification of aac(6′)-aph(2″) gene (Mahdiyoun et al. 2016). The reaction mixture for the *aac(6′)-aph(2″)* gene was 25 µL and consisted of 21 µL of 1X Qiagen master mix, 0.5 µL of 10 picomolar primer (forward and reverse) and 3 µL of extracted DNA template. Amplification was performed following the conditions which were initial denaturation at 95°C for 15 minutes, denaturation at 94°C for one minute, annealing at 53°C for one minute 30 seconds, extension at 72°C for one minute of 35 cycles and final extension at 72 for seven minutes. The PCR amplification products were fractionated by electrophoresis through 2.5% agarose gel visualized by staining with ethidium bromide. The PCR product size were 163 base pair (bp) for *mecA* gene and 222 bp for *aac(6′)-aph(2″)* gene.

Quality control

For quality control of biochemical tests, purity plate was used. Similarly, for the standardization of the culture and antimicrobial susceptibility testing, *S. aureus* (ATCC 25923) was used as a control strain

RESULTS

Prevalence of *Staphylococcus* spp and MRSA

In this study, 130(6.6%) staphylococci isolates were obtained from 1969 various clinical samples. Among them, 66 (50.7%) were *S. aureus* whereas remaining 49.2% were CoNS. Among 66 *S. aureus,* 50 (75.7%) were MRSA. Only 15(22.7%) isolates were recognized as GRSA. Figure 1 depict overall prevalence of different bacterial genera.

Association of different variables with *S. aureus* isolates

Of the 66 isolates of *S. aureus,* 50 (75.7%) were MRSA and remaining 24.2% were MSSA. Most of the MRSA isolates were obtained from wound swab 43(78.2%) whereas least number of MRSA were recovered from pus samples 5(55.6%) ($p > 0.05$). Similarly, GRSA were also more prevalent in wound swab 14 (22.5%) and tissue 1 (100%) ($p<0.05$). Gender wise, 73.1% females were found to be harboring MRSA which is slightly less than that of the male (77.5%). Meanwhile, prevalence of GRSA among men is much higher 12(30%) as compared to females 3(11.5%) but the result was statistically insignificant ($p>0.05$) in both cases. Moreover, age group 31-45 accounted highest 9(100%) MRSA isolates as compared to other age group and the data was statistically significant as well. We found that 8(27.6%) of the GRSA isolates were from age group 16-30 which is highest among all age groups. But, none of the *S. aureus* isolates from patients aged ≥60 was GRSA ($p>0.05$). Prevalence of MRSA was higher in patients enrolled at IPD 17(89.5%) as compared to OPD visiting patients which was 70.2% although statistically it was not proved ($p>0.05$). Contrary to this result, GRSA was more prevalent in OPD visiting patients 11(23.4%). This data was also not significant ($p>0.05$). (Table 1)

Antimicrobial resistance patterns of the isolates

We found that all MRSA isolates were resistant to

ampicillin but, gentamicin resistance rate was quite low (24% MRSA). Doxycycline was resisted by 32% MRSA isolates, tetracycline by 32% and chloramphenicol by 14%. Of the 16 MSSA isolates, high level of resistance was observed against ampicillin (81.3%) and least resistance was observed with both tetracycline and clindamycin (6.3%). Gentamicin resistance was found in 18.8% of MSSA isolates (Table 2)

Possession of *aac(6')-aph(2")* and *mecA* gene by MRSA and MSSA isolates

All the 48 *mecA* gene harboring isolates were MRSA whereas 10 *aac(6')-aph(2")* gene harboring isolates were MRSA and remaining 2 were MSSA. Altogether, 10(20%) isolates were found to have both type of gene, of which all were MRSA (Table 3). Also, 10 out of 12 *aac(6')-aph(2")* gene harboring isolates were found to have *mecA* gene as well and there was a strong association between the occurrence of these genes ($p<0.01$).

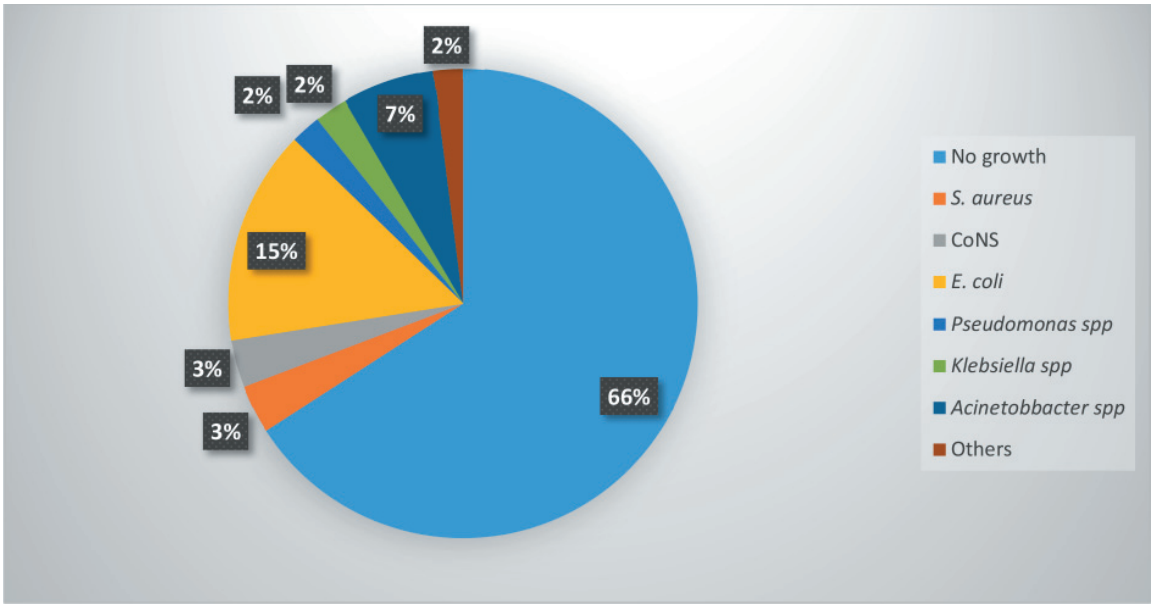

Figure 1: Distribution of different bacterial isolates in the clinical isolates

Table 1: Distribution of staphylococci with different attributes

Attributes	Parameters	Total (n=66)	GRSA(n=15)	p-value	MRSA (n=50)	p-value
Gender	Female	26	3(11.5%)	0.122	19(73.1%)	0.682
	Male	40	12(30%)		31(77.5%)	
Age group	<15	8	2(25%)	0.778	8(100%)	0.01*
	16-30	29	8(27.6%)		21(72.4%)	
	31-45	9	2(22.2%)		9(100%)	
	46-60	13	3(23.1%)		9(69.2%)	
	More than 60 years	7	0		3(42.9%)	
Sample	CVP Tips	1	0	0.012*	1(100%)	0.386
	Pus	9	0		5(55.6%)	
	Tissue	1	1(100%)		1(100%)	
	Wound swab	55	14(25.5%)		43(78.2%)	
Patient status	IP	19	4(21.1%)	0.908	17(89.5%)	0.08
	OPD	47	11(23.4%)		33(70.2%)	

* significant at 1% level of significance

Table 2: AST pattern of the isolates

Antibiotics	MRSA (n=50)			MSSA (n=16)		
	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)
Amikacin	16(32%)	27(54%)	7(14%)	14(87.5%)	2(12.5%)	0(0%)
Gentamicin	26(52%)	12(24%)	12(24%)	13(81.3%)	3(18.8%)	0(0%)
Ciprofloxacin	2(4%)	45(90%)	3(6%)	11(68.8%)	5(31.3%)	0(0%)
Cotrimoxazole	11(22%)	35(70%)	4(8%)	13(81.3%)	3(18.8%)	0(0%)
Levofloxacin	8(16%)	41(82%)	1(2%)	10(62.5%)	6(37.5%)	0(0%)
Erythromycin	6(12%)	39(78%)	5(10%)	6(37.5%)	4(25%)	6(37.5%)
Clindamycin	12(24%)	25(50%)	13(26%)	12(75%)	1(6.3%)	3(18.8%)
Cefoxitin	-	50(100%)	-	16(100%)	-	-
Doxycycline	32(64%)	16(32%)	2(4%)	13(81.3%)	3(18.8%)	0(0%)
Tetracycline	14(28%)	36(72%)	0(0%)	15(93.8%)	1(6.3%)	0(0%)
Chloramphenicol	40(80%)	7(14%)	3(6%)	14(87.5%)	1(6.3%)	1(6.3%)

Table 3: Acquisition of different genes among staphylococci isolates

		Presence of			
	Isolates(n)	*mecA* gene only	*aac(6')-aph(2")* gene only	Both	p-value
MRSA	50	38(76%)	0	10(20%)	0.000*
MSSA	16	0	2(12.5%)	0	

* significant at 1% level of significance

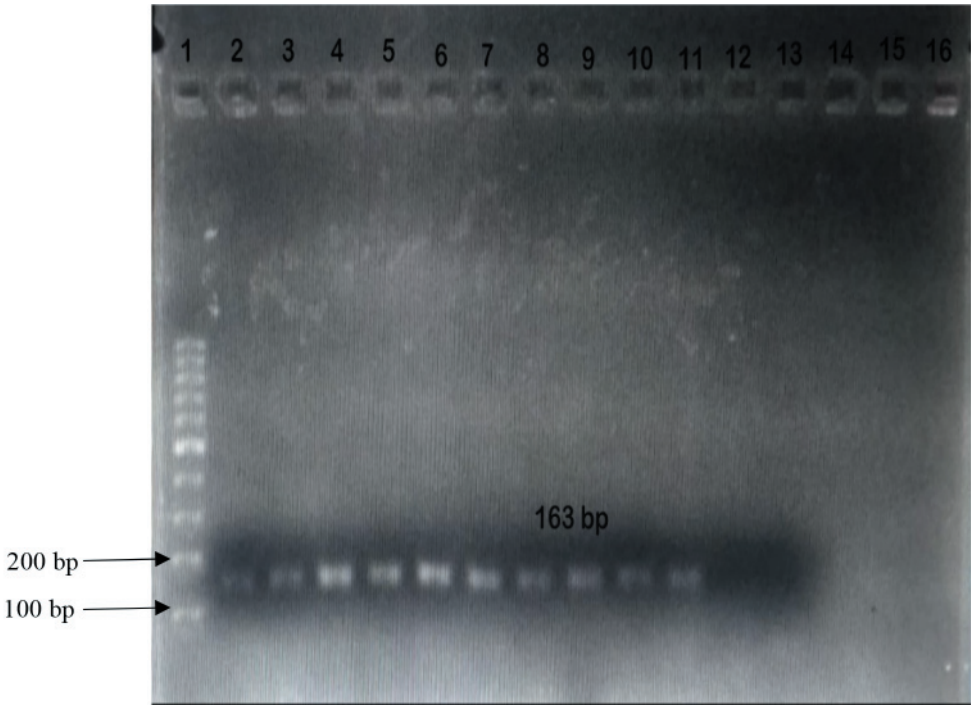

Figure 2: Confirmation of *mecA* gene by gel documentation of PCR products

Lane 1: 100 bp DNA Ladder, Lane 2: Positive control, Lane 3-11: Positive isolates, Lane 12: Negative isolate, Lane 13: Negative control (ATCC 25923)

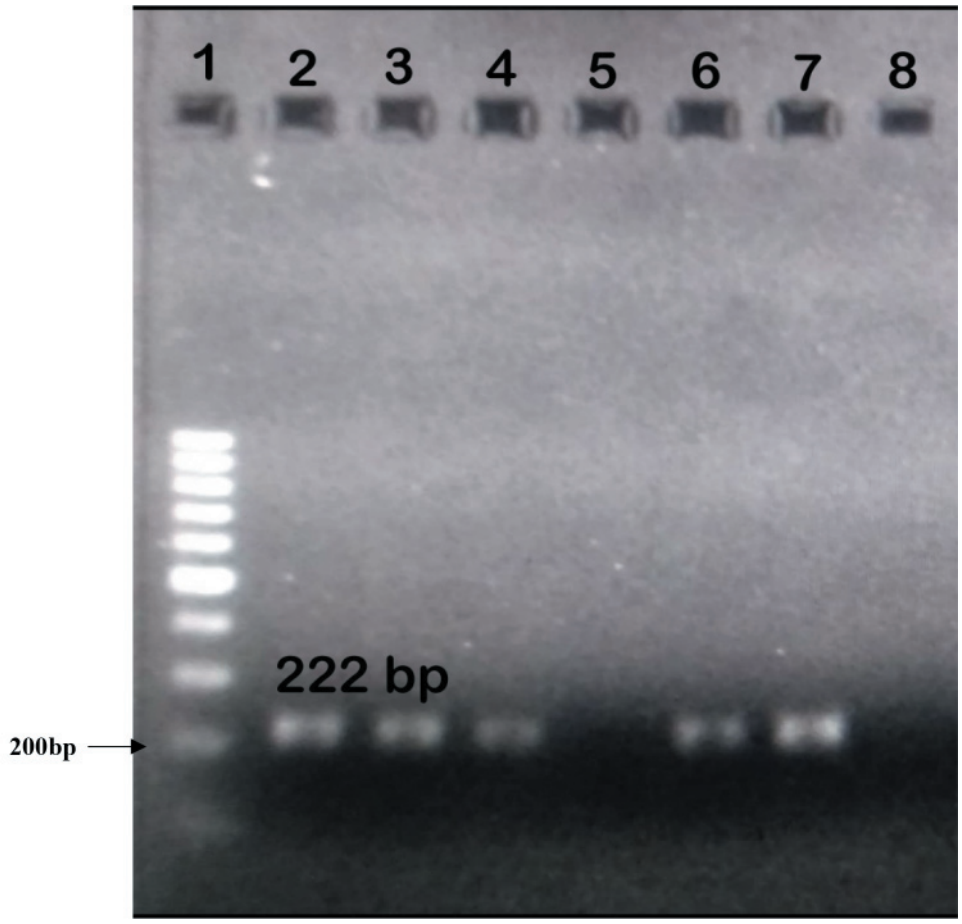

Figure 3: Confirmation of *aac(6')-aph(2")* gene by gel documentation of PCR products
(Lane 1:100 bp DNA Ladder, Lane 2: Positive control, Lane 3-4 & 6-7: Positive isolate, Lane 5: Negative isolate, Lane 8: Negative control)

DISCUSSION

The main objective of this study was to determine the status of MRSA and occurrence of *aac(6')-aph(2")* gene in gentamicin resistant MRSA as well as MSSA. Since there are limited studies done on the detection of *aac(6')-aph(2")* gene in gentamicin resistant MRSA as well as MSSA, the analyses and conclusion drawn from this study helps in understanding of this pathogen in present context and will help to adopt better clinical practices in clinical settings.

Overall prevalence of staphylococci in this study was 6.6%. A research done in Palpa, Nepal has also reported the rate of staphylococci in clinical sample around 6.7% (Raut et al. 2017). Meanwhile, higher prevalence (19.9%) was found in one study done by (Sapkota et al. 2019). Contrary to this result, a study done in Kathmandu has found very low (4.1%) staphylococci prevalence (Kandel et al. 2020). Biofilm forming ability on many uninhabitable surfaces, ubiquitous in nature may have contributed to high prevalence of *Staphylococcus aureus* (Sherchand et al. 2016). Among 66 isolates of *S. aureus,* 50 (75.8%) isolates were screened as methicillin resistant. Indiscriminate use of antibiotics might be one of the reason for high prevalence of MRSA. Our findings is consistent with previous reports from Chitwan (Khanal and Jha, 2010), Pakistan (Brig et al. 2004; Jan et al. 2010), Kathmandu (Sapkota et al. 2019) and Eritrea(Garoy et al. 2019). However previous studies from Nepal reported low prevalence (<50%) of MRSA (Kumari, Mohapatra and Singh, 2008; Sah et al. 2013; Bhatta et al. 2016). There might be various explanations for variation in prevalence of MRSA in different studies such as difference in study site, length of the study period, infection control measures, antibiotic prophylaxis and treatment in each hospital and epidemic nature of these microorganisms (Hassoun, Linden and Friedman,

2017). This study showed higher prevalence of MRSA in wound swab (78.2%) and least in pus (55.6%). This finding is supported by previous report from (Nsofor, Nwokenkwo and Ohale, 2016), (Obiazi et al. 2007) and (Nwoire et al. 2013) where incidence was high from wound swab. However our finding is in opposite with some other results where high incidence was from pus (Khanal and Jha, 2010; Sapkota et al. 2019).The high incidence in wound swab might be due to poor personal hygiene and exposure of wound, which might have made it prone to contamination and infection. Other reason might be due to high percentage of wound swab being processed in comparison to pus sample (Bowler, Duerden and Armstrong, 2001). In this study, we analyzed socio-demographic as well as clinical characteristics of patients but none of them except age group for MRSA and types of sample for GRSA were found significantly associated. However, this finding are not new in our community. In recently published papers, it was also reported that community-associated MRSA infection and resistance to gentamicin were not associated with any socio-demographics characters of patients (Herold et al. 1998; Raut et al. 2017).

Regarding antimicrobial resistance, all MRSA were resistant to ampicillin. A study from few years ago has also reported that ampicillin was completely resisted by these bacteria (Khanal, Adhikari and Guragain, 2018). Similarly, resistance was quite higher towards some other drugs such as third line therapy drug ciprofloxacin (90%). Only 24% isolates were found resisting gentamicin. These findings are similar to some previous report by (Belbase et al. 2017; Khanal, Adhikari and Guragain, 2018). There is reason for concern because MRSA often are or can readily become resistant to multiple antibiotics, especially to some second and third line drugs which can lead to higher morbidity rate due to MRSA infection (Chambers and DeLeo, 2009). PCR reaction was carried out by a single set of primers for the amplification of *mecA* gene and *aac (6')-aph (2") gene*. Only 2 isolates were not harboring *mecA* gene among 50 MRSA isolates whereas the gene was found in remaining 48 isolates. There has been various report of MRSA not harboring *mecA* gene (Ariza-Miguel et al. 2014; Cikman et al. 2019). When Ariza-miguel performed genomic sequence analysis of that isolate, he found that it carried a *mecA* homolog gene sequence with approximately 69% similarity to the classical *mecA* gene, and this newly identified gene encoded a protein with approximately 63% similarity to the PBP 2a protein and was named mecC in 2012 (Paterson et al. 2014). This same gene may be present in 2 *mecA* negative MRSA isolates which causes resistance to methicillin. We found that 24% MRSA isolates were gentamicin resistant. The result was found consistent with some previous studies performed by Pandey (Pandey, Raza and Bhatt, 2013) and Khanal (Khanal, Adhikari and Guragain, 2018) separately. High prevalence (83.3%) of *aac(6')-aph(2")* gene was found among gentamicin resistant MRSA which is similar to the work done by Rahimi (Rahimi, 2016) and Udo and Dashti (Udo and Dashti, 2000). In this study, *aac(6')-aph(2")* gene was present in only 66.7% gentamicin resistant MSSA. In contrary to this, (Udo and Dashti, 2000) reported *aac(6')-aph(2")* gene in 100% gentamicin resistant MSSA isolates. *aac(6')-aph(2")* gene was not detected in some of gentamicin resistant *S. aureus*. This abnormality can be explained as there are various other mechanism such as reduced permeability of cells (Mingeot-Leclercq, Glupczynski and Tulkens, 1999; Aslangul et al. 2006; Bennett, Dolin and Blaser, 2010; Garneau-Tsodikova and Labby, 2016), mutation and modification of ribosome (Wilson, 2013) which causes resistance to gentamicin and not only due to presence of that particular gene.

CONCLUSIONS

More than three quarter of *S. aureus* isolates were MRSA, this shows that resistivity is increasing every passing year while almost a quarter of MRSA isolates were resistant to gentamicin, one of the important drugs of choice for MRSA infection. The results of this study ensure the necessity for increased dedication to infection control methods that adhere to established protocols and are intended to stop the spread of MRSA infection among susceptible people. To stop the rapidly spreading of resistant bacteria, it can be advised to improve diagnostic facilities and practice antimicrobial stewardship.

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Ethical approval and consent to participate

As the sampling process involves human volunteers, ethical consent was required prior to executing the

research work. Ethical clearance was taken from the Institutional Review Committee (IRC) of the Public Health Concern Trust, Nepal (IRC application number: 009-2020). Patients were recruited after obtaining written informed consent.

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