Prevalence of Staphylococci in Environmental Surfaces and Characterization of Isolates by Antibiotic Susceptibility

Charu Arjyal[1*], Prabhu Raj Joshi[2], Divya Nepal[1], Rachana Kafle[1], Anuja Panthi[1], Radhika Thapa[1], Puspa Pandey[1]

[1]Department of Microbiology, Tri-Chandra Multiple Campus, Ghantaghar, Kathmandu, Nepal

[2]Nepalese Farming Institute, cmilanjoshi@gmail.com, Kathmandu, Nepal

***Corresponding author:** Charu Arjyal, Department of Microbiology, Padma Kanya Multiple Campus, Bagbazar, Kathmandu, Nepal; Email: carjyal@gmail.com

ABSTRACT

Objectives: The purpose of the study was to determine the extent of staphylococcal contamination in various environmental sites and to characterize the isolates by antibiotic susceptibility.

Methods: A cross-sectional study was conducted and 123 samples were collected from 9 different sites around Kathmandu valley. Isolation of *S. aureus* was done through cultural and biochemical analysis. Kirby-Bauer disc diffusion test was employed to test the susceptibility of isolates to antibiotics.

Results: A total of 25 *S. aureus* (20.33%) were isolated; among which 12 isolates exhibited methicillin resistance i.e. 48% (MRSA) and 13 isolates were methicillin susceptible, 52% (MSSA). Similarly, 53 Coagulase Negative Staphylococci (CoNS) were isolated; among which 17(32.07%) were resistant to methicillin. The antibiotic resistance patterns of MRSA were reported as: erythromycin(n=2;16.6%.), clindamycin (n=2;16.6%), cotrimoxazole (n=2;16.6%), ciprofloxacin (n=2;16.6%) and gentamicin (*n* = 1;8.3%). MRCoNS showed high resistance to erythromycin (n=6; 35.2%), followed by co-trimoxazole (n=4; 23.5%), novobiocin (n=4; 23.5%) and ciprofloxacin (n=3; 17.6%). All MRSA and MRCoNS isolates were susceptible to linezolid and clindamycin.

Conclusion: This study reports relatively high prevalence of MRSA on environmental surfaces, predominating in areas having heavy crowds. There may be a likely connection between humans and the environment to share MRSA and MSSA.

Key words: *S. aureus*, environment, antibiotic, susceptibility

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium that produces uniform sized cocci that can be found individually or in pairs. They are non-motile and non-capsulated, but some virulent strains are encapsulated. They've been linked to everything from pimples, impetigo, boils, cellulitis, scalded skin syndrome, folliculitis, furuncles, carbuncles, and abscesses to life-threatening conditions like pneumonia, osteomyelitis, meningitis, Toxic Shock Syndrome, endocarditis, and septicaemia (Tong et al 2015). However, these infections appeared to be under control with the discovery of penicillin; unfortunately, the respite from resistance was short-lived.

S. aureus has acquired determinants by horizontal gene transfer of mobile genetic elements, which has resulted in resistance to a variety of drugs (Jensen and Lyon 2009) and referred to be Methicillin-resistant *Staphylococcus aureus* (MRSA) (Gurusamy et al 2015). MRSA strains initially described in the 1960s, emerged as a leading source of nosocomial infections in the last decade (Monecke et al 2011).

MRSA began as a hospital-acquired infection, but it has already spread to the community and livestock. Different sources of acquiring Methicillin Resistant Staphylococcus aureus have been named as hospital-associated MRSA

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(HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA). Hospitalized patients, particularly the elderly, are generally weakened and vulnerable to infection, including MRSA (Jacobs et al 2014). Meanwhile, in the late 1990s and early 2000s, CA-MRSA strains appeared, infecting healthy people who had not been exposed to hospital environments. Compared to HA-MRSA, community-acquired MRSA is more easily treated and more pathogenic (Calfee et al 2011) making it a global threat even in this sophisticated era of medication.

In general, antibiotic resistance is described as bacteria's ability to develop resistance genes that counteract the inhibitory impact of prospective antibiotics, allowing them to survive (Blair et al 2015). In the case of regular Antibiotic Susceptibility Test (AST) procedures, it typically takes at least 24 hours to establish bacterial colonies and another 24 hours to characterize isolates, including identification by biochemical tests and phenotypic Antibiotic Susceptibility Tests (Altaie and Dryja 1994; Faro et al 2016). Antimicrobial resistance is a major global health concern, and drug-resistant *Staphylococcus aureus* represents a substantial issue among Gram-positive bacteria. Additionally, the epidemiology of MRSA has been reported to be changing due to the emergence of community-acquired MRSA (CA-MRSA) (L'Heriteau et al 1999).

The principal agents that cause nosocomial infections are Methicillin-resistant coagulase-negative staphylococci (MRCoNS). The expression of the *mecA* gene, which produces an alternative penicillin-binding protein (PBP2a) with a low affinity for these antibiotics, is the main mechanism of resistance to β-lactam antibiotics in CoNS (Geha et al 1994). Vancomycin is usually the drug of choice for the treatment of infections caused by MRCoNS (Srinivasan et al 2002).

Community Acquired MRSA is found to be a common cause of skin and soft tissue infection and might be common in an overcrowding population where there is limited access to clean water (Loewen et al 2017). This ignites the necessity of this research. Given that staphylococci survive on inanimate objects for

prolonged periods, ambient surfaces such as shrines and parks, schools/colleges, restaurants, bank ATMs, and vegetable and fruit markets may serve as vectors for staphylococci acquisition and dissemination among the community.

In Nepal, no extensive environmental evaluations have been conducted to determine which ambient surfaces are staphylococci reservoirs. Identifying major staphylococci reservoirs will help guide future measures to lower the prevalence of MRSA in the population and the risk of infection and transmission.

METHODS

Study design, study site and sample size

The study was qualitative, and primary data were collected from August 2019 to December 2019. The variables of this study were the occurrence of *S. aureus*, CoNS, MRSA, MRCoNS, and their antibiotic susceptibility profiles. The study was cross-sectional comprising of field and laboratory based procedures. The samples were collected from 9 different environmental sites which were relatively crowded i.e. Kalimati vegetable market, Maitidevi temple, Pashupatinath temple, Swayambhunath stupa, Bus station, Basantapur Durbar Square, ATM booths, and a public Campus area of Kathmandu valley. A total of 123 samples (environmental swabs) were collected randomly from 9 different sites around Kathmandu valley. Samples were processed in the laboratory of Nepalese Farming Institute, Maitidevi, Kathmandu.

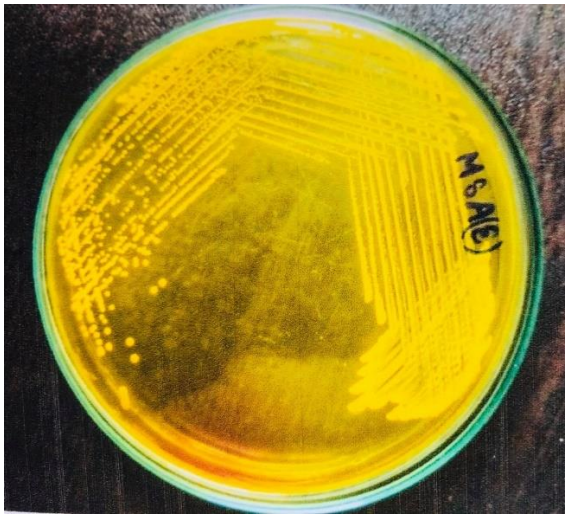
Sample collection and transportation

Several surfaces (approximately 1 meter) around the spot often handled by humans were gently swabbed using a normal sterile swab (sponge swabs) wet with buffered peptone broth. To avoid contamination, the collected swabs were placed in a vial containing M-Staphylococcus broth (supplemented with a final concentration of 75 mg/L polymyxin B, 0.01 percent potassium tellurite, and either with or without 12.5 mg/L nystatin), screw-capped, clearly labeled, and transported to the laboratory right away.

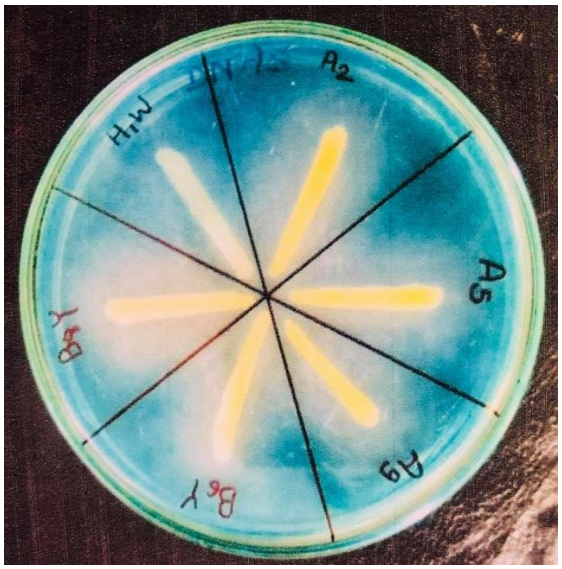
Isolation of *S. aureus*/CoNS

Environmental swabs enriched in M-Staph broth were cultured in a CO2 enhanced atmosphere for 48 hours at 37°C. The dark black precipitate-containing vials were directly cultured in Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hours. MSA colonies that

fermented mannitol (yellow colonies) and colonies that did not ferment mannitol were sub-cultured on nutrient agar and incubated at 37°C for 24 hours. Pigmented colonies having round, raised, opaque, smooth, and shiny surface with a diameter of about 2-3 mm were indicative of *S. aureus*/CoNS (Photograph 1). Further phenotypic identification of *S. aureus*/CoNS was made by Gram staining, catalase test, oxidase test, oxidative/fermentative, and coagulase/DNase test. The key test for the isolation of *S. aureus*/CoNS was the coagulase test/DNase test; *S. aureus* was identified based on a positive coagulase and DNase test (Photograph 2) that differentiates *S. aureus* from CoNS (DNase negative and coagulase-negative) (CLSI 2018).



Photograph 1- Isolated colonies of *S. aureus* in mannitol salt agar



Photograph 2- DNase test

Detection of MRSA/MRCoNS

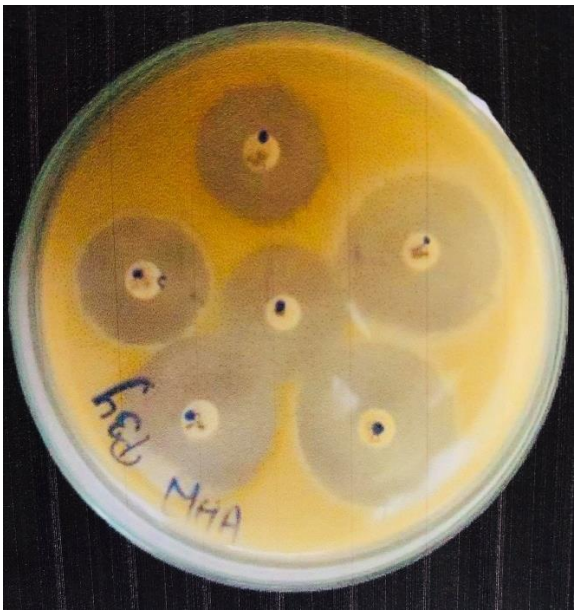
All the isolates of *S. aureus*/CoNS were subjected to cefoxitin disc diffusion testing on Mueller-Hinton agar (MHA) using a 30 µg cefoxitin disc. Isolates having an inhibition zone diameter of ≤ 21 mm were reported as methicillin-resistant *S. aureus* (MRSA) and ≥ 22 mm were reported as methicillin-susceptible *S. aureus*. Furthermore, isolates having an inhibition zone diameter of ≤ 24 mm were reported as methicillin-resistant CoNS (MRCoNS) and ≥ 26 were reported as methicillin-susceptible CoNS (CLSI 2018).

Antibiotic susceptibility testing by disc diffusion method

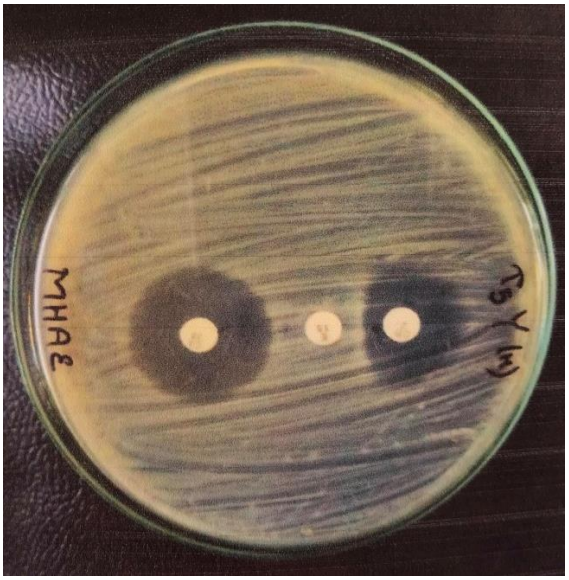
The modified Kirby-Bauer disc diffusion method was used to assess in vitro antibiotic susceptibility of all reported *S. aureus*/CoNS/MRSA/MRCoNS isolates. Gentamicin (10 g), erythromycin (15 g), ciprofloxacin (5 g), tetracycline (30 g), clindamycin (2 g), cotrimoxazole (1.25/23.75 g), novobiocin (5 g), penicillin (10 g), and linezolid (30 g) were the antibiotics examined. In order to make the inoculums, 3–4 similar colonies were transferred from nutrient agar to sterile normal saline. The turbidity of the inoculums was adjusted to meet the McFarland criterion of 0.5. Swabbing on MHA with a sterile cotton swab soaked in inoculums was used to prepare the grass culture of the test inoculums. Antibiotic discs were placed on the inoculated MHA plate and left to incubate for 18 hours at 37°C. The inhibition zone around the discs was measured after incubation, and the results were interpreted as sensitive, moderate, or resistant (CLSI 2018) (Photograph 3).

Detection of inducible clindamycin resistance in *S. aureus*

The D-zone test was used to detect inducible clindamycin resistance in *S. aureus* that was erythromycin (15 g) resistant but clindamycin (2 g) susceptible. Erythromycin and clindamycin were placed 15–26 mm apart in the lawn culture of test inoculums on MHA and incubated at 37°C for 18 hours. The flattening of the clindamycin zone of inhibition close to the erythromycin disc (known as a D-zone) during incubation indicated inducible clindamycin resistance (CLSI 2018) (Photograph 4).



Photograph 3- Antibiotic susceptibility pattern of *S. aureus*



Photograph 4- Inducible Clindamycin Resistance Test (D-test)

Detection of β-lactamase

The penicillin disc diffusion zone-edge test was employed to detect the production of β-lactamase enzyme. McFarland standard of 0.5 was used to compare the turbidity of the inoculum for standardization. A sterile cotton swab was dipped into the inoculums and the lawn culture of the test inoculums was prepared by swabbing on MHA.

The detection of β-lactamase synthesis was done using a penicillin (10 g) disc (CLSI 2018).

RESULTS

Occurrence of *S. aureus*/CoNS in the environment

Out of 123 samples collected from 9 different sites within Kathmandu valley, a total of 25(20.3%) *S. aureus* along with 53(43.1%) CoNS were isolated (Figure 1).

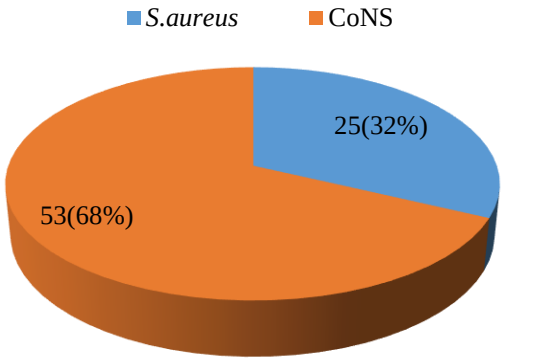


Figure 1: Occurrence of *S. aureus*/CoNS in the environmental samples

Occurrence of MRSA/MRCoNS in the environment

Twelve of the 25 *S. aureus* isolates tested positive for MRSA (48 %). Similarly, 17 (32.1%) of the 53 CoNS isolates tested positive for methicillin resistance (MRCoNS) (Figure 2).

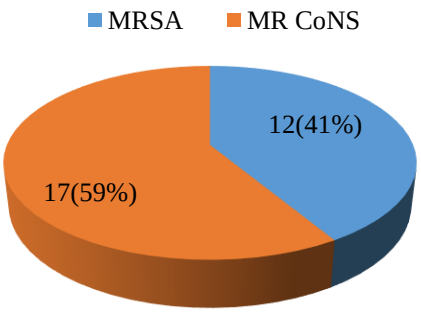


Figure 2: Occurrence of MRSA/MR CoNS in the environmental sample

Distribution of *S. aureus* and MRSA among different sites

The majority of the *S. aureus* were isolated from Pashupatinath temple (n=6; 24%) and Swayambhunath stupa (n=6; 24%), with the least amount found in vegetable market (n=1; 4%), Maitidevi (n=1; 4%) temple and campus areas (n=1; 4%). Meanwhile, no traces of *S. aureus* were found in cafes. MRSA was isolated in large numbers from Pashupatinath (n=3; 25%), the bus station (n=3; 25%), and ATM booths (n=3; 25%).

One MRSA isolate was found in each of the following locations: vegetable market (n=1; 8.3%), Maitidevi temple (n=1; 8.3%), and Basantapur Durbar Square (n=1; 8.3%). MRSA was not detected in Swayambhunath, campus area and cafes (Table 1).

Distribution of CoNS/MRCoNS among different sites

The high numbers of CoNS were detected from bus stations (n=10; 18.8%), while low numbers from Maitidevi temple (n=4; 7.5%). The distribution of MRCoNS is high in bus station (n=4; 23.5%) and ATM booths (n=4; 23.5%), followed by Durbar Square (n=3; 17.6%) and cafes (n=3; 17.6%). Two isolates from Pashupatinath areas (n=2; 11.8%) and only one isolate from college premises (n=1; 5.9%) were also detected. MRCoNS were not detected in samples from vegetable markets and Swayambhunath (Table 2).

Antibiotic Susceptibility profile of *S. aureus*/MRSA

The antibiotic resistance pattern of *S. aureus* was as follows: erythromycin (n=2; 8%), clindamycin (n=2; 8%), cotrimoxazole (n=2; 8%), ciprofloxacin (n=2; 8%) and gentamicin (n = 1; 4%) as shown in Table 4. All the isolates were susceptible to linezolid, and tetracycline. Gentamicin (n=2; 8%) and ciprofloxacin (n=2; 8%) resistance was intermediate in two isolates. Likewise, the resistance patterns of MRSA were reported as follows: erythromycin (n=2; 16.6%), clindamycin (n=2; 16.6%), cotrimoxazole (n=2; 16.6%), ciprofloxacin (n=2; 16.6%) and gentamicin (n = 1; 8.3%). Tetracycline and linezolid were totally effective against MRSA isolates.

Antibiotic Susceptibility profile of CoNS/ MR CoNS

The antibiotic resistance pattern of CoNS was as follows: erythromycin (n=13; 24.5%), clindamycin (n=1; 1.9%), cotrimoxazole (n=12; 22.6%), ciprofloxacin (n=4; 7.5%), linezolid (n=0;0%), novobiocin (n=12; 22.6%) and gentamicin (n =1;1.9%). Similarly, MRCoNS were resistant to erythromycin 6(35.2%), followed by co-trimoxazole (n=4; 23.5%), novobiocin (n=4;23.5%) and ciprofloxacin (n=3;17.6%). Isolates showed low resistant to tetracycline (n=1;5.8%). All the isolates were susceptible to clindamycin and linezolid while one isolate showed intermediately resistance to gentamicin (n=1;5.8%) (Table 4).

Inducible clindamycin resistance in MRSA and MRCoNS

MRSA isolates did not show the inducible clindamycin resistant pattern. In contrast, 3 out of 17 MR CONS (17.7%) showed a positive D-test, indicative of inducible clindamycin resistance (Figure 3).

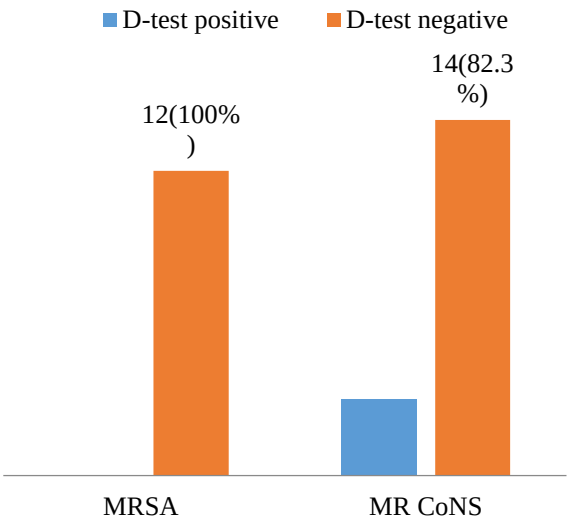


Figure 3: Inducible clindamycin resistant pattern in MRSA and MR CoNS

β-Lactamase production in MRSA and MSSA isolates

Nine out of 12 MRSA (75%) isolates produced β-lactamase enzymes. Similarly, 12(70.5%) out of 17MSSA isolates produced β-lactamase enzymes.

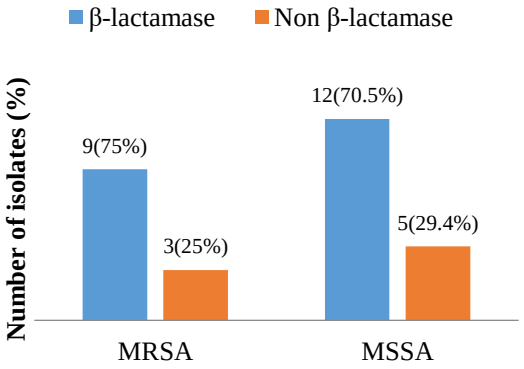


Figure 4: β-lactamase enzyme production in MRSA and MSSA isolates

DISCUSSION

The study provides an analysis of MRSA isolated from different sites in Kathmandu valley and their antibiotic susceptibility patterns. In comparison to clinical samples, a small number of studies have been undertaken on various environmental samples.

The environmental carriage rate of *S. aureus* and CoNS was found to be comparatively higher than the study conducted in shrine areas of Kathmandu valley (Arjyal et al 2020), where 120 samples were collected from shrines among which 17.5% *S. aureus* were isolated. Using swab sampling with broth enrichment, we evaluated the recovery of different concentrations of MRSA from typical ambient surface types in a systematic manner.

Table 1: Distribution of *S. aureus* and MRSA among different sites

Sample collection sites	Number of samples	Number of *S. aureus* isolated (%)	Number of MRSA isolated (%)
Vegetable market	10	1(4)	1(8.3)
Maitidevi temple	15	1(4)	1(8.3)
Pashupatinath temple	23	6(24)	3(25)
Swayambhunath	15	6(24)	0(0)
Bus Station	10	3(12)	3(25)
Basantapur Durbar Square	9	2(8)	1(8.3)
ATM booths	20	5(20)	3(25)
Campus area	10	1(4)	0(0)
Cafes	11	0(0)	0(0)
Total	123	25(20.3)	12(48)

Table 2: Distribution of CoNS/MRCoNS among different sites

Sample collection sites	Number of samples	Number of CoNS isolated (%)	Number of MRCoNS isolated (%)
Vegetable market	10	8(15.1)	0(0)
Maitidevi Temple	15	2(3.7)	0(0)
Pashupatinath temple	23	4(7.5)	2(11.8)
Swayambhunath stupa	15	6(11.3)	0(0)
Bus station	10	10(18.8)	4(23.5)
Basantapur Durbar Square	9	6(11.3)	3(17.6)
ATM booths	20	5(9.4)	4(23.5)
Campus area	10	6(11.3)	1(5.9)
Cafes	11	6(11.3)	3(17.6)
Total	123	53(43.1)	17(32.1)

Table 3: Antibiotic Susceptibility pattern of *S. aureus*/MRSA

Antibiotics (µg)	Susceptibility Pattern of *S. aureus*			Susceptibility Pattern of MRSA		
	Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Cefoxitin (30)	13(52)	-	12(48)	0(0)	-	12(100)
Erythromycin (15)	23(92)	-	2(8)	10(83.4)	-	2(16.6)
Clindamycin (2)	23(92)	-	2(8)	10(83.4)	-	2(16.6)
Ciprofloxacin (5)	21(84)	2(8)	2(8)	8(66.7)	2(16.7)	2(16.6)
Tetracycline (30)	25(100)	-`	0(0)	12(100)	-	0(0)
Co-trimoxazole (25)	23(92)	-	2(8)	10(83.4)	-	2(16.6)
Linezolid (30)	25(100)	-	0(0)	12(100)	-	0(0)
Gentamicin (10)	22(88)	2(8)	1(4)	9(75)	2(16.7)	1(8.3)

Table 4: Antibiotic Susceptibility Testing (AST) of CoNS/MRCoNS

Antibiotics (µg)	Susceptibility pattern of CoNS			Susceptibility pattern of MRCoNS		
	Sensitive (%)	Intermediate (%)	Resistant (%)	Sensitive (%)	Intermediate (%)	Resistant (%)
Erythromycin (15)	40(75.5	-	13(24.5)	11(64.8)	-	6(35.2)
Clindamycin (2)	52(98.1)	-	1(1.9)	17(100)	-	0(0)
Ciprofloxacin (5)	49(92.5)	-	4(7.5)	14(82.4)	-	3(17.6)
Tetracycline (30)	50(94.4)	-	3(5.7)	16(94.2)	-	1(5.8)
Co-trimoxazole (25)	41(77.4)	-	12(22.6)	13(76.5)	-	4(23.5)
Linezolid (30)	53(100)	-	0(0)	17(100)	-	0(0)
Gentamicin (10)	52(98.1)	1(1.9)	1(1.9)	16(94.2)	1(5.8)	1(5.8)
Novobiocin (5)	41(77.4)	-	12(22.6)	13(76.5)	-	4(23.5)

The high prevalence of *S. aureus* in our study could be attributed to the use of enrichment media, as well as the disparity in sample numbers and collection sites. On the other hand, even with broth enrichment, no *S. aureus* was detected in cafes using sampling methods that successfully recovered the same dilution from other sites.

Comparing our results to several other studies conducted, we found that the transmission rate of MRSA varied depending on the location. Our findings demonstrated a higher occurrence of MRSA (48%) than a study conducted near temples in Kathmandu (Roberts et al 2018), in which 59 saliva samples from wild monkeys were obtained, with 6.8% of macaque MRSA being isolated. On the other hand, the first study, which looked at the prevalence of CoNS in an environmental sample from a Tunisian hospital and correlated it with antibiotic resistance, contradicted our findings, showing a high prevalence of CoNS, with 83 (41.5%) of 200 tested samples being CoNS (including 63/150 (41.3%) inanimate surface samples) (Dziri et al 2016). To our knowledge, this is the first study conducted in Kathmandu that determines the prevalence of both MRSA and MRCoNS in multiple sites at the same time.

The diverse distribution of *S. aureus* and CoNS, which led in substantial variations of MRSA and MRCoNS, were directly influenced by the place where they occurred. The highest staphylococcal contamination was seen in Pashupatinath and Swayambhunath area (24%) followed by ATM booths (20%). Notably, MRSA was most frequently detected on the commonly touched item on surfaces like railings, number pad of ATMs, seats and the handles of buses in the heavily crowded places (45% of the positive samples) which is higher than the study conducted by (Simoes et al 2011) reporting MRSA in public urban buses. The closed chambers with limited ventilation could be one factor for the high number of MRSA in ATMs. Despite the high occurrence of *S. aureus* in the Swayambhunath area, no MRSA was detected which might be indicative of proper sanitation around the site, yet other staphylococcal species such as CoNS were reported. Moreover, unlike Pashupatinath, Swayambhunath does not have a cremation site, which appears to have contributed considerably in the rise of MRSA. There were no traces of *S. aureus* in cafes, which could have been due to the sample collection period, although certain MRCoNS strains were found. The results showed that those in cafes and college locations were the least likely to contract MRSA, which could be owing to the sites' regular sanitation and decent hygiene.

Furthermore, in all the sampling sites the predominance of CoNS was observed which was expected since those are ubiquitous bacteria. Meanwhile, the samples collected from bus terminals and ATM booths revealed that the highest number of CoNS (23.5%) was resistant to methicillin. In our investigation, the prevalence of MRCoNS on campus was relatively low (5.9%), compared to a study conducted in a university context in Thailand, where 41/200 samples (20.5%) were MRCoNS (Seng et al 2017). This could imply that patients are less likely to develop staphylococcal skin disorders like miliaria and atopic dermatitis, as well as bacteremia and prosthetic valve endocarditis.

We discovered that MRSA and MRCoNS isolates were resistant to multiple antimicrobial agents. The percentage of MR staphylococci isolates (MRSA and MRCoNS) counters the result of Kitti et al (2018) which shows 96.8% MR CoNS and 82.6% MRSA occurrences. MRCoNS showed the highest resistance to erythromycin whereas MRSA showed the same resistance pattern to erythromycin, clindamycin, co-trimoxazole and ciprofloxacin resembling the study by Lyytikäinen et al (1996) that showed a dramatic increase in the percentage of isolates resistant to penicillin, erythromycin, ciprofloxacin, clindamycin and oxacillin. The

rising rate of antibiotic resistance and MDR among pathogenic, commensal, and opportunistic bacteria necessitates a more thorough examination of CoNS prevalence and drug profiles (WHO 2014).

Linezolid was found to be the most sensitive drug against MRSA as well as MRCoNS. This demonstrates its limited application in MRSA treatment. It could also be utilized as a second-line or salvage treatment (Choo et al 2016). Resistance to tetracycline observed in our study is similar to the study carried out by Belbase et al (2017) which showed that few strains were resistant to tetracycline and clindamycin.

Despite the fact that our study has some unique strength and is one of the very first attempts to directly compare the multiple sites for *S. aureus* and CoNS simultaneously, this study is not without its limitations. The application of antibiotics is our main emphasis; however, the data does not allow for phylogenetic study of samples. Using these data as the primary indicator for clinical purposes cannot be considered as a good idea. Therefore, the use of molecular techniques such as Polymerase Chain Reaction (PCR), nucleic acid sequencing for the detection of *S. aureus* could be employed to get better results.

CONCLUSION

The occurrences of *S. aureus*/CoNS and their methicillin-resistant phenotypes were slightly high in comparison to other studies. All the isolates were fully susceptible to linezolid, tetracycline, which suggest their effectiveness under in vitro condition. Surfaces of environments, including shrines, schools/colleges, vegetable and fruits market, restaurants and ATM of banks may be the potential sources of staphylococcal contamination.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

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