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EDITORIAL

COVID-19 Pandemic: Lesson Learned and Next Plan of Actions

The whole world has witnessed one of the worst pandemic of the history with over 534 million cases and 6.3 million deaths due to COVID-19 globally, and the pandemic is still ongoing.

Pre-pandemic preparedness in countries with weaker health system was not adequate to combat with a disastrous pandemic like COVID-19. In fact, there has to be sustained and well-coordinated health care system from center to local level contexts ready for any public health emergency of international concern (PHEIC).

A balanced media coverage on science behind such pandemic and promt and proper message delivery to the community is another prerequisite to addess the pandemic during its early phase. In many instances, misinformation, misconception, disregarding the authorized information remained 'clicking time bomb' in abruptly spreading the cases in an exponential manner.

Lack of public-private partnership (PPP) remained another limitation observed during the early pandemic when a perception deeply rooted among the stakeholders who assumed that government is solely responsible in a fight againt pandemic. In reality, community and private sector involvement and ownership, coordination and reporing worked significantly. Effective implementation of public health and social measures was not possible without these joint efforts from all sectors.

Open public database systems helped tracking the disease progressing and interrupting it at right pomt which often prevented the already weakened health care systems becoming non-functional due to over-burden.

On technological perspectives, availability of sequence data quite early in the pandemic and the knowledge obtained from the past Coronavirus outbreaks of PHEICs (SARS/ MERS) helped extra-ordinarily in the development of reliable diagnostics and, safe and effective vaccines. This pandemic has proved how rapidly a technology can be transferred from one corner of the world to another, one pertinent example is the 'realtime PCR based facilities' beimg available in resource-limited settings too.

With all these lessons learnt from the COVOD-19 pandemic, each nation, public health agencies like WHO, R&D sectors, humanitarian organizations and phillathropists need to work on developing applicable plan of actions (not just a pre-pandemic preparedness!) to combat against the future outbreaks, PHEICs, and pandemins and safeguard the people worldwide.

Komal Raj Rijal, PhD
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Editor

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

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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.

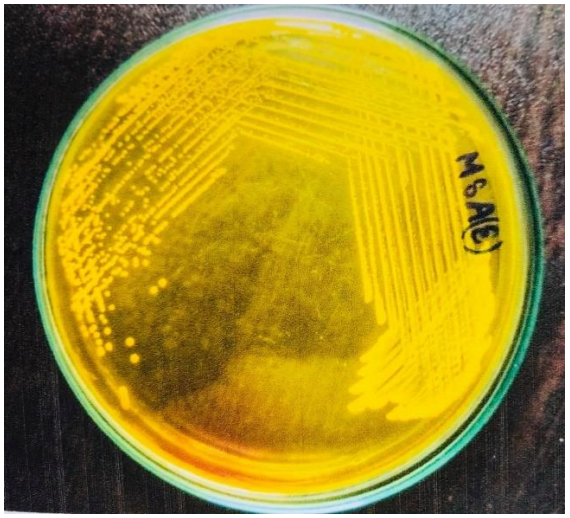
Sample collection and transportation

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.

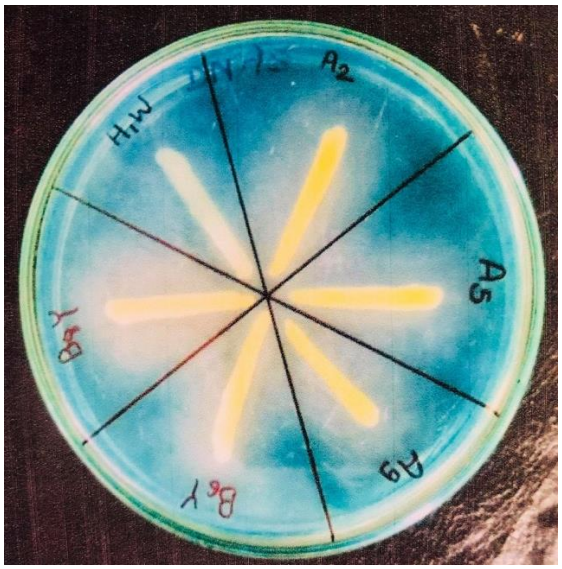
Isolation of *S. aureus*/CoNS

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

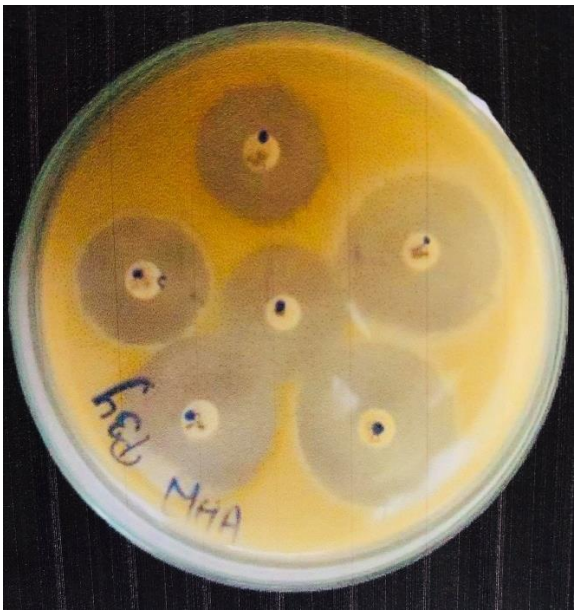
All the isolates of *S. aureus*/CoNS were subjected to cefoxitin disc diffusion testing on Mueller-Hinton agar (MHA) using a 30 µg cefoxitin disc. Isolates having an inhibition zone diameter of ≤ 21 mm were reported as methicillin-resistant *S. aureus* (MRSA) and ≥ 22 mm were reported as methicillin-susceptible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

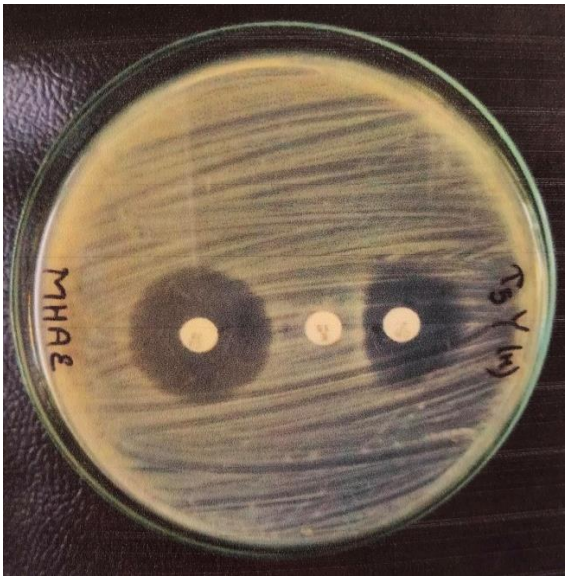
The modified Kirby-Bauer disc diffusion method was used to assess in vitro antibiotic susceptibility of all reported *S. aureus*/CoNS/MRSA/MRCoNS isolates. Gentamicin (10 g), erythromycin (15 g), ciprofloxacin (5 g), tetracycline (30 g), clindamycin (2 g), cotrimoxazole (1.25/23.75 g), novobiocin (5 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).

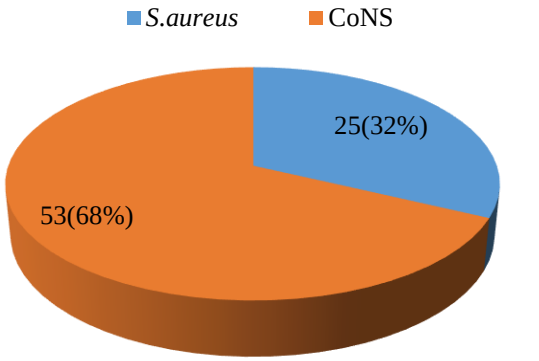


Figure 1: Occurrence of *S. aureus*/CoNS in the environmental samples

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).

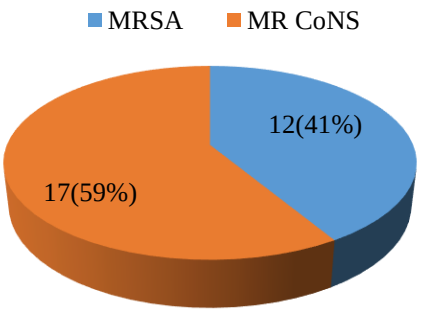


Figure 2: Occurrence of MRSA/MR CoNS in the environmental sample

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).

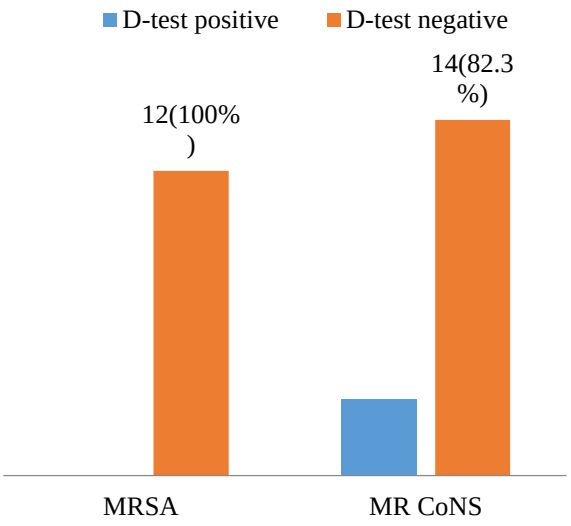


Figure 3: Inducible clindamycin resistant pattern in MRSA and MR CoNS

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.

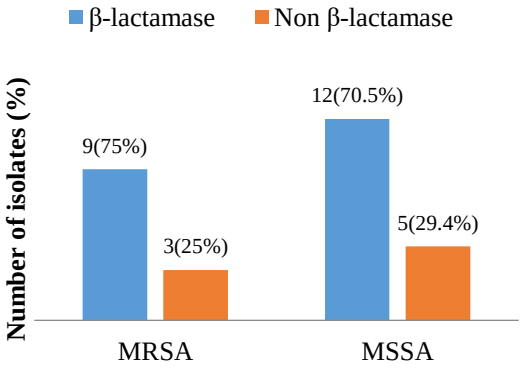


Figure 4: β-lactamase enzyme production in MRSA and MSSA isolates

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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Knowledge and Perception of COVID-19 Pandemic during the First Wave: a Cross-Sectional Study among Nepalese Healthcare Workers

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ABSTRACT

Objectives: This study aimed to assess knowledge and perception of COVID 19 among frontline health care workers in Nepal.

Methods: A web-based cross-sectional study was conducted among frontline healthcare workers involved in managing and treating COVID-19 in Nepal by adapting the questionnaire from the World Health Organization. Data were analyzed using the Statistical Package for Social Science version 26.0. A chi-square test was used to investigate the association level among variables at 95% level of significance.

Results: A total of 285 health professionals participated in this study, among them male (50.5%), and female (49.5%). The mean (± SD) age of participants was 27.21 ± 5.42 years old. Most of the participants got information on COVID-19 from social media (50.5%) and online news/newspapers (40.3%). On the other hand, a significant proportion of Health care workers (HCWs) had poor knowledge about its transmission (n=170, 59.6%) and an incubation period (n=129, 45.3%).

Conclusion: There was a significant gap in knowledge and perception, particularly about the transmission and incubation period. A concerned national initiative to respond the pandemic could help better compliance with behavioral guidelines to respond to this public health emergency.

Keywords: COVID-19, knowledge, perception, pandemic, Nepal

INTRODUCTION

Health Care Workers (HCWs) are in primary contact with the patient and are an essential source of exposure to others in a health care setting. HCWs were anticipating being at high risk of infection and the inception of transmission in the community. Some prior studies found a lack of knowledge and attitude of HCWs towards MERS CoV (Alsahafi & Cheng, 2016; Althomairy et al., 2018) and SARS (Deng et al., 2006). Inadequate knowledge and perception among HCWs can directly impact behaviors, resulting in delayed diagnosis, poor infection control, and disease transmission (Omrani & Shalhoub, 2015; Wixom et al., 1971).

The World Health Organization (WHO) has also implemented multiple workshops and COVID-19 materials in dozens of languages to improve safety precautions, including awareness-raising and web-based training of HCWs in preparedness activities (COVID-19, 2021). Misunderstanding among HCWs has resulted in delayed control efforts to provide the appropriate care in many instances (Hoffman & Silverberg, 2018), which ultimately contributed to the rapid spread of infection in hospitals and places where they live (McCloskey & Heymann, 2020.; Selvaraj et al., 2018).

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Based on their experience and values, knowledge may affect the perception of HCWs (Oppenheim et al., 2019.; Piltch-Loeb et al., 2019). The knowledge and perception regarding pandemic among healthcare workers have been reported differently in different studies (Abdel Wahed et al., 2020; Amin et al., 2020; Upadhyaya et al., 2020).

Due to its novel origin and rapid spread all over the world, healthcare workers must be well informed about COVID-19 to protect themselves and their patients. Therefore, the study was conducted to evaluate the knowledge and perception of COVID-19 among HCWs in Nepal.

METHODS

Study design and setting

A cross-sectional web-based -study was conducted, and a convenience sampling method was adopted. Data were obtained by taking the help of HCWs (Health Care workers) at various COVID-19 dedicated health centers in all seven provinces of Nepal, including private hospitals, government hospitals, and others, using a self-administered questionnaire.

Study participants

The study population included in this study was all registered HCWs (doctors, nurses, pharmacist, paramedics, diagnostic personnel, and public health professionals). All HCWs were working in the health sector of Nepal. Health care workers of all professions directly or indirectly involved in the screening, diagnosis, and management of COVID-19 infection was included in the study.

Sample size

The sample size was calculated using the formula.

Sample size (n) = $\frac{Z^2pq}{d^2}$

Where, n= sample size z= reliability level (1.96) p= estimated proportion in population= 0.78 q=1-p= 1-0.78= 0.22, d=maximum tolerable error (0.05) Nepal. Reference taken from a similar study on COVID-19 done in Iran (Bhagavathula et al., 2020). From the above formula, the minimum required sample size (n) calculated was 285.

Variables

Independent variables

The independent variables comprised of socio-demographic characteristics (age, gender) and work

profile (working experience- in years, working province, working sector, educational qualification) of HCWs.

Dependent variables

The dependent variables included knowledge, perception regarding COVID-19 pandemic among health care workers working in Nepal. Based on previous research conducted in other countries, we picked knowledge and perception as the dependent variables. Therefore, this study will generate a reference point of information on HCWs' knowledge and perception of COVID-19, which will aid in the development of future interventional studies that will eventually assist HCWs in preparing for COVID-19 and other infectious diseases.

Data collection tool

The questionnaire included questions on knowledge and perception about COVID-19. There were questions and statements in three sections (demographics, knowledge and perception).

The questionnaire was divided into three-part, section 1 including 12 items related to socio-demographic, awareness items and source of information: age, gender, profession, working place, types of job, work experience, heard about COVID-19, attended any of the lectures/discussions about Novel Coronavirus disease. Source of information was assessed by using 4 statement/4 point, Likert scale 1 is least used and 4 is most used.

Section 2 included 7 items of knowledge about symptoms of COVID-19-affected patients (2 items), different modes of transmission (2 items), precautions and risk prevention (3 items). A score of '1' was given a 'correct' response and a score of '0' for a 'wrong' response. Section 3 included 7 items perceptions of COVID-19 (7 items/true or false questions). A score of '1' was given for response 'good' and a score of '0' was given for responses 'poor' (Bhagavathula et al., 2020).

Ethical approval

Ethical approval was obtained from the Ethical Review Board of Nepal Health Research Council (NHRC) (IRB reference number 2883). The objective of the study was mentioned and the consent for participation was also obtained from the participants to participate in the survey and they were allowed to leave the study form anytime during the study if they don't want to continue.

Data collection procedure

A Web-based, cross-sectional study was conducted from 19 June to 16 August, 2020 (One month and twenty-eight days), among frontline health workers. Validated 23-item survey instrument was used from WHO course material on an emerging respiratory virus, including COVID-19

(Bhagavathula et al., 2020). Unfortunately, the Nepali language version of this tool has not yet been prepared. A total of 310 responses 25 were redundant.

The questionnaire was developed using Google form and sent to the health workers via different messenger groups and other social media including Facebook, Twitter and Viber. Only the core study member has access to the data archive to protect data privacy.

Content of the survey instrument and scoring system

Knowledge was assessed by the questions focusing on COVID-19 etiology, signs and symptoms, transmission, and risk prevention. Each response was scored as "1" (correct) and "0" (wrong), with scores ranging from 1 to 7. A cutoff level of ≤4 was considered to indicate poor knowledge about COVID-19 whereas >4 was considered adequate knowledge about COVID-19 (Bhagavathula et al., 2020).

Perceptions towards COVID-19 were assessed using 7 items, and each question was labelled as good (scored as "1") or poor perception (scored as "0"). Scores ranged from 0 to 7. The participants' perceptions are classified as good (score >5) or poor (score ≤5) (Bhagavathula et al., 2020).

Statistical analysis

The obtained data were checked, coded and exported to Microsoft Excel and then imported in Statistical Package for Social Sciences (SPSS) version 26 statistical software for cleaning and analysis. Descriptive statistics were applied to compute frequency, standard deviation and percentage with a 95% confidence interval. The chi-square test was performed to investigate the association level among variables.

RESULTS

Demographic characteristics of the participants

A total of 285 HCWs were involved in this study. Almost half of the participants (50.5%) were males. Mean (±) SD age of participants was 27.21 ± 5.42 years and ranged from 18 to 50 years (Table 1).

Table 1. Socio-demographic characteristics of health care workers (n=285)

Characteristics	Participants, n (%)
Sex	
Male	144 (50.5)
Female	141 (49.5)
Age in year	
< 20	17 (6.0)
20-25	108 (37.9)
25-30	113 (39.6)
30-35	18 (6.3)
35-40	20 (7.0)
> 40	9 (3.2)
Profession	
Allied Health workers	190 (66.7)
Doctor	73 (25.6)
Public health	22 (7.7)
Working Province	
Province 1	29 (10.2)
Province 2	14 (4.9)
Bagmati	175 (61.4)
Gandaki	21 (7.4)
Lumbini	29 (10.2)
Karnali	3(1.1)
Sudhur paschim	14 (4.9)
Working Sector	
Private	173 (60.7)
Government	112 (39.5)

Source of knowledge

In this study, the source of knowledge on COVID-19, the primary source of information, was obtained from the official website of the Government (n= 56 19.6 %) and family members and colleague (n= 34 11.9%). The source of information was more often from a family member and colleague (n=91 31.9%). Among the study participants most of the participants reported that we mostly used social media for the source of knowledge 50.5% and only 11.6% of the participants used social media least for the source of knowledge of COVID-19 (Table 2).

Knowledge of COVID-19

The level of knowledge regarding COVID-19 among HCWs is shown in Table 3. For instance, 73 doctors (82.2%) and 141 allied health workers (66.5%), thought that COVID-19 derived from bats which was reported to be significantly associated at 95% level of significance. Majority of the participants correctly responded that hand hygiene, covering nose and mouth while coughing, and avoiding sick contacts can help in the prevention of COVID-19

Perception of COVID-19

Table 4 lists the perception about COVID-19, Majority of the respondants (88.4%) (health workers) has a positive perception regarding COVID-19.

The majority of participants (88.8%) thought COVID -19 is not fatal, and the flu vaccine is not adequate against COVID-19 85.6%. The Flu vaccine was not sufficient against COVID-19 85.6%. Over 90.9% of the HCWs knew that properly cooked meat products are acceptable for consumption.

Association between sex, age and profession

Items related to perceptions of COVID-19 among HCWs were analyzed separately using a chi-square test to examine their association with age and sex and across different professions. (Table 5)

The majority of the participant <25 years old (n=114, 91.2%) and 84.9% (n=62) of the doctors believed that the symptoms of the COVID-19 appear 2-14 days. Many doctors correctly believed that sick patients should share their travel history (n=71, 97.3%). Nurses were found to perceive that flu vaccination is insufficient to prevent COVID-19 transmission (n=60, 90.9%). The false perception that washing hands with soap and water can help prevent COVID-19 transmission was 18.5% in other health workers than doctors and nurses. Flu vaccinated is sufficient for preventing COVID-19 was reported to be significantly associated with the age group categorized in table 5".

Table 2. Participants Source of knowledge about (COVID-19) (n=285).

Response	Source of knowledge about COVID 19			
	News media, n (%)	Social Media, n (%)	Government website, n (%)	Family member and colleague, n (%)
Least used	34 (11.9)	33 (11.6)	56 (19.6)	34 (11.9)
Sometimes	61 (21.4)	35 (12.3)	71 (24.9)	73 (25.6)
More often	75 (26.3)	73 (25.6)	85 (29.8)	91 (31.9)
Most used	115 (40.3)	144 (50.5)	73 (25.6)	87 (30.5)

Table 1.Knowledge about coronavirus disease 2019 (COVID-19) among health care worker (n=285)

Questions	Doctors (n=73), n (%)	Allied health workers/ Public health Profession (n=212), n (%)	Total correct responses, (n=285) n (%)	p value
COVID-19 is thought to originate from bat	60 (82.2)	141 (66.5)	201 (70.5)	**0.01**
COVID-19 transmitted through air, contact, feco-oral route	26 (35.6)	89 (42)	115 (40.4)	0.4
Headache, fever, cough, sore throat, and flu are symptoms of COVID-19	46 (63)	145 (68.4)	191 (67)	0.5
The incubation period of COVID-19 (2-14 days)	30 (41.1)	99 (46.7)	129 (45.3)	0.4
COVID-19 leads to pneumonia respiratory failure, and death	56 (76.7)	166 (78.3)	222 (77.9)	0.8
Supportive care is the current treatment for COVID-19	50 (68.5)	158 (74.5)	208 (73.0)	0.4
Hand hygiene, covering nose and mouth while coughing, and avoiding sick contacts can help in the prevention of COVID-19 transmission	63 (86.3)	192 (90.6)	255 (89.5)	0.4

Table 2. Health care worker's perceptions toward coronavirus disease 2019 (COVID-19) (n=285).

Statement	Yes, n %	No, n %
COVID-19 symptoms appear in 2-14 days	252 (88.4)	33 (11.6)
COVID-19 is fatal	32 (11.2)	253 (88.8)
Flu vaccination is sufficient for preventing COVID-19	41 (14.4)	244 (85.6)
During the outbreak, eating well-cooked and safely handled meat is safe	259 (90.9)	26 (9.1)
Sick patients should share their recent travel history with health care providers	275 (96.5)	10 (3.5)
Disinfect equipment and working area in wet markets at least once a day	253 (88.8)	32 (11.2)
Washing hands with soap and water can help in the prevention of COVID-19 transmission	37 (13.0)	248 (87.0)

Table 3. Association between Sex, Age and Profession

Question and response	Sex			Age in years			Profession			
	Male (n=144), n (%)	Female (n=141 n (%)	P value	<25 (n=125), n (%)	25-50 (160), n (%)	P value	Doctor (n=73), n (%)	Nurse (n=66), n (%)	Other health worker(n=146), n (%)	P value
COVID-19 symptoms appear in 2-14 days			0.1			0.2				0.4
Yes [a]	123 (85.4)	129 (91.5)		114 (91.2)	138 (86.3)		62 (84.9)	61 (92.4)	129 (88.4)	
No	21 (14.6)	12 (8.5)		18 (14.8)	22 (13.8)		11 (15.1)	5 (7.6)	17 (11.6)	
COVID-19 is fatal			0.7			0.1				0.4
Yes	15 (10.4)	17 (12.1)		18 (14.4)	14 (8.8)		5 (6.8)	9 (13.6)	18 (12.3)	
No [a]	129 (89.6)	124 (87.9)		107 (85.6)	146 (91.3)		68 (93.2)	57 (86.4)	128 (87.7)	
Flu vaccinated is sufficient for preventing COVID-19			0.2			0.01				0.3
Yes	25 (17.4)	16 (11.3)		25 (20)	16 (10)		10 (13.7)	6 (9.1)	25 (17.1)	
No [a]	119 (82.6)	125 (88.7)		100 (80)	144 (90)		63 (86.3)	60 (90.9)	121 (82.9)	
During the outbreak, eating well-cooked and safely handled meat is safe			0.7			0.3				0.8
Yes [a]	132 (91.7)	127 (90.1)		116 (92.8)	143 (89.4)		66 (90.4)	59 (89.4)	134 (91.8)	
No	12 (8.3)	14 (9.9)		9 (7.2)	17 (10.6)		7 (9.6)	7 (10.6)	12 (8.2)	
Sick patients should share their recent travel history with health care providers			0.7			0.7				0.8
Yes [a]	138 (95.8)	137 (97.2)		120 (96)	193(87.3)		71 (97.3)	64 (97.0)	140 (95.9)	
No	6 (4.2)	4 (2.8)		5 (4)	28 (12.7)		2 (2.7)	2 (3.0)	6 (4.1)	
Disinfect equipment's and working area in wet markets at least once a day			0.3			0.08				0.4
Yes [a]	125 (86.8)	128 (90.8)		104(83.2)	149(93.1)		66 (90.4)	61 (92.4)	126 (86.3)	
No	19 (13.2)	13 (9.2)		21(16.8)	11(6.9)		7 (9.6)	5 (7.6)	20 (13.7)	
Washing hands with soap and water can help in prevention of COVID-19 transmission			0.2			0.5				0.2
Yes [a]	23 (16.0)	14 (9.9)		18 (14.4)	19 (11.9)		5 (6.8)	5 (7.6)	27 (18.5)	
No	121 (84)	127 (90.1)		107 (85.6)	141 (88.1)		68 (93.2)	51 (92.4)	119 (81.5)	

[a] Insicates correct answer

DISCUSSION

This study provides evidence of the knowledge and perception of COVID-19 among individual health workers working in Nepal. Currently rapidly growing COVID-19 transmission for everyone and health workers, a crucial question arises how we manage information to help health workers in public health crises. The findings of this study suggest a significant gap between the amount of information available on COVID-19 and the depth of knowledge among HCWs, particularly about the mode of transmission and the incubation period of COVID-19.

In this study, the level of knowledge of HCWs about COVID-19 was low, but perceptions of COVID-19 transmission prevention were reported positive, which was similar to the Bhagavathula et al. finding (Bhagavathula et al., 2020). A similar cross-sectional study from China recorded that 90% of the health profession had a strong knowledge of COVID-19 and that 70% of the participants had adequate knowledge in the Uganda study (Olum et al., 2020; Zhong et al., 2020). This study reported that 25.6 % (n=73) of HCWs used the official government website as the primary source of knowledge on COVID-19.

This suggests that COVID-19 related information posted online by official government authorities had a significant effect on improving the level of awareness of HCWs. More than half (50.5%) of HCWs use social media as a source of knowledge. Currently, there is a wide range of information available via the internet that leads to debunk information that can spread rapidly and misguide HCWs. The World Health Organization warns of a potential "infodemic" of fraudulent news ("WHO Says Fake Coronavirus Claims Causing 'Infodemic,'" 2020). HCWs should be proactive in selecting the source of information about COVID-19 related information and should use a more scientific and authentic source of information. Similarly, virtual (online) seminars, online training, and courses can help educate and raise awareness about COVID-19 and other emerging infectious diseases (Shrestha et al., 2020).

The outcomes of this study indicate that lower-level knowledge of COVID-19, in particular, the mode of transmission and the incubation period of COVID-19, less than 50 % of the participants only gave an accurate answer to the question. We found significant knowledge gaps between doctors and other HCWs. Knowledge of healthcare worker's is more valuable than the public.

HCWs are one of the critical sources of information about COVID-19 and their role in treating the disease and prevention. If HCWs do not have sufficient knowledge of COVID-19, they also contribute to the spread of COVID-19 to the general population because they are more likely to be exposed to the virus.

Educational status is a powerful indicator of knowledge and perception. The literacy rate in Nepal is only 66 per cent, which is even lower in rural areas (Central Bureau of Statistics – CBS, 2011). Our findings indicate that greater support from health authorities is required to spread COVID-19-related information across all categories of HCWs.

The majority of HCWs had a positive perception of COVID-19 as a preventable disease. Although, discordance was identified in the perceptions of different kinds of HCWs. Concerning perception related to the symptoms, the possibility of survival and vaccination was more than 80% of participants' found to respond to these items correctly. Most of HCWs aged 25-50 years (n=149, 93.1%) recommended that disinfect the equipment at least once a day. More than 95% of HCWs had agreed that a sick person should share their travel history. More than a quarter of the doctors thought that eating meat during the outbreak was unsafe. That could be because COVID-19 was closely linked to a wet market in China, and other viral infections such as SARS, MERS, and Ebola emerged from zoonotic pathogenic agents (Carnero et al., 2018; Zhu et al., 2020). Primary cases were reported in 58 districts, even during the lockdown. The government of Nepal approved the import of everyday most important goods such as gasoline, LP gas, food items, and grocery items from India, which resulted in a major spike in COVID-19 instances, primarily in bordering regions with India. India had the highest number of COVID-19 cases and CFR in South Asia; COVID-19 infection among Indian drivers and car owners who travelled to Nepal could have spread the virus to other parts of Nepal (Paudel et al., 2020). Finally, a vast majority of HCWs fully agreed that maintaining hygiene practice, reporting recent travel history when individuals are sick and cleaning the equipment used in wet markets are strongly recommended. Until date, many new SARS-CoV-2 variants (alpha, beta, delta etc.) were emerging in the world with change in disease pattern, severity, nature and symptoms of COVID 19 in compare to original SARS-CoV-2. Therefore, this study unable to measure the knowledge and perception of COVID 19 caused by new variants of SARS-CoV-2. A newer study on knowledge and perception level of HCWs of COVID 19 caused by new emerging variants at present context should be carried out for in depth understanding of the changing pattern of COVID 19.

CONCLUSIONS

The knowledge of the study participant regarding preventive strategies or techniques in this study found insufficient knowledge level, a significant gap in information and different perception of COVID -19 among participant.

Data presented in this study are self-reported and partly dependent on the participant's honesty and recall ability. Participants not using the internet could not participate in this study, so it cannot be generalized for all the health workers. Despite the limitation, the study was conducted using the WHO developed the questionnaire and which was already used. Our findings provide valuable information about the knowledge and perceptions of HCWs during peak period of the pandemic.

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CONFLICT OF INTEREST

The authors declare that there is no potential conflict of interest with respect to this paper.

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Microbiological Study of Food Packaging Paper of Kathmandu Valley

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ABSTRACT

Objectives: The purpose of this study was to isolate and identify microorganisms of food packaging papers of Kathmandu valley and determine antibiotic susceptibility of the isolates.

Methods: A total of 34 food packaging paper samples were collected aseptically from hotels, bakeries and sweet shops (considered as closed shop) and open street vendors and were transported to microbiology laboratory of Golden Gate International College for processing. The isolates were identified by standard microbiological procedures and subjected to antimicrobial susceptibility testing by modified Kirby-Bauer disk diffusion method following CLSI guidelines. The rate of Extended Spectrum Beta- lactamase (ESBL) producing and multiple drug resistant (MDR) isolates were also determined.

Results: All 34 samples yielded microbial growth with average microbial count of 4.145×10^5 CFU/g. Among 103 microbial isolates, 78 were bacteria, 15 molds and 10 yeasts. The predominant bacterial and mold isolates were *Bacillus* spp (43.59%) and *Cladosporium* spp (46.67%) respectively. Ciprofloxacin (42/43) and Amikacin (42/43) were the most effective and ampicillin (39/43) was most resistant antibiotics for Gram negative bacteria. A total of 9.30% Gram negative isolates were identified as ESBL producing and MDR strains.

Conclusion: This result indicates that potential pathogens are found in food packaging papers which can be threat to health of consumers as they may act as a source of food borne infection.

Keywords: Food packaging papers, antibiotic susceptibility testing, MDR, ESBL

INTRODUCTION

Enormous number of people consume several varieties of foods which are generally served in recycled papers such as abandoned recycled newspapers (Hladikova et al. 2015). The main ingredient of all paper is biodegradable plant material cellulose fibers, hemicellulose and lignin. Besides, loading or filling materials like $CaCO_3$, Talc and other several other chemicals depending on the type of paper may be used (Guzińska et al. 2012).

The biodegradable constituents can enhance microbial growth in paper and paperboard packaging whereas contamination can occur a result of contaminated raw materials used in paper production, during processing of raw materials, during transportation and during handling (Mohammadzadeh-Vazifeh et al. 2015).

As a packaging material, newspapers, academic papers, hospital report papers are also used. These papers often come in direct contact with food like Samosa, Chatpate, Paratha, pakoda, bakery products and other Nepali street foods. These re-used papers may be already contaminated when stored in dirty and damp places (Rana et al. 2019). The contaminating microbes can decay food (e.g., *Enterobacter cloacae*, *Bacillus subtilis*), generate odorous compounds (e.g., actinomycetes, *Clostridium* spp), produce slime (e.g., *Bacillus* spp, *Klebsiella* spp) and impact human health when they encounter food (e.g., *Proteus* spp, *Salmonella* spp, molds) (Raaska et al. 2002).

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Many studies have reported spore-bearing Gram-positive bacteria *Bacillus* as the maximum protruding families for paper and paperboard contaminant. Other commonly found bacteria are Klebsiella spp, Citrobacter spp, Proteus spp, Pseudomonas spp, Salmonella spp, Enterobacter spp, Staphylococcus aureus, etc. (Vaisanen et al. 1991).

The consumption of such contaminated food through various food packaging could result in outbreak of food borne illness. Health organizations of several countries have recognized microbial content value of the paper and paperboard in food packaging but still there is no thoughtful global consideration to the bio-hazardous exposures that may arise from microbial pollution in food packaging. The regular monitoring of total bacterial count and the presence of fecal coliforms in paperboards is needed to reduce such illness. Therefore, this study aimed to determine the microbial load with their antibiotic susceptibility pattern. The outcome of this study would be helpful to reduce microbial load by suggesting good hygiene practices to all food handlers including consumers.

METHODS

Sample collection

A total of 34 food packaging paper samples from different places of Kathmandu and its vicinity were collected in steam sterilized polythene bags and transported to laboratory of Goldengate International College. Sample collection was done during study period of April to June 2019.

Microbial load detection of paper samples

Sample preparation was done by defibering method in which Ringer's solution can easily dissolve fibers containing microorganisms (Mohammadzadeh-Vazifeh et al. 2015). The bacterial load was determined by using Plate Count Agar (PCA) and fungal load was determined by using Potato Dextrose Agar (PDA) with 10-fold dilution in normal saline. One gram of each paper sample was weighed followed by serial dilution up to 10^{-5} and then inoculated aseptically on Plate Count Agar by using pour plate technique.

For selective isolation, a loopful of diluted sample (10^{-1}) was inoculated on selective media like MacConkey Agar, Mannitol Salt Agar, Eosin Methylene Blue Agar, Salmonella-shigella agar and Tryptose Citrate Bile Salt Sucrose Agar and incubated at 37°C for up to 48 hours. The isolated colonies from these media were identified by observing colony morphology followed by Gram staining and biochemical tests.

PDA plates incorporated with chloramphenicol ($0.05gl^{-1}$) were observed for fungal growth. Yeasts and molds were differentiated by observing colony morphology and microscopic study. Molds were further identified following standard microbiological procedures (*Fungal Descriptions and Antifungal Susceptibility*, n.d.).

Antibiotic susceptibility testing

Modified Kirby-Bauer disk diffusion test based on the guidelines of Clinical and Laboratory Standard Institute (CLSI 2012) method was used to evaluate the antimicrobial susceptibility pattern of the isolates to a set of antibiotics and determination of methicillin resistance *S. aureus* and ESBL producing strains. The antimicrobial agents tested for Gram negative bacteria were Ampicillin (AMP,10µg), Imipenem (IMI,10µg), Gentamycin (GEN,10µg), Cefotaxime (CTX,30µg) Ceftazidime (CAZ,30µg)), ciprofloxacin (CIP ,5µg) , Cefixime (CFM,5 µg) and Piperacillin/ Tazobactam (PIT) and for Gram positive bacteria were: Amikacin (AK,30µg), Chloramphenicol (C,30 µg), Cloxacillin (COX,10µg), Cotrimoxazole (COT,25µg) Ciprofloxacin (CIP,5µg), Erythromycin (E,15µg), Tetracycline (TE,30µg), Gentamycin (GEN,10µg).

The multidrug resistance was tested among the isolates and interpreted by using the standard guideline (Magiorakos et al. 2011)

Screening of ESBL producing and MDR organisms

ESBL producers were detected from Ceftazidime and/or Cefotoxime resistant isolates using standard combined disc-diffusion method. ESBL producer was detected by more than 5 mm distance difference in zone size between ceftazidime/ceftazidime with clavulinic acid (CAZ/CAC) and cefotaxime/ceotaxime with clavulinic acid (CTX/CEC) (CLSI 2014).

The multidrug resistance was tested among the isolated and interpreted by using the standard guideline (Magiorakos et al. 2011).

RESULTS

Among 34 paper samples collected from closed shop and open street vendors, closed shop used paper and paperboards (PPBs) whereas open street vendors extensively used reused newspaper, academic papers, office documents, printed papers and even hospital papers for food packaging. Due to this although all the samples had equal probability of getting contaminated, samples obtained from open street vendors had significantly higher microbial yield.

Microbial load detection

The food packaging paper was found to be most contaminated with an average bacterial and fungal load of 1.53×10^5 CFU/g. The obtained average microbial count obtained in open (n=21) and closed (n=13) paper samples were 3.62×10^5 CFU/g and 4.67×10^5 CFU/g respectively (Figure 1).

Microbial diversity

All the samples tested were found to be contaminated. Among the 103 microbial species identified, predominant isolates were bacteria followed by molds and yeasts (Figure 2).

Distribution of bacteria

A total of 78 bacterial isolates of 9 different species were identified, of which 4 were coliform group of bacteria, 3 were Gram negative bacteria other than coliforms and 2 Gram positive isolates. *Bacillus* spp 34 (43.59%) was the predominant isolate followed by K*lebsiella* spp 16 (20.51%). Majority of the isolates 46 (58.97%) were detected from the samples of street vendors (open retailer). Only 32 (41.03%) isolates were detected from the samples of closed retailers (Table 1).

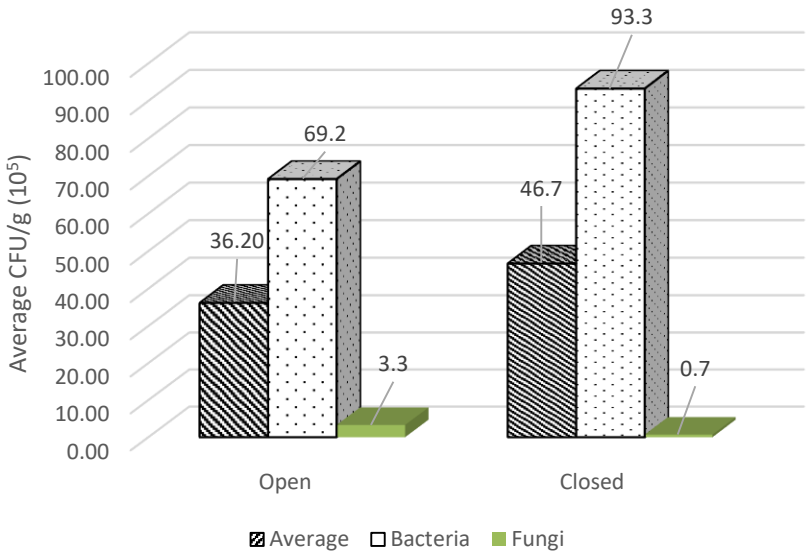

Figure 1: Enumeration of microorganism in paper samples

Distribution of fungi

Among 25 isolates of fungi isolated, 15 (60%) were molds and 10 (40%) were yeasts. Among molds identified, *Cladosporium* spp 7/15 (46.67%) was the dominant one followed by *Aspergillus* spp*, Mucor* spp and *Fusarium* spp.

Antibiotic susceptibility pattern of coliforms

The coliform isolates were most resistant against ceftazidime and ampicillin.

Antibiotic susceptibility pattern of Gram negative bacteria other than coliforms

The non-coliform isolates were resistant against ceftazidime, ampicillin and cefotaxime.

Antimicrobial susceptibility of *Staphylococcus aureus* isolates

The single isolate of *Staphylococcus aureus* was sensitive towards Gentamicin, Clindamycin, Chloramphenicol, Tetracycline and Erythromycin i.e., 1 (100%) and resistant against Cefoxitin, Penicillin and Ciprofloxacin i.e. 0 (0%).

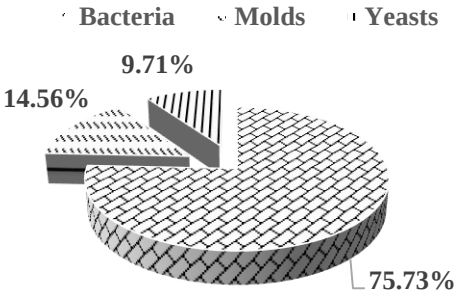

Figure 2: Microbial diversity of food packaging paper

Table 1: Distribution of bacterial isolates according to the retailer type

Category	Organisms	Retailer Type		Total (n)(%)
		Open (n)	Closed (n)	
Coliforms	*E. coli*	0	1	1(1.28)
	Klebsiella spp	11	5	16(20.51)
	Citrobacter spp	4	4	8(10.26)
	Enterobacter spp	1	5	6(7.69)
Sub-total		**16**	**15**	**31**
Gram negative bacteria other than coliforms	*Pseudomonas* spp	5	3	8(10.26)
	Salmonella spp	1	1	2(2.56)
	Proteus spp	2	0	2(2.56)
Sub-total		**8**	**4**	**12**
Gram positive bacteria	*Staphylococcus aureus*	1	0	1(1.2)
	Bacillus spp	21	13	34(43.59)
Sub-total		**22**	**13**	**35**
Total		**46**	**32**	**78**

Table 2: Distribution of fungi in paper samples

S.N.	Sample type	Sample	Fungi	Number	Percentage
1	**Open**	21	*Aspergillus* spp	2	9.52
			Cladosporium spp	3	14.28
			Mucor spp	2	9.52
			Yeasts	7	33.33
	Sub-total			**14**	
2	**Closed**	13	*Cephalosporium* spp	1	7.69
			Penicillium spp	1	7.69
			Cladosporium spp	4	30.77
			Fusarium spp	2	15.38
			Yeasts	3	27.27
	Sub-total			**11**	
	Total			**25**	

Distribution of ESBL-producing organisms

Out of total 78 isolates, 44 isolates were subjected for ESBL screening test. A total of 35(79.55%) isolates were screened positive. ESBL production by ceftazidime 5(14.28%), cefotaxime 11(31.43%) and both 19(54.28%) of them. 0(0%) were confirmed to be ESBL producer.

Table 3: Antibiotic Susceptibility Test of coliforms

Antibiotics	*Klebsiella* spp(N=16) n (%)	*Citrobacter* spp(N=8) n (%)	*Enterobacter* spp(N=6) n (%)	*E. coli*(N=1) n (%)
GEN	14 (87.5)	8 (100)	6 (100)	1 (100)
AK	16 (100)	8 (100)	5 (83.3)	1 (100)
PIT	15 (93.7)	7(87.5)	6 (100)	1 (100)
IPM	16 (100)	7 (87.5)	6 (100)	0 (0)
CTX	9 (56.2)	3 (37.5)	3 (50)	1 (100)
CFM	10 (62.5)	7 (87.5)	2 (33.3)	1 (100)
CIP	16 (100)	8 (100)	6 (100)	1 (100)
CAZ	14 (87.5)	0 (0)	0 (0)	1 (100)
AMP	1 (6.25)	0 (0)	0 (0)	1 (100)

GEN-Gentamicin, AK-Amikacin, PIT-Piperacillin/Tazobactam, IPM-Imipenem, CTX-Cefotaxime, CFM-Cefoxime, CIP-Ciprofloxacin, CAZ-Ceftazidime AMP- Ampicillin

Table 4: Antibiotic Susceptibility Test of Gram-negative bacteria other than coliform

Antibiotics	*Pseudomonas* spp(N=8) (n%)	*Salmonella* spp(N=2) (n%)	*Proteus* spp(N=2) (n%)
GEN	7 (87.5)	2 (100)	2 (100)
AK	8 (100)	2 (100)	2 (100)
PIT	7 (87.5)	2 (100)	1 (50)
IPM	7 (87.5)	2 (100)	2 (100)
CTX	2 (25)	1 (50)	0 (0)
CFM	7 (87.5)	2 (100)	0 (0)
CIP	8 (100)	1 (50)	2 (100)
CAZ	2 (25)	1 (50)	1 (50)
AMP	1 (12.5)	1 (50)	0 (0)

GEN-Gentamycin, AK-Amicakin, PIT-Pipercillin/Tazobactam, IPM-Imipenem, CTX- Cefotaxime, CFM- Cefoxime, CIP-Ciprofloxacin, CAZ-Ceftazidime, AMP- Ampicillin.

Table 5: Distribution of ESBL-producing organisms

Isolate	Screened positive			Confirmed
	CAZ only	CTX only	Both	
Klebsiella spp (n=16)	0	9	5	2
Citrobacter spp (n=6)	1	1	3	0
Salmonella spp (n=2)	0	0	1	0
Enterobacter spp (n=6)	4	0	2	1
Pseudomonas spp (n=8)	0	1	7	1
Proteus spp (n=2)	0	0	1	0

Table 6: MDR profile of the isolates

Resistance towards drug	Number of isolates	Number of Antibiotic classes	Organism
AMP, CTX, PIT	1	3	*Klebsiella* spp
AMP, CAZ, CFM, PIT, CTX	1	3	*Enterobacter* spp
AMP, GEN, CAZ, CFM, CTX	1	3	*Klebsiella* spp
AMP, CFM, IPM	1	3	*Pseudomonas* spp

Multidrug resistance

Two species of *Klebsiella* spp, one *Enterobacter* spp and one *Pseudomonas* spp were confirmed to be multi drug resistant (Table 6).

DISCUSSION

Paper being biodegradable and environment friendly, they are the most commonly used food packaging materials in comparison to plastic and other method of food packaging. Paper packaging is not only prevalent among street vendors even sweet shops, bakeries, etc. also use them commonly. As food remains in contact with these papers, microbiological study of them can be considered as an important aspect as it's a matter of health of general people.

During this study, the total number of 34 food packaging paper samples were collected from different places of Kathmandu valley during 3 months of study from April to June 2019. Each of the 34 samples yielded microbial growth. This may have occurred as a result of contaminated raw materials used in paper production, during processing of raw materials, during transportation and during handling. The microbes were enumerated, isolated and identified for microbial analysis.

The average bacterial load obtained from defibering method was (2.65×10^2 -5.4×10^6) CFU/g which was comparable with study performed by Mohammadzadeh-Vazifeh et al. (2015) which was in the range of (0.2×10^3 to $> 1.0\times10^5$) CFU/g and comparatively less than studied

by Rana et al. (2019) which was in the range of (1.9×10^8-7.5×10^8) CFU/g.

Higher number of bacterial isolates were detected with range between (2.7×105-3.01×105) CFU/g which exceed the given permittable range of 2.5×102 CFU/g for paper materials used for food packaging defined by FDA (Food and Drug Administration) (Sood and Sharma 2019).

Lower number of isolates were found to be at the range of 0.2×102 to 0.4 ×102 CFU/g which is accordance to the value defined by FDA and can be considered as safe for packing food.

The total number of microbial isolates detected were 103 of which *Bacillus* spp(43.59%) was the predominant bacteria followed by *Klebsiella* spp. This may be due to their ubiqutous and spore forming nature. Study conducted by Sood and Sharma (2019) also reported *Bacillus* spp as dominant bacteria. In paper industry these *Bacillus* spp are primary organisms to accumulate slime by themselves which starts by formation of monomolecular layer. These bacteria also enhance growth of secondary organisms such as *Klebsiella* spp and *Pseudomonas* spp (Blanco et al. 1996). The growth of other bacteria isolated also have potential to cause food borne illness leading to complications (Bennett et al. 2013).

Molds like *Cladosprium*, *Aspergillus*, *Fusarium* were identified which have potential to produce mycotoxin directly affecting the consumers' health (*Mycotoxins: Risks in Plant, Animal, and Human Systems*, 2003).

Ciprofloxacin (42/43) and Amikacin (42/43) were most effective and ampicillin (39/43) was most resistant antibiotics towards Gram negative bacteria. No MRSA isolates and four ESBL producers *Klebsiella* spp (2), *Pseudomonas* spp (1) and *Enterobacter* spp (1) were confirmed from paper samples. Similarly, all the ESBL producers were MDR. Presence of MDR isolates suggests spread of community-associated (CA) MDR bacteria related to high mortality and morbidity (van Duin and Paterson, 2016). The high resistance to the commonly used antibiotics may be due to random source of the papers including hospital. This result indicates that potential pathogens are found in food packaging papers which can be threat to health of consumers.

CONCLUSION

All of the 34 samples were contaminated with bacteria and fungi among which *Bacillus* spp was the most predominant bacteria. Also, the bacterial load in open paper used by street vendors exceeded the permissible limit provided by FDA.

Mostly reused newspaper, academic papers, office documents, printed papers and even hospital report papers were used as packaging materials. Microbial contamination depends on the type of papers used by them. The presence of such microbial contaminants is uncommon and unsafe for human health. So, the reliable safe supply of food is important for people's general health. The result confirmed that the microbial contamination of paper-based foodstuff may impose health hazard or infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antimicrobial Activity of Some Common Spices

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ABSTRACT

Objective: Antibiotic toxicity and multi drug resistant pathogens are the two greatest challenges that today's medical world has been facing. As a consequence of the haphazard use of antimicrobials, the spread of antimicrobial resistance is now a global issue. This study aimed to investigate antimicrobial activity of some common spices.

Methods: During the study period Five commonly used spices were collected from local market of Lagankhel, Lalitpur. The antimicrobial activity of selected naturally grown spices was done against two gram positive and three gram-negative pathogenic bacteria. The extracts of the spices were obtained by using absolute ethanol (99.9%) to carry out the antibacterial susceptibility assay using agar well diffusion method.

Results: The result of agar well diffusion method showed Clove and Cinnamon were found to possess relatively higher antimicrobial activities by preventing the growth of all 5 tested bacteria. Gram positive bacteria were found to be more sensitive to spices than Gram negative bacteria.

Conclusion: The finding of this study showed that extract of spices can be alternative to synthetic drugs to control infectious diseases.

Key words: Spices, absolute ethanol, dimethyl sulfoxide, antibacterial susceptibility assay, agar well diffusion method

INTRODUCTION

Spices have been used for many centuries by various cultures to enhance flavor and aroma of our foods as our ancestors have recognized the usage of spices in food preservation and in treatment of clinical ailments and there are several reports on development of antibiotic resistance in diverse bacterial pathogens (Gold and Moellering 1996). There is no particular definition of spices, mostly because they are derived from different parts of the plants, such as clove from flower bud, pepper from fruit, cinnamon from bark or ginger from rhizome. Spices and aromatic vegetable materials have long been used in food not only for their flavour and fragrance qualities and appetizing effects but also for their preservative and medicinal properties. Since the ancient times, they have been used for preventing food spoilage and deterioration and also for extending the shelf life of foods (Shan et al 2007).

The antioxidant and antimicrobial property of spices are very important to preserve the quality of food material and at the same time provide safety to consumer (Singh et al 2007).

The present study aimed to investigate the antibacterial properties of locally available spice extracts of *Zingiber officinale, Circuma longa, Piper nigrum, Syzygium aromaticum* and *Cinnamonum verum* using agar well diffusion method against some gram negative bacteria (*Escherichia coli, Pseudomonas* sps*, Salmonella* sps) and gram positive bacteria (*Staphylococcus aureus, Bacillus* sps). Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased (Bernhoft 2010). The use of alternatives and natural sources of antimicrobials hence becomes important to tackle with the current situation.

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Present investigation was carried out to report antimicrobial activity of the extract of commonly used spices against microorganisms that can cause infectious disease.

METHODS

In this study five commonly used spices were randomly sampled from the local market of Lagankhel, Lalitpur. Clean and dirt free spices were dried in laminar flow biological safety cabinet. Among the wet spices, turmeric and ginger were skinned. They were oven dried at 55^0C.The spices extract was prepared by ethanol extraction method. The sticky mass of the extract was dissolved in an aliquot of 1ml of 10% v/v Dimethyl sulfoxide (DMSO). The phytochemical yield was estimated according to the method described by Prasani et al (2005).

Five microorganisms, two gram positive (*Staphylococcus aureus* and *Bacillus* sps) and three gram negative (*Pseudomonas* sps. *Escherichia coli* and *Salmonella* sps) were used for antimicrobial susceptibility test. All strains were hospital isolates preserved at Department of Microbiology, D.A.V. College. For bacterial growth nutrient agar and nutrient broth (Himedia Laboratories Ltd. Mumbai, India) were used. Muller Hinton agar (Himedia Laboratories Ltd. Mumbai, India) was used for sensitivity assay and Dimethylsulphoxide (DMSO) (HiMedia Laboratories Ltd. Mumbai, India) was used for solvent for tested extracts.

The crude extracts of plants were screened for its antimicrobial activity against the organisms by agar well diffusion method. The bacterial culture was taken from NA and inoculated in nutrient broth and incubated at 37°C for 3 hours and turbidity was compared with 0.5 McFarland standard dilutions having cell suspension of about $1.5x10^8$ CFU/ml. This was used to inoculate by carpet culture on the surface of MHA plates. The 6mm sterile cork borer was used to make wells. Sample extract of 50µl was used in 6mm well and DMSO as a negative control. The plates were then left for half an hour with the lid closed. Then the plates were incubated at 37°C for 24 hrs then observed for the zone of inhibition which is suggested by the clear area around the well (WHO 1991).

Determinations of MICs of the spice extracts were determined by well diffusion and agar dilution techniques and the concentrations of the extracts used were 0.25, 0.05, 0.1 and 0.2 mg/ml. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration. MBC was determined for those extract which showed the antibacterial activities by two-fold dilution method.

RESULTS

There existed a difference in the percentage yield of the extracts obtained from different spices. Among the spices, the yield percentage of the spices was obtained from *Cinnamonum verum* (21%) and the lowest percentage of spice extract was obtained from *Piper nigrum* (12.85%) (Figure 1). Stalikas (2007) in his study found that extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances.

Among the five spices, clove and cinnamon were found to be effective against tested bacteria (Table 1). It was found during the present investigation that the spice extract of *Syzygium aromaticum* was the most effective spice among the five chosen spices. It had the maximum zone of inhibition against *E. coli* (20.67mm). The result of *Syzygium aromaticum* supports the results obtained by Agaoglu (2006) and Agnihotri and Vaidya (1995). In case of MIC (Table 2) the spices performed, C4 (Clove) showed the lowest concentrations in which no growth was observed in comparison to all the other spices. The MIC ranged from 0.025 mg/ml to 0.2 mg/ml in case of ethanol extracts. Black Pepper (C3) showed turbidity in most of the concentrations observed and hence it was considered to be the least effective in inhibiting the microorganisms at low concentration of the extract.

The MBC test (Table 3) results were found to be comparable to the MIC test results. The MBC ranged from 0.025 mg/ml to 0.2 mg/ml in case of ethanol extracts. Clove showed highest bactericidal activity even at lowest concentrations being the most effective among all the spices. Black pepper didn't show bactericidal activity even athigherconcentrations used in the study and showed bacterial growth on the culture media.

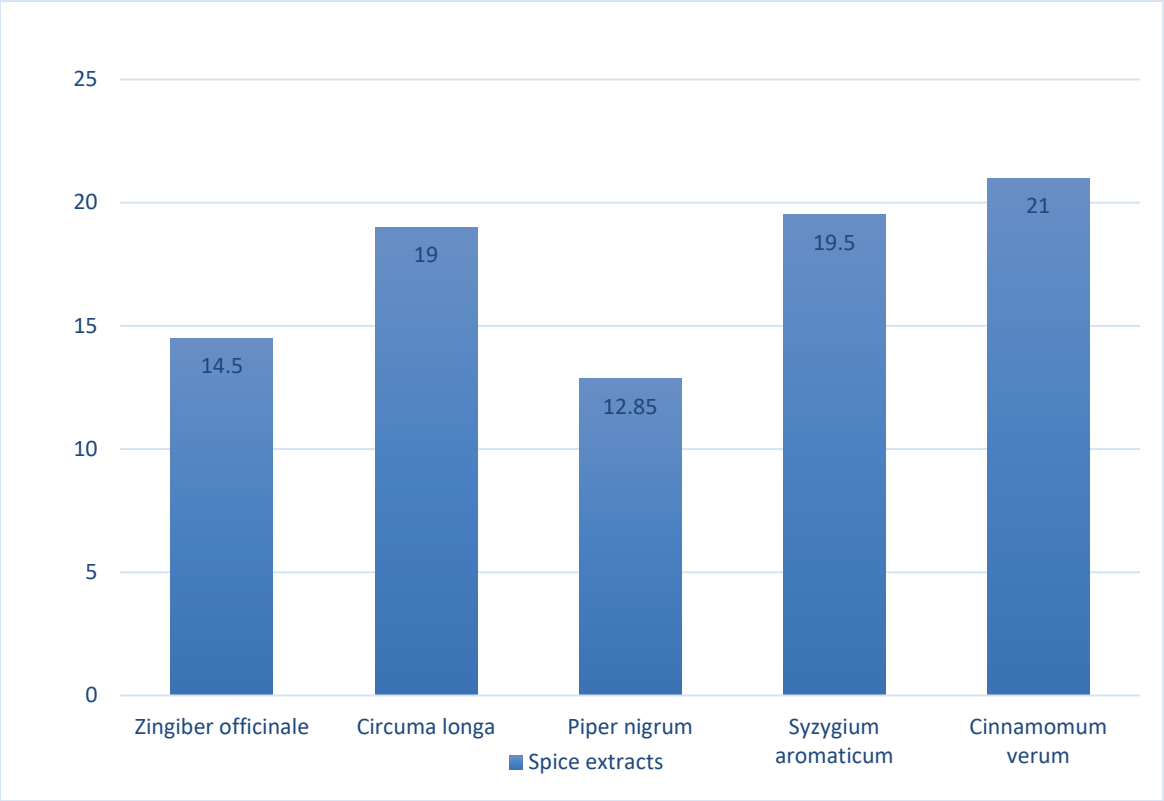

Figure 1: Percentage yield of extracts

Table 1: Antibacterial activity of spice extract against selected bacterial strains

Spices	**Minimum inhibitory concentration of spice extract (in mg/ml)**				
	E. coli	***S. aureus***	***Pseudomonas* sps**	***Salmonella* sps**	***Bacillus* sps**
Ginger	>0.2	0.2	0.2	>0.2	>0.2
Turmeric	0.05	0.025	0.2	0.1	0.05
Black pepper	>0.2	0.2	>0.2	>0.2	>0.2
Clove	0.05	0.025	0.05	0.05	0.025
Cinnamon	0.05	0.1	0.05	0.05	0.025

Table 2: Minimum inhibitory concentration of spice extracts

Spices	Zone of Inhibition(in mm)				
	E. coli	***S . aureus***	***Pseudomonas* sps**	***Salmonella* sps**	***Bacillus* sps**
Ginger	-	11.67	12	-	-
Turmeric	12	12	11.67	11.33	11.67
Black pepper	-	12.33	-	-	-
Clove	20.67	18.33	19.67	18.33	20
Cinnamon	13	12.67	15	11.33	11

Table 3: Minimum bactericidal concentration of spice extracts

Spices	Minimum bactericidal concentration of spice extract (in mg/ml)				
	E. coli	***S . aureus***	***Pseudomonas* sps**	***Salmonella* sps**	***Bacillus* sps**
Ginger	>0.2	0.2	0.2	>0.2	>0.2
Turmeric	0.1	0.05	>0.2	0.1	0.05
Black pepper	>0.2	0.2	>0.2	>0.2	>0.2
Clove	0.05	0.025	0.1	0.05	0.025
Cinnamon	0.1	0.1	0.2	0.1	0.025

DISCUSSION

This study was carried out with the aim that spices possess antimicrobial agents. In this study, commonly used spices was taken and analysed for the phytochemicals. Among the five spices used for study Cinamon (*Cinnamonum verum*) was found to be the most efficient. For the study the ethanol extraction method was used which has the capacity to extract the secondary metabolites for the antimicrobial phytochemicals like flavonoids, polyphenyl, tannis, terpenoids and alkaloids. (Tiwari et al 2011)

Antimicrobial activity of the extract also tallies with the above results as in case of cinnamon showed highest microbial activity and its MIC value also less in compare to others.

From this it ws aobserved that if proper extraction was done we can extract the active phytochemicals from the spices and use it to treat the patients with infectious diseses.

CONCLUSION

The present study provides an important basis of antimicrobial activity of these spices extracts. This guided that pure form of extract is more effective agent and can be used as alternative for the treatment of infections associated with the studied microorganisms.

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Biofilm Producing *Pseudomonas aeruginosa* in Patients with Lower Respiratory Tract Infections

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ABSTRACT

Objectives: To determine the prevalence of Gram-negative bacteria in lower respiratory tract infections and study the biofilm producing *Pseudomonas aeruginosa.*

Methods: This study was conducted at B & B Hospital Pvt. Ltd., Lalitpur, Nepal from February to September 2018. The samples were collected from the patients (n=420) with signs and symptoms suggestive of LRTIs. The isolated organisms were identified and antimicrobial sensitivity was performed. Among all the isolates, *P. aeruginosa* isolates were subjected for biofilm detection by microtitre plate method.

Results: Out of 420 specimens, 90 (21.6%) were culture positive. *Klebsiella pneumoniae* (42.9%) was found to be the predominant organism with higher rate of resistance to antibiotics. A total of 25 isolates of *P. aeruginosa* were isolated among which 15 (60%) were biofilm producers. Biofilm-producing isolates of *P. aeruginosa* were found more resistant to the tested antibiotics.

Conclusion: Gram-negative bacteria were found to be the predominant etiological agents in causing the LRTIs; *K. pneumoniae,* being the most commonly isolated bacteria. Most *P. aeruginosa* were capable of producing the biofilm. The biofilm producers were more resistant to the antibiotics. The biofilm may help increase the resistivity nature of the bacteria.

Key words: LRTI, Gram-negative bacteria, biofilm, antibiotic resistance, MDR

INTRODUCTION

Lower respiratory tract infection is one of the major health problems, being the leading cause of morbidity and mortality in many developing countries (Prajapati and Talsania 2011; Jacobs et al. 2009). The etiology, signs, and symptoms of respiratory infections vary with age, sex, season, immune status of an individual (Mishra et al. 2012). The etiologies of respiratory infections play a significant role in the diagnostic procedure. A variety of organisms are usually involved in LRTIs, Gram-negative bacteria being the most common bacteria (Goel et al. 2009; Kumari et al. 2007). *P. aeruginosa* is a commonly isolated pathogen from LRTIs (Samad et al. 2017). The microbiological investigations play important role in minimizing the complications in LRTIs. The increasing antimicrobial resistance among respiratory pathogens including *P. aeruginosa,* as it adds up the economic burden for the treatment process (Ahmed et al. 2013). *P. aeruginosa* colonizes and produces the biofilm in the respiratory tract of patients with impaired host defense mechanisms (Bentzmann et al. 1996). Biofilm formation is an important mechanism for the survival of *P. aeruginosa* which acts as the protection factor of bacteria against the host immune system and antibiotic therapy, thereby rendering the antibiotic resistance and favoring the chronicity of the infection (Lima et al. 2018).

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METHODS

This study was conducted at B & B Hospital Pvt. Ltd., Gwarko, Lalitpur in collaboration with GoldenGate International College, Kathmandu, Nepal from February to September 2018. A total of 420 non-duplicate respiratory specimens including expectorated sputum, suction tube, tracheostomy tube and pleural fluid from all patients with symptoms of LRTIs were studied. The samples which were not collected and labeled properly and with visible contaminations were excluded. The samples were inoculated onto Mac Conkey Agar (MA), Blood Agar (BA) and Chocolate Agar (CA). The MA and BA have been incubated aerobically at 37°C for 24-48 hours while CA plates were incubated in a candle jar at 37°C for 24-48 hours (Bhatta et al. 2019). The bacterial isolates were identified by standard microbiological procedures including microscopy, colony morphology and biochemical tests as described by the American Society of Microbiology (ASM). Antibiotic Susceptibility tests of the bacterial isolates were performed by Modified Kirby-Bauer Disk Diffusion technique using Mueller Hinton Agar (CLSI 2015). Among all the isolates, *P. aeruginosa* isolates were subjected to biofilm detection by the microtiter plate method.

Detection of biofilm by microtiter plate method

Microtiter plate culture as described by Christensen (Christensen, 1989) was performed to detect biofilm formation by the *P. aeruginosa* isolates. The suspension of *P. aeruginosa* isolates was prepared in Tryptic Soya Broth (TSB) supplemented with 1% glucose. The suspension was diluted at 1:100 with fresh TSB. Then 200 µl of suspension was loaded into wells of 96 well sterile flat-bottom polystyrene micro-titer plate. A set of 3 such microtitre plates were prepared. *P. aeruginosa* strain PA01 and TSB with 1% glucose were used as the positive and negative control respectively. The micro-titer plate with bacterial suspensions was then incubated at 37°C for 24 hours. After incubation, the suspension was removed by gentle tapping and each well was washed with 200 µl of Phosphate Buffer System (pH 7.3) four times. Subsequently, 2% sodium acetate was used for fixation followed by staining with 100µl of 0.1% crystal violet. Excess stain was removed by washing the plate with de-ionized water and dried. The microtiter plate was then rinsed with 0.2 ml of ethanol-acetone (80:20 v/v). The ELISA reader was used to obtain the absorbance at a wavelength of 570 nm. The value of optical densities for each isolate was calculated from the average of three wells. The value was compared to the optical density of the negative control (ODc). The isolates were classified based on mean optical densities (Stepanovic et al. 2000):

OD ≤ ODc (≤0.658): non-biofilm producer

ODc < OD ≤ 2× ODc (>0.658-1.361): weak biofilm producer

2× ODc < OD ≤ 4 × ODc (>1.316-2.632): moderate biofilm producer

4× ODc < OD (>2.632): strong biofilm producer

RESULTS

Culture of samples

A total of 420 specimens including 349 sputum, 31 pleural fluid, 28 suction tip, and 12 tracheostomy specimens were processed. Among them, 90 specimens showed positive culture including 68 (75.65%), 1 (1.19%), 13 (14.26%) and 8 (8.9%) from sputum, pleural fluid, suction tube and tracheostomy tip respectively (Figure 1). The male inpatients showed the highest culture positive (56 ; 62.3%) while female outpatients showed the least culture positive (2; 2.2%).

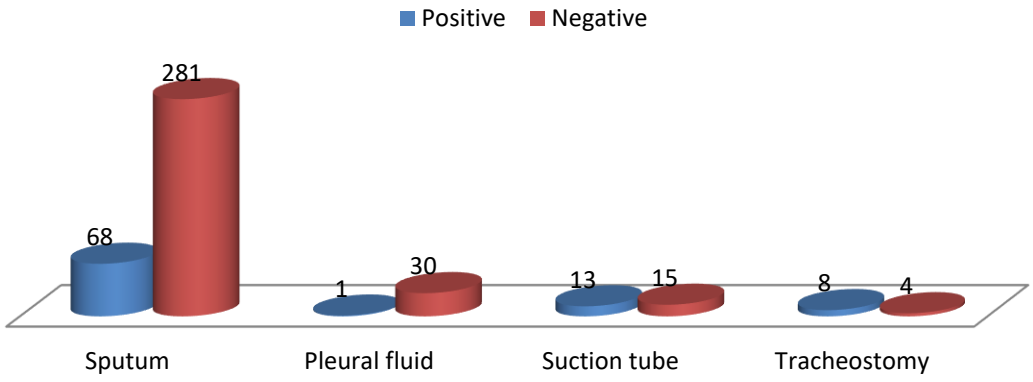

Figure 1: Barchart showing culture positivity in different specimens

Monomicrobial and polymicrobial growth

Out of 90 culture positive samples, a single bacterium was isolated from each of 66 (73.33%) specimens whereas polymicrobial infection was detected in 24 (26.67%) specimens; including 11 (45.83%), 1 (4.16%), 7 (29.17%), and 5 (20.84%) from sputum, pleural fluid, suction tube and tracheostomy tube respectively (Figure 2).

Distribution of different organisms in clinical samples

A total of 114 Gram-negative bacteria were isolated from 90 culture-positive specimens. *K. pneumoniae* was the most predominant bacteria (n=49, 42.9%), followed by *P. aeruginosa* (n=25, 21.9%) (figure 3).

Resistance pattern of Gram-negative respiratory pathogens

The highest number of *K. pneumoniae* was resistant to ceftriaxone (93.8%) followed by cefepime (87.8%). Similarly, the highest number of *Acinetobacter* spp. was resistant to ceftriaxone and cefepime (87%).

The majority *of P. aeruginosa* isolates showed the highest resistance to cefeperazone/sulbactum followed by piperacillin/tazobactum. A total of 11 *P. aeruginosa* isolates was found to be the multidrug resistant (MDR).

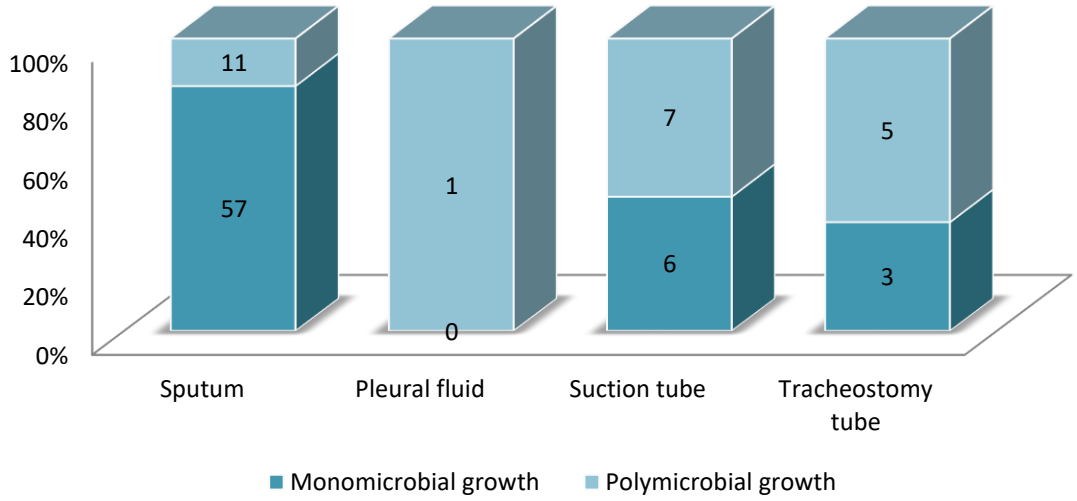

Figure 2: Barchart showing monomicrobial and polymicrobial growth in different specimens

Table 1: Distribution of bacterial isolates

Organism	Number of isolates (%)
K. pneumoniae	49 (42.98)
P. aeruginosa	25 (21.92)
E. coli	18 (15.8)
Acinetobacter spp.	16 (14.03)
K. oxytoca	2 (1.75)
P. vulgaris	1 (0.88)
P. mirabilis	1 (0.88)
C. freundii	1 (0.88)
H. influenzae	1 (0.88)
Total	**114**

Table 2: Resistance pattern of Gram-negative respiratory pathogens

Antibiotics	*K. pneumoniae* (%)	*P. aeruginosa* (%)	*E. coli* (%)	*Acinetobacter* (%)	*K. oxytoca* (%)
Amikacin	73.4	32	55.5	50	50
Gentamicin	71	28	50	50	100
Ciprofloxacin	69.4	28	77.8	31.3	50
Ceftriaxone	93.8	-	83.3	87	100
Ceftazidime	-	28	-	-	-
Cefepime	87.8	48	88.9	87	100
Imipenem	79.5	44	61.7	50	50
Meropenem	75.5	48	50.6	43	50
Piperacillin/Tazobactum	77.5	56	55.5	56	100
Cefeperazone/Sulbactum	75.5	68	61.1	56.	100
Colistin	0	0	0	0	0

Biofilm producers among *Pseudomonas aeruginosa*

Among the 25 *P. aeruginosa* isolates, 15 isolates were found to be a biofilm producer; out of which 2 (13.33%) were strong, 4 (26.67%) were moderate and 9 (60%) were weak biofilm producer. The higher biofilm producers were isolated from sputum specimens (n=11; 73.33%) followed by suction tube (n=3; 20%) and tracheostomy tube (n=1; 6.67%) (Table3). Similarly, 14 isolates from the inpatients and 1 isolate from the outpatient were biofilm producers (Figure 4).

Antibiotic resistance pattern of biofilm producing and non-producing *P. aeruginosa*

The overall antimicrobial resistance patterns of *P. aeruginosa* showed that biofilm producers were more resistant than biofilm non-producers. The highest number of biofilm-producing *P. aeruginosa* was found to be resistant to Cefeperazone/Sulbactum. Similarly, the highest number of biofilm non-producers was found to be resistant to Cefepime.

Biofilm and MDR

Out of 15 biofilm producing *P. aeruginosa,* 8 (53.3%) were MDR while among 10 biofilm negative isolates, 3 (30%) were MDR.

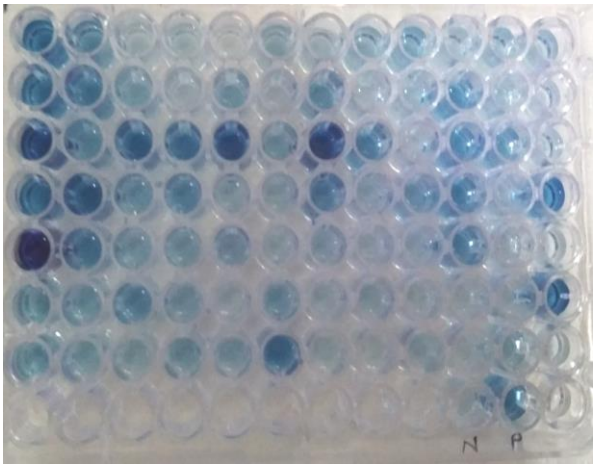

Figure 4: Detection of biofilm by Microtiter Plate method (H_{10}- Negative Control, H_{11}- Positive Control)

Table 3: Biofilm producing *P. aeruginosa*

Specimens	Number of biofilm producer (n=15)			
	Strong (%)	**Moderate (%)**	**Weak (%)**	**Total (%)**
Sputum	2	2	7	11 (73.33)
Suction tip	-	2	1	3 (20)
Tracheostomy tube	-	-	1	1 (6.67)
Total	**2 (13.33)**	**4 (26.67)**	**9 (60)**	**15 (100)**

Table 4: Antibiotic resistance pattern of biofilm producing and non-producing *P. aeruginosa*

Antibiotics	Resistance pattern	
	Biofilm producer (%)	Biofilm non-producer (%)
Amikacin	8 (53.3)	2 (20)
Cefepime	7 (46.7)	6 (60)
Ciprofloxacin	8 (53.3)	5 (50)
Gentamicin	5 (33.3)	2 (20)
Imipenem	6 (40)	3 (30)
Meropenem	7 (46.7)	3 (30)
Piperacillin/tazobactum	6 (40)	4 (40)
Cefeperazone/Sulbactum	10 (66)	5 (50)
Colistin	0	0
Ceftazidime	5 (33.3)	3(30)

DISCUSSION

Gram-negative bacteria were the major pathogens isolated from lower respiratory tract infections and *K. pneumoniae* was the most predominant one. The increased rate of resistance of those pathogens to routinely used antibiotics were obsereved in our study. This situation shows a huge problem in management of LRTIs caused by such bacteria pathogens. In addition, we also reported most of *P. aeruginosa* isolated from the patients with LRTIs to have the ability to produce biofilm. The increased resistance was observed among biofilm producers as compared to biofilm non-producers which might add more challenge in antimicrobial therapy to treat the infections.

The prevalence of LRTI was found to be 21.4% which was similar to the study by Nepal et al. 2018(24.6%). However, the rate of culture positivity was higher in the studies by Mishra et al. 2012 (44.4%) and Ieven et al. 2018 (59%). The prior use of antibiotics, exclusion of viral and other atypical bacteria in this study may have resulted in a lower prevalence rate of LRTIs (Ahmed et al. 2018; Nepal et al. 2018). The suction tube culture showed the highest rate (66.67%) which was supported by the study conducted by Nepal et al. 2018, at Kathmandu Model Hospital. Kathmandu with 100% culture positivity. The culture positivity was relatively higher in the specimens from inpatients (92.3%) than outpatients (6.7%). A similar result was obtained in the study by Khan et al. 2015. The hospitalized patients with long-term stay and who are under medication and steroids are susceptible to LRTIs due to weaker immune status (Guzek et al. 2014). The surgical manipulations, intubations also furnish the suitable environment for opportunistic bacteria to cause LRTIs (Bajpai et al. 2013).

The male patients were more susceptible to LRTIs with 66.7% culture positivity in this study. The result coincided with the study by *Olugbue et al. 2011.* A higher rate of LRTIs in the male may be attributed to a high incidence of smoking and alcohol consumption (Ziyade et al. 2010). Poly-microbial infection was observed in 26.7% of the specimen. The studies by Mishra et al. 2012, Khan et al. 2015 and Nepal et al 2018 also revealed a lower rate of poly-microbial infection i.e. 9%, 20% and 15.36% respectively. The identification of the polymicrobial infection is very important for treatment strategies since the polymicrobial infection mightn't be managed with an antibiotic.

Among the heterogeneous bacterial etiological agents of LRTIs, member of the Enterobacteriaceae family remains the predominant pathogens. All the isolated bacteria were Gram-negative. *K. pneumoniae* (42.9%) was the most predominant organism followed by *P. aeruginosa* (21.9%) which corresponds to the study by Nepal et al. 2018. Similar results were present in other studies as well (Ahmed et al. 2018; Okesola and Oni 2009). The higher prevalence of *K. pneumoniae* may be due to their ubiquitous presence and their ability to cause nosocomial infections (Paczosa et al. 2016). Among the isolated *P. aeruginosa*, 44% were found to be MDR. Vishwanath et al. 2013 reported 5.7% MDR *P. aeruginosa*. However, Goel *et al*. 2009 found higher rates of MDR *P. aeruginosa* which may be due to the inclusion of specimens from the Intensive Care Unit. The spread of antimicrobial resistance genes among the clinical pathogens, irrational use of antimicrobials, etc. contribute to the development of MDR nature in bacteria (Ahmed et al. 2013)

The increasing rate of MDR among different bacterial pathogens are of great concern since the infections caused by those pathogens might have longer hospital stay with higher morbidity and mortality.

In this study, 60% of *P. aeruginosa* were reported to be biofilm producers which were similar to the study conducted by Yekani et al. 2017. Lima et al. 2018 reported 77 *P. aeruginosa* as the biofilm producer. Among the biofilm producers, the highest producers were isolated from sputum. Similarly, the inpatients harbored the maximum biofilm producers which may be due to the increasing use of invasive diagnostic procedures and patients' association with indwelling devices (Dash et al. 2013). The biofilm-producing *P. aeruginosa* showed greater resistance to cefeperazone/ sulbactum (66%). Other study by Saha et al. 2018 showed the different antibiotic-resistant patterns of biofilm-producing *P. aeruginosa*. The biofilm producers render the inefficient antibiotics treatment thereby promoting chronic infectious diseases (Saha et al. 2018; Sanchez et al. 2013; Alves et al. 2014; Rao et al. 2008). Since biofilm production not only contribute the pathogens to adopt in the different environmental niche but also help them to resist towards many antimicrobial agents. Most of hospital equipments were colonized with such pathogens. Therefore, it is equally important to routinely screen the bacterial pathogens for biofilm production with their antibiogram patterns.

This study couldn't observe biofilm production and compare with antibiogram pattern of all bacteria isolates from LRTIs. As a main objective, we just focus on *P. aeruginosa*. Moreover, due to limited time and budget, the MIC of all antimicrobial agents to *P. aeruginosa* couldn't be performed. The overall results were based on disc diffusion method.

CONCLUSION

This study reveals the predominance of Gram-negative bacteria in LRTIs and their increased rate of resistance to routinely used antibiotics. *K. pneumoniae* was found to be the most predominant organism in LRTI. The study also reported most of *P. aeruginosa* isolated from the patients with LRTIs to have the ability to produce biofilm. The increased resistance was observed among biofilm producers as compared to biofilm non-producers.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Microbial Quality Analysis of Panipuri Samples Collected from Different Parts of Bhaktapur

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ABSTRACT

Objectives: The study was aimed to identify potential bacterial pathogens from the panipuri samples collected from different vendors of Bhaktapur district and determine their antibiogram patterns.

Methods: Altogether 120 (40 puri, 40 stuffing and 40 pani) samples of panipuri were collected from different vendors of Bhaktapur district in a cold chain and were transported to the microbiology laboratory. Puri and stuffing were then pre-enriched, enriched and cultured while pani samples were processed using the MPN method. All the isolates were identified following standard microbiological procedure and subjected to antibiotic susceptibility testing following CLSI guidelines.

Results: The result revealed contamination of 77.5% stuffing, 67.5% puri and 52.5% pani samples with pathogenic bacteria. Among bacterial pathogens, the highest number was *Staphylococcus aureus* followed by *Escherichia coli* and *Salmonella* spp. 100% of *S. aureus* were found to be resistant to ampicillin and novobiocin. Similarly, 94.1% of *E. coli* were resistant to ampicillin followed by ciprofloxacin (64.7%). A very few isolates of *Salmonella* spp*, Shigella* spp and *Vibrio* spp were resistant to tetracycline. The highest number of multidrug-resistant bacteria were *S. aureus*, followed by *Klebsiella* spp and *E. coli.*

Conclusion: The study showed that the panipuri samples from street vendors were found to be highly contaminated with pathogenic bacteria which might affect consumers' health. Thus, to prevent any food-borne illness in the future, frequent evaluation and regulation of the quality of such foods should be carried out.

Keywords: Street foods, panipuri, bacterial pathogens, antimicrobial-resistant, multidrug-resistant

INTRODUCTION

According to (FAO 1997), street food is "ready-to-eat foods and beverages prepared and/or sold by vendors and hawkers especially in the street and other similar public places". People nowadays want to save time and money, whenever possible, thus the consumption of street food is growing enormously (Tuladhar and Singh 2012). Consequently, in developing countries, drinks, meals, and snacks sold by street food vendors are widely consumed (FAO 1988).

Street foods are becoming more popular these days in Nepal and the most popular street foods in Nepal are "Panipuri" or "Phulki" and "Chatpate". Although it is very popular, easily available and cheap, it is frequently associated with various food-borne diseases in India and elsewhere (FAO 1988, Estrada-Garcia et al. 2004, Chumber et al. 2007, Ghosh et al. 2007).

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Panipuri and its constituents support growth of microorganisms since these are suitable media for the microorganisms (Dassana 2010; Pearce 2016; Ladage 2017). Panipuri is usually sold in open unhygienic surroundings often dwelt with houseflies and air-borne dust and vending places are usually congested. Water is a very critical raw material for street food but its supply is also limited. Many times, biological, physical and chemical contamination is also common in water that is used for processing street food, washing the containers and utensils and even water used for drinking (WHO 1996). Contact with sewage water contaminates street food with bacteria like *Salmonella* spp*, Shigella* spp, *Campylobacter* spp*.* and *E. coli* (Frelund et al. 1987; Blostein 1993; Beuchat 1996 and Gayler et al. 1955). Apart from this, it is often sold at roadsides without running water sources that require utensils and hands to be washed in a single bucket, unhygienic preparation and handling, negligence like they do not use gloves while serving the food also easily contaminate the food and open-drain area (Tambekar et al. 2009) can cause food-borne disease like diarrhoea, vomiting, etc. Hence, due to a lack of knowledge about health and hygiene between vendors, inappropriately prepared, stored and served food products have raised a question regarding their microbiological quality (Nyenje et al. 2012).

Food-borne illness associated with the consumption of street food has been reported in various places of India (Das et al. 2012). Food-borne illnesses caused by microorganisms are a major national and international health problem and an important cause of death in developing countries (Garode et al. 2012). Studies conducted in America, Asian and African countries have revealed increased bacterial pathogens in the food (Das et al. 2010). Panipuri is displayed openly in the streets and its preparation is also questionable. Thus, from the consumer health point-of-view, the microbial quality of street vended food becomes very important as food acts as a major source for transmission of food intoxications (Barro et al. 2006). Street food vendors are mostly uninformed of good hygiene practices and causes of diarrheal diseases (Mensah et al. 2002), which can increase the risk of street food contamination (Bhaskar et al. 2004; Tambekar et al. 2009). Therefore, Street foods are the major cause of health problems due to the lack of basic infrastructure and services, and difficulty in controlling a large number of vendors because of their diversity, mobility and temporary nature (De sausa 2008). To analyze the microbial quality of the panipuri, this research has been conducted to isolate the potential bacterial pathogens in those samples and to determine their antibiogram patterns.

METHODS

Study site and duration: The laboratory investigation of this project was carried out in Sainik Awasiya Mahavidhyalaya, Sallaghari: Bhaktapur, Nepal. The study was conducted from October 2017 October to 2018 February.

Sample collection, transport and processing: A total of 120 panipuri samples were collected from different parts of Bhaktapur district, Nepal. The samples were collected separately as pani, puri and stuffing (every 40 samples) in sterile containers containing ice packs; and were transported to the laboratory within an hour and were processed immediately following standard procedure.

Laboratory procedure

Pre-enrichment and enrichment: For the bacterial analysis, the solid samples (puri and stuffing) were pre-enriched on buffered peptone water and further enrichment was done on selenite F broth and alkaline peptone water.

Isolation and identification of bacterial pathogens: For the isolation of *S. aureus* and non-fastidious Gram-negative pathogens, the pre-enriched samples were cultured on Mannitol Salt Agar (MSA) and Mac-Conkey agar (MA). The isolated colonies from MSA and MA were identified based on standard microbiological procedures including colony morphology and biochemical tests. Whereas the enriched samples on selenite broth were cultured on XLD for isolation and identification of *Salmonella* spp and *Shigella* spp. Likewise, the enriched samples in alkaline peptone water for cultured on TCBS agar plates. The bigger golden-yellow colonies were further processed by biochemical tests and serotyping for identification of *V. cholerae* (Cheesbrough 2006).

The pani samples were subjected to the MPN method for isolation and identification of coliforms (Cappuccino and Sherman 2009). The colonies obtained from the culture on EMB agar were identified by using standard microbiology methods including colony morphology, Gram staining and biochemical tests.

Antibiotic susceptibility testing and multidrug-resistant: The identified bacterial isolates were subjected

to antibiotic susceptibility testing by modified Kirby Bauer disc diffusion method on MHA plates following CLSI guidelines (CLSI 2017). Similarly, the isolates that were resistant to 3 or more different classes of antibiotics were considered multidrug-resistant strains (Magiorakos et al. 2011).

RESULTS

Growth on panipuri samples: Out of 40 stuffing, 40 puri samples, 77.5% of stuffing and 67.5% of puri samples showed bacterial growth on different culture media. The pH range of pani was 2.0 to 3.9. Out of 40 samples processed by the MPN method, 21 samples from pani were contaminated with faecal coliforms, *E. coli*. Because of the acidic nature of pani samples, only *E. coli* was studied in pani samples (Figure 1).

Frequency of bacterial pathogens: A total of seven different types of bacterial pathogens have been identified from 120 panipuri samples. The highest number of bacterial pathogens were *S. aureus* (37/117) followed by *E. coli* (34/117) and *Salmonella* spp (19/117). Only 2 *V. cholerae* isolates were confirmed from stuffing samples (Table 1).

Antibiotic susceptibility pattern: All *S. aureus* were resistant to ampicillin followed by chloramphenicol (67.6%) and cotrimoxazole (67.6%). Among Gram-negative pathogens, the highest number of isolates were resistant to ampicillin. 100% of *Klebsiella* spp and *V. cholerae* were non-susceptible to ampicillin. Likewise, 94.1% of *E. coli* isolates were resistant to ampicillin. Among *Salmonella* isolates, 42.9% of *S.* Typhi and 66.7% of *S.* Paratyphi were resistant to ampicillin respectively. All *Salmonella* isolates were sensitive to methicillin and novobiocin. Erythromycin, gentamicin and tetracycline were found to be effective drugs against Gram-negative bacterial pathogens isolated in this study (Table 2)

Multidrug-resistant pathogens: The highest number of multidrug-resistant bacteria were *S. aureus* which was 67.6% followed by *Klebsiell*a spp (57.1) and *E. coli* (52.9%). Out of 2 *V. cholerae* isolates, 1 isolate was found to be MDR strain (Table 3).

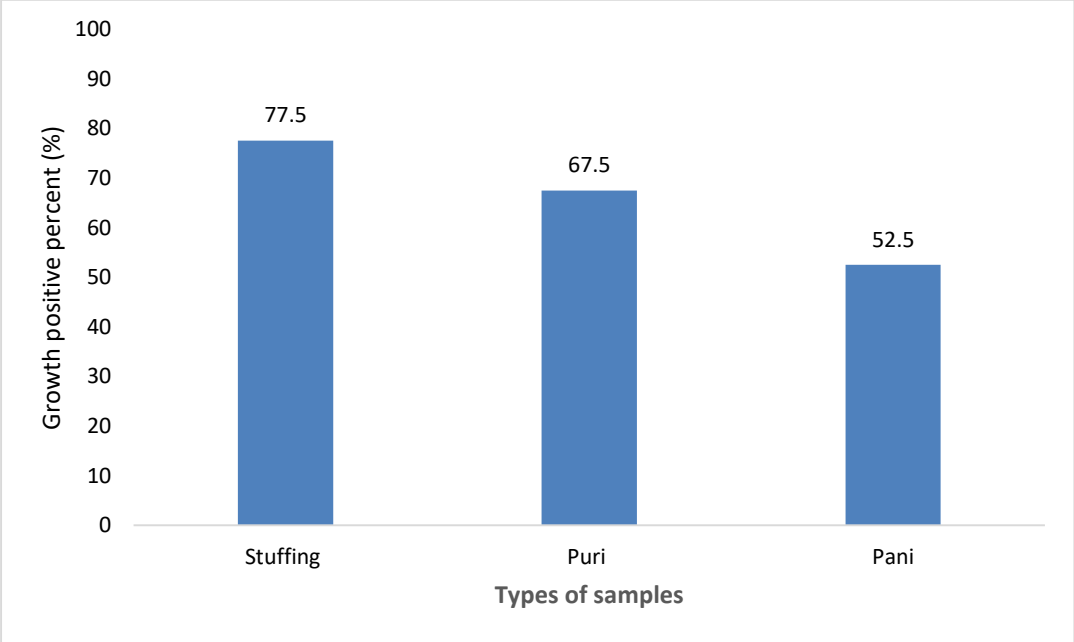

Figure 1: Bacterial growth percentage on different samples

Table 1: Number of bacterial pathogens isolated from different samples

Bacterial pathogens	Stuffing samples (n=40)		Puri samples (n=40)		Pani samples (n=40)		Total no.
	No.	Percent %	No.	Percent %	No.	Percent %	
Staphylococcus aureus	25	62.5	12	30.0	ND	ND	37
Klebsiella spp	11	27.5	3	7.5	ND	ND	14
Salmonella Typhi	7	17.5	0	0	ND	ND	7
Salmonella Paratyphi	9	22.5	3	7.5	ND	ND	12
Shigella spp	8	20.0	3	7.5	ND	ND	11
Vibrio cholerae	2	5.0	0	0	ND	ND	2
E. coli	8	20.0	5	12.5	21	52.5	34
Total no. of isolates	**70**		**26**		**21**		**177**

n= no of samples used, ND: not done

Table 2: Antibiotic susceptibility testing of bacterial pathogens isolated from the samples

Antibiotics used	Number of resistant bacteria (%)						
	S. aureus	*Klebseilla* spp	*S.* Typhi	*S.* Paratyphi	*Shigella* spp	*V. cholerae*	*E. coli*
	n=37	n=14	n=7	n=12	n=11	n=2	n=34
Ampicillin	37 (100)	14 (100)	3 (42.9)	8 (66.7)	9 (81.8)	2 (100)	32 (94.1)
Chloramphenicol	25 (67.6)	7 (50)	1 (14.3)	1 (8.3)	2 (18.2)	0 (0)	8 (23.5)
Ciprofloxacin	23 (62.2)	8 (57.1)	4 (57.1)	11 (91.7)	3 (27.3)	0 (0)	22 (64.7)
Cotrimoxazole	25 (67.6)	12 (85.7)	1 (14.3)	2 (16.7)	1 (9.1)	2 (100)	18 (52.9)
Erythromycin	7 (18.9)	6 (42.9)	0 (0)	1 (8.3)	0 (0)	1 (50)	13 (38.2)
Gentamycin	17 (45.9)	7 (50)	0 (0)	1 (8.3)	0 (0)	0 (0)	18 (52.9)
Methicillin	11 (29.7)	3 (21.4)	0 (0)	0 (0)	1 (9.1)	0 (0)	7 (20.6)
Novobiocin	4 (10.8)	5 (35.7)	0 (0)	0 (0)	0 (0)	0 (0)	8 (23.5)
Tetracycline	16 (43.2)	3 (21.4)	1 (14.3)	2 (16.7)	2 (18.2)	0 (0)	16 (47.1)

Table 3: Multidrug-resistant strains among the bacterial pathogens

Organisms	Total no. of isolates	No. of MDR (%)
Gram-negative organisms		
Escherichia coli	34	18 (52.9)
Klebsiella spp	14	8 (57.1)
Salmonella Typhi	7	1 (14.3)
Salmonella Paratyphi	12	2 (16.7)
Shigella spp	11	2 (18.2)
Vibrio cholerae	2	1 (50)
Gram-positive organisms		
Staphylococcus aureus	37	25 (67.6)

DISCUSSION

Readily prepared food in the street for consumption varies from place to place according to their cultural practices and social traditions. Among them, panipuri is one of the popular street foods sold in South- Asian countries (Pearce 2016). It originated in India and is very popular in Nepal as well. The present research work was undertaken to find out the presence of pathogenic bacteria, especially *S. aureus*, *E. coli, Klebsiella* spp*, Salmonella* spp*, Shigella* spp, and *Vibrio* spp from panipuri collected from different places of Bhaktapur district of Nepal. Our studies show higher bacterial growth in stuffing and puri than in pani; this result is in agreement with the study done by Tambeker et al (2008). It is explained by the low acidity (6.1) and more nutrient content of stuffing (Campbell 2017). In most cases running tap water is not available at vending sites; hand and dishwashing are usually done in the same buckets and without soap water. The serving plates or the container are not properly washed. Unhygienic food handling practices increase contamination.

Of 120 panipuri samples collected from 40 vendors during the study period at Bhaktapur district were categorized into stuffing, puri and pani samples. More than 50% of each sample was found to be contaminated with bacterial pathogens including *S. aureus, Klebsiella* spp*, Salmonella* spp*, Shigella* spp*, V. cholerae* and *E. coli.* Out of 80 samples of stuffing and puri, 62.5% of both stuffing and 30% of puri were found contaminated with *S. aureus* which was the highest rate among bacterial pathogens. This indicated cross-contamination of the food with the skin flora of vendors due to their poor hygiene and improper handling of food. During sample collection, we observed more than 90% of vendors (37/40) did not wear globes during panipuri preparation. The panipuri might have been also contaminated by consumers and the environment since that stuff was kept open in those vendors. The presence of *Klebsiella* spp in our study was 27.5% and 7.5% on stuffing and puri respectively, which might be due to carrier vendors as found in the study conducted by Gieser et al. (2011) or improperly washing of raw ingredients used on potato stuffing with contaminated water which is supported by the result of the study conducted by Podschun et al. (2001) in which *Klebsiella* spp were isolated from the surface water sample. A total of 16 *Salmonella* spp on stuffing and 3 isolates on puri samples were isolated. Out of which, 7 were *S.* Typhi and 12 were *S.* paratyphi. The presence of *Salmonella* spp, *Shigella* spp and *V. cholerae* were directly associated with the use of faecal contaminated water during panipuri preparation. This statement was supported by our study on pani samples by MPN method. The MPN analysis of pani samples indicated 52.5% contamination with *E. coli.* In the earlier study on analysis of street foods of Kathmandu carried out by Tuladhar and Singh (2012) showed that all the food samples analyzed were contaminated with bacteria including *S. aureus* followed by *Bacillus alvei, Escherichia coli, Enterobacter aerogenes, Bacillus subtilis, Serratia* spp, *S. saprophyticus*. However, the result of this study is different from ours as the bacteria like *Salmonella* spp; *Shigella* spp, *Vibrio* spp and *Klebsiella* spp were not isolated in this study. This variation may be due to the environmental condition around the food stalls or due to the hygienic condition of the food vendors. Even the type of ingredients used on the stuffing i.e stuffing may cause variations in the result as suggested by the study conducted (Nyenje et al. 2012).

On antibiotic susceptibility testing, 100% isolates of *S. aureus* from both stuffing and puri were found to be resistant to ampicillin; this result was in agreement with the study done by Bouza et al (2002). The study conducted in India by Das et al. (2010) showed 92.3% and 72.4% antibiotic resistance on *Salmonella* spp and *Shigella* spp respectively. Our result showed that 100% of both *Salmonella* Typhi and *Shigella* spp isolated were sensitive to gentamicin which contradicts with the findings of Kumar et al. (2017) as their study showed that 20.88% and 2.08% *Salmonella* spp and *Shigella* spp respectively were resistant to gentamicin. Similarly, none of *V. cholerae* isolates were found to be resistant to tetracycline; however, the result shown by Raissy et al. (2012) contradicted our result in which 18.1% of *Vibrio* isolates were found to be resistant to tetracycline. It might be due to non-cholera *Vibrio* isolates in their study (Intyre and Feely 1965).

Out of 40 samples of pani, MPN test revealed that 52.5% of the samples were contaminated with *E. coli*. The absence of other bacteria in pani is due to the low pH of the pani, as most bacteria favor growing in neutral conditions (Thompson 2017). *E. coli* isolated from pani was found to be highly resistant to ampicillin, ciprofloxacin and cotrimoxazole; however, the study done by Nazir et al. (2007) showed that *E. coli* isolates were sensitive to chloramphenicol and ciprofloxacin. The presence of *E. coli* in pani indicates the use of fecal contaminated and untreated water. In addition, the pH of the water samples was found to be in the range of 2.0-3.9 which correlates with the pH range of tamarind water pH (range, 1.8 to 3.7; mean, 2.8) (Nassereddin and Yamani 2005).

All different classes of antibitics as recommended by CLSI guidelines couldn't be used in this study.

CONCLUSION

This study indicated panipuri of different parts of Bhaktapur district were highly contaminated with pathogenic bacteria which can contribute to potential health risks for consumers. Both vendors and consumers should be aware of the possible infections and good food hygiene practices for prevention.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antimicrobial Resistance in *Escherichia coli*: a Cross Sectional Study in Chicken Poultry of Kirtipur, Nepal

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ABSTRACT

Objectives: The study was conducted to determine antimicrobial susceptibility pattern and prevalence of Extended Spectrum Beta Lactamase (ESBL) producing *E. coli* from fecal sample of different chicken poultry farm located at Kirtipur.

Methods: The cross sectional study was conducted from November 1st 2019 to February 29th 2020. The samples were collected from 27 different poultry farms and processed at Microbiology laboratory at Trichandra Multiple Campus. Identified *E. coli* were subjected to antimicrobial susceptibility test by using Kirby and Bauer Disc Diffusion technique and Combined disk method was used to determine ESBL *E. coli*.

Results: From all 27 poultry farms, *E. coli* was isolated from broiler (n=13), layer (n=10), and local (n=4) breeds, of which 23 (85.18%) were not registered. The chickens were fed with vitamin and calcium as growth promoter along with antibiotics; Piperacillin, Colistin and Doxycycline. Altogether 85.18% (n=23) isolates showed sensitivity towards Nitrofurantoin, Cefotaxime and Ceftazidime followed by Tigecycline 77.7% (n=21). Among these isolates 66.6% (n=18) were resistance towards Piperacillin followed by Ampicillin 37% (n=10). A statistically significant correlation was seen in resistance rate between broiler and layers. Among total isolates 37.03% (n=10) were Multi Drug Resistance (MDR) and 14.81% (n=4) were ESBL producer. Unregistered poultry farms were associated with MDR and ESBL Ec isolates.

Conclusion: Unregistered poultry farms and irrational use of antibiotics has influenced development of MDR and ESBL isolates. Timely monitoring and surveillance is suggested to decrease the trend of antimicrobial resistance (AMR) in poultry system.

Key words: Chicken feces, *E. coli*, AST, MDR, ESBL

INTRODUCTION

Rapid increment in poultry production in low and middle income countries with high demand for broiler and layer with massive antibiotic abuse has increased in persistence and cross transmission of Antimicrobial resistance (AMR) and Extended Spectrum Beta Lactamase (ESBL) isolates (Hedman et al. 2020; Widodo et al. 2020). Drug resistant profound in infection associated bacteria has led each of these species to give rise to antimicrobial resistance (AMR) against varied antibiotics used in treatment as seen in *E. coli* (Ec) (Dunachie et al. 2020). Since the 1st use of antibiotic drug in poultry in 1946, 20-52% of antibiotics are used only for production in poultry which has resulted in MDR and ESBL Ec colonization in poultry birds (Yang et al. 2019; Ilyas et al. 2021).

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Since the start of commercial production of poultry in 1980, these industries are known to contribute 3-4% of total Gross domestic product (GDP) of the country Nepal where programs prioritizing poultry farms with aim to alleviate poverty are increasing in different provinces of the country (Food and Agriculture Organization 2014). The chicken meat production has increased from 16,662 Metric tons to 255,001 Metric tons from 2008/09 to 2019/2020 (Ministry of Agriculture and Livestock Development 2021) also with indispensible use of antibiotics as growth promoter (Koirala et al. 2021). At one hand Kathmandu valley has been recognized as a center for poultry production where daily consumption of 350,000kg chicken is reported (Anonymous 2021) and on another hand MDR and ESBL Ec isolates from rectal swab (Subramanya et al. 2021) and meat sample (Shrestha et al. 2017) from Kathmandu valley have been reported.

ESBL Ec has been recognized as an indicator organism for ESBL producing Enterobacteriaceae which is regarded major global threat indwelling in and between human, animal and environment hosts (World Health Organization 2021). Consumption of antibiotics is known to be highest in poultry industries aiding in increment of AMR bacteria (Agyare et al. 2019). As the poultry industries are flourishing in Nepal, this study was done to determine the prevalence of MDR and ESBL Ec in poultry farm of Kirtipur, Nepal.

METHODS

Excreted fresh fecal sample of chicken were collected aseptically in wide mouth container from the ground inside of the poultry farm (Ybanez et al. 2018; Cox et al. 2015). Samples were kept in ice box with ice during transportation and reached to laboratory within 3 hours of collection (Zhuang et al 2017).

All the samples were processed for identification of *E. coli* at microbiology laboratory of Trichandra Multiple College, Kathmandu. The fecal samples were suspended in buffered peptone water in 1:10 (w/v) for enrichment and incubated at 37°C for 24hr (Stromberg et al. 2017). After 24 hr, a loop full of sample was streaked onto Mac Conkey Agar plate and incubated for 24hr at 37°C. After 24hr the Lactose fermenting pink colonies were further streaked onto Nutrient Agar plate and incubated for 24hr at 37°C. The colonies from NA plates were taken for Gram Staining, enzymatic (catalase and oxidase) and biochemical (IMViC), TSI, Citrate, O/F and Urease tests and also streaked onto Eosin Methylene Blue Agar (EMB) (Quinn et al 2011). Antibiotic susceptibility test was performed using Kirby and Bauer disc diffusion technique (Hudzicki 2016). The panel of ten different antibiotics categories was used for performing AST, as follows: Ampicillin (10µg), Piperacillin (30µg), Cefoxitin (30µg)„ Ceftriaxone (30 µg), Ceftazidime (30 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Nitrofurantoin (300 µg), Nalidixic acid (30 µg) and Tigecycline (15 µg). Multi Drug Resistance (MDR) isolates were categorized according to European Centre for Disease Prevention and Control (ECDC) guidelines (Wolfensberger et al. 2019). The ESBL was detected by using combined disk method using Cefotaxime (30µg) and Ceftazidime (30µg) alone and in combined with Clavulanic acid (10µg) as in Clinical Laboratory Standards Institute guidelines (2020).

The correlation coefficient was calculated by IBM SPSS Statistics 21 software as a test for significance. The level of significance was set al $p \leq 0.05$ with 95% confidence intervals.

RESULTS

All of the 27 chicken fecal samples collected showed growth of *E. coli*. Among this 13 were from boiler, 10 from layers and 4 local breed. Out of 27 poultry farms, 23 (85.18%) were not registered whereas 4 (14.82%) were registered (Fig1).

Vitamins and calcium were used as growth promoter in 25 (92.5%) poultry farms, whereas 2 (7.4%) local poultry farm fed zinger and garlic. The local and layer poultry farms 23 (85.15%) fed chickens with antibiotics viz., Pipericillin, Colistin and Doxycycline. Antibiotic Susceptibility test profile showed *E. coli* were highly sensitive towards Nitrofurantoin, Ceftriaxone and Ceftazidime (85.18%). Highest resistance rate was seen against Piperacillin (66.6%) followed by Ampicillin (37.03%). The results showed 22.2% (n=6) isolate resistance to Tigecycline (Table1).

E. coli isolate from Broiler and Layer showed resistance against Piperacillin 76.9% and 70% followed by Ampicillin 53.8% and 60% respectively. The resistance percentage showed by broiler and layer for different antibiotics showed statistically significant pairwise correlation at 0.01 level of significance (p=0.03) (Table 2).

E. coli isolate from broiler 69.2% (n=9) and Layer 10% (n=1) were found to be MDR whereas 23.7% (n=3) and 7.6% (n=1) *E. coli* isolate from broiler and Layers were found to be ESBL producers respectively. The *E. coli* isolates

from the poultry farm showed 10 (37.03%) MDR and 4 (14.81%) were ESBL producers. The MDR isolates in unregistered farm (33.33%) were comparatively higher than registered farm (3.71%). All the ESBL isolates (n=4) were identified from unregistered farm only. None of the isolates from local breeds were MDR or ESBL producer (Table 3). A significant association is seen in between MDR and ESBL isolated and unregistered poultry ($p<0.05$). The MDR isolates showed resistance against various types of antibiotics as shown in Table 4.

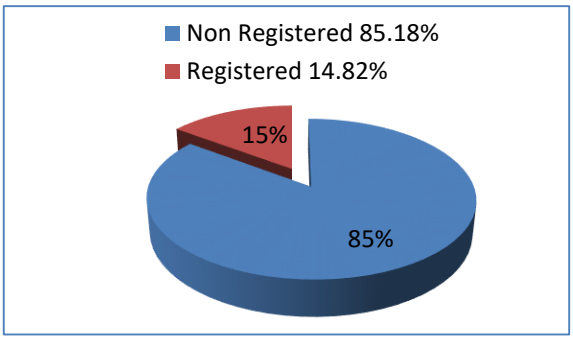

Figure 1: Distribution of poultry farms

Table 1: Antibiotic Susceptibility profile of *E. coli*

Antibiotics	Sensitive		Intermediate		Resistance	
	No.	%	No.	%	No.	%
Ampicillin	8	29.6	9	33.3	10	37
Cefoxitin	19	70.3	0	0	8	29.6
Ceftriaxone	23	85.18	2	7.4	2	7.4
Ceftazidime	23	85.18	2	7.4	2	7.4
Ciprofloxacin	14	51.8	5	18.5	8	29.6
Gentamicin	19	70.3	0	0	8	29.6
Nalidixic acid	17	62.9	5	18.5	5	18.5
Nitrofurantoin	23	85.18	2	7.4	2	7.4
Piperacillin	5	18.51	4	14.81	18	66.6
Tigecycline	21	77.7	0	0	6	22.2

Table 2: Antibiotic sensitivity and resistant profile among breeds of chicken

Antibiotics	Broiler				Layer				Local			
	Sensitive		Resistance		Sensitive		Resistance		Sensitive		Resistance	
	No	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ampicillin	3	23	7	53.8	3	30	6	60	3	75	0	0
Cefoxitin	7	53.8	6	46	8	80	2	20	4	100	0	0
Ceftriaxone	9	69.2	2	15.3	8	80	1	10	4	100	0	0
Ceftazidime	9	69.2	2	15.3	8	80	1	10	4	100	0	0
Ciprofloxacin	4	30.7	7	53.8	1	10	6	60	4	100	0	0
Gentamicin	9	69.2	4	30.7	6	60	4	40	4	100	0	0
Nalidixic acid	5	38.4	5	38.4	8	80	0	0	4	100	0	0
Nitrofurantoin	9	69.2	2	15.3	10	100	0	0	3	75	1	25
Piperacillin	1	7.6	10	76.9	1	10	7	70	3	75	1	25
Tigecycline	9	69.2	4	30.7	9	90	1	10	3	75	1	25

Table 3: MDR and ESBL profiles among breeds in two types of farms

Breed/ Farm	Isolate n (%)		MDR n (%)		ESBL n (%)	
	Registered (n=4)	Unregistered (n=23)	Registered (n=1)	Unregistered (n=9)	Registered (n=0)	Unregistered (n=4)
Broiler	3 (11.11)	10 (37.04)	1 (3.71)	8 (29.62)	0	3 (11.1)
Layer	1 (3.71)	9 (33.33)	0	1 (3.71)	0	1 (3.71)
Local	0	4 (14.81)	0	0	0	0

Table 4: MDR isolate showing resistance against various types of antibiotics

SN	Sample no.	Breed	Antibiotics
1	15	Broiler	CN, CX, PI
2	27	Broiler	CN, CIP, TGC
3	10	Broiler	CN, CIP, NX, PI
4	19	Broiler	CN, CX, CTX, TGC
5	5	Broiler	CN, CIP, NX, PI, AMP
6	12	Layer	CN, CX, CTX, PI, AMP
7	14	Broiler	CN, CX, CIP, NIT, PI
8	18	Broiler	CN, CX, CIP, PI, AMP, TGC
9	23	Broiler	CN, CX, CIP, NX, PI, AMP, TGC
10	24	Broiler	CN, CX,CTX, CIP, NIT, NX, PI, AMP, TGC

AMP: Ampicillin, CIP: Ciprofloxacin, CN: Gentamicin, CX: Cefoxitin, NX: Nalidixic acid, NIT: Nitrofurantoin, CTX:Ceftriaxone, PI: Piperacillin, TGC: Tigecycline

DISCUSSION

Nepal being an agriculture based country, in recent years is known to have increasing production and rise in population dependency on chicken poultry products for meat, egg and manure (Poudel et al. 2020). The study shows less than one fourth (14.82%) of the operating poultry farms are registered. The commercial poultry survey 2071/72 has recorded 14% of the registered poultry farm in Kathmandu valley having 16% of trained owner (Central Bureau of Statistics 2015). The chickens (92.5%) were fed with vitamins and calcium as growth promoter. The vitamin and minerals are generally regarded as nutraceuticals which helps to boost the poultry production by promoting health and development of skeletal muscles (Manikandan et al. 2020). Local chicken breed (n=2) were fed with ginger and garlic. These natural supplements are known to substitute the commercial growth promoter enhancing the longevity and health status of chicken (Karangiya et al. 2016). The antibiotics Piperacillin, Doxycycline and Colistin are used irrationally by the farmers as fed supplement in poultry farms (85.1%) although the dose regimen for various antibiotics has been suggested for use in poultry production (Castanon 2007). Since 1940, the discovery of antibiotics as growth promoter, doxycycline is used for disease prevention, Piperacillin for increasing weight and Colistin for growth enhancement (Manikandan et al. 2020). The population feeding on such commercially grown meat products is known to suffer from obesity (Angulo et al 2005). The study done by Shrestha et al. (2020) showed increase in prevalence of obesity from 4.7% in 1975 to 13.1% in 2014; and consumption of poultry product of 5% in 1980 to 46% in 2021 (Anonymous 2021).

The *E. coli* showed broad range of AMR pattern and were highly resistant towards Piperacillin (66.6%) followed by Ampicillin (37%). Intensification of the poultry production in LMIC for economic upliftment has compromised in quality of poultry system and massive use of growth promoter and antibiotics as fed supplement has resulted in

increased burden of AMR population in poultry (Hedman 2020). A common resistance towards Ampicillin, Tetracycline, 3GC and development of wide range of resistance varieties has been reported (Food and Agriculture Organization 2016). The highly resistance against Piperacillin in our study was due to its use as feed supplement. Lower resistance rate was seen against third generation Cephalosporins (7.4%) which was similar to study done by Bushen et al (2021). Tigecycline resistance was seen in 22.2% of isolates in this study. Tigecycline is the last drug of choice for MDR producing Gram negative isolates and plasmid mediated resistance towards this antibiotic in poultry production has created baleful situation for human population in South Asia (Moshin et al 2021).

Among total isolates about one fourth were MDR among which one fourth were ESBL producer. Food animals are known to be reservoirs of ESBL and ampicillinase producing *E. coli* (FAO 2016) where MDR with 52.5% (Bushen et al. 2021), 75.06% (Rahman et al. 2020) and 43.2% (Manishimwe et al. 2017) have been reported. The lower rate of MDR in our study was due to the study in small sample. Similarly, 37.8% of ESBL Ec has been recorded in Nigeria by Mwambete and Stephen (2015) which were higher than our study but 13.91% of ESBL Ec was reported by Rahman et al. (2020) which was almost similar to our study. A significant correlation was seen in resistant rate of Broiler and Layers in our study which was related to study done by Rahman et al. (2020) and Manishiwe et al. (2017). None of the isolate from local breed was MDR or ESBL producer as antibiotics were not given as feed supplements. The major contributing factor for AMR resistant development is prolonged use of sub therapeutic level of antibiotics as growth promoter in poultry production (Food and Agriculture Organization 2016). ESBL producers are known to bear diverse antibiotic resistance gene. Meanwhile poultry products are recognized as potential source for ESBL producing bacteria burgeoning MDR related bacterial infection in human community (Falgenhauer et al 2019). The presence of an antibiotic residue in consumable poultry product above the tolerance level is known to cause health severity in human ranging from rise of MDR to cell toxicity (Trieiber and Knaeuer 2021). The poultry farms are to be regulated timely for any unforeseen hazards in near future.

CONCLUSION

The poultry industries are one of the contributors to National GDP of Nepal but lack of regulation system has increased unregistered poultry farm. The major breed in poultry industries are broiler and layers. The poultry farmers are dependent mostly on the artificial growth promoter and irrational use of antibiotics which have resulted in the increased burden of AMR with ESBL Ec in poultry system.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antifungal Susceptibility and Biofilm Formation of *Candida albicans* Isolated from Different Clinical Specimens

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ABSTRACT

Objective: Increasing antifungal resistance and biofilm formation among *Candida* species is an intimidating public health concern, especially at the hospital settings. In light of this, the current study was designed to assess the biofilm-forming ability of clinically isolated *Candida albicans* and determine their antifungal susceptibility against both the planktonic and sessile forms.

Methods: A total of 58 *Candida* isolates from different clinical samples received at the Microbiology laboratory of KIST Medical College and Teaching Hospital, Lalitpur, Nepal in between April to October 2018 were included in the study. Isolation and identification of *C. albicans* was done following standard microbiological procedures that comprised of microscopic observations along with germ tube formation and biochemical tests. Besides qualitative investigation of biofilm by tube method, it was also investigated quantitatively by crystal violet staining method and metabolic activity of the biofilm was assayed by tetrazolium (XTT) salt reduction method. Antifungal susceptibility pattern against common antifungal drugs was determined as planktonic and sessile Minimum Inhibitory Concentrations (MICs) by broth micro-dilution method.

Results: Out of 58 *Candida* recovered from the total samples, 21(36.2%) were identified as *C. albicans.* The vaginal swabs showed a higher prevalence (57.14%, 4/7) of *C. albicans* whereas none were recovered from the wound swabs. Qualitative study of biofilm formation showed that 4 (19.1%) *Candida albicans* were strong biofilm producers, 11 (52.3%) isolates were moderate and 6 (28.6%) produced weak or none biofilms, whereas a majority (85.7%) of the isolates gave biofilm positive test in microtiter plate assay. The metabolic activity of the biofilm revealed that the average absorbance following the metabolic reduction of tetrazolium salt was 0.577. Interestingly, both the methods used for assessing biofilm productions correlated well ($r=0.569$, $p=0.007$). Most of the isolates were susceptible to Fluconazole (80.9%) at MIC 0.12 µg/mL, Amphotericin B (76.19 %) at MIC 0.25 µg/mL and Clotrimazole (80.9%) at MIC 0.25 µg/mL. In addition, sessile forms of *C. albicans* was found to have 2 to 8 fold increases in MIC compared to the planktonic cells.

Conclusion: High prevalence of *C. albicans* in vaginal swabs may implicate that the women are more prone to vaginosis. The sessile forms are more resistant to antifungal agents and proper administration of antifungal targeting the biofilms should be prioritized only with susceptibility result interpretations.

Key words: *Candida albicans*, biofilm, antifungal resistance, minimum inhibitory concentration

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INTRODUCTION

Candidiasis is a fungal infection which is caused by the fungi of genus *Candida*, predominantly by *Candida albicans. C. albicans* is one of the major fungal pathogens causing invasive fungal infections (Horn et al 2012) and occupy top ranking among the infectious agents. *C. albicans* can invade wide range of anatomical sites including muco-cutaneous deep tissues and organs and superficial sites such as skin, nail and mucosa; oral cavity and vagina being the most frequently encountered (Ramage et al 2001a). *C. albicans* imposes a greater public health challenge because of its high mortality rates and increasing costs of care and duration of hospitalization and this is why *C. albicans* is considered to be of higher medical and economic importance (Almirante et al 2005; Lai et al 2012; Sardi et.al 2013).

Three among four women during their lifetime are affected from candidiasis especially during their child bearing age (Bongomin et al 2017). About 6% of Nepalese women suffer from recurrent vulvovaginal candidiasis where 'recurrent' refers to four episodes per year (Khwakhali and Denning 2015). Moreover, *C. albicans* and also minority of other *Candida* species were the second highest colonization to infections and the overall highest crude mortality along with being the third leading cause of catheter-related infections (Crump and Cillingon 2000; Ramage et al 2001a). The alteration of *Candida* from commensal of an anatomical sites to an opportunistic pathogen is mediated by host's weakened immune system and various virulence factors such as adherence, biofilm formation and hydrolytic enzymes that comes into play at suitable host environment for pathogen (Sardi et al 2013).

A biofilm is an organized community that is regulated by molecular mechanism of regulation of gene expression in response to fluctuations in cell density that is the exchange of chemical signals among cells in a process known as quorum sensing (Yu et al 2012). The formation of the biofilm *in vivo* mediates the important work in pathogenesis. Moreover, development of biofilm is also related to the developmentof the resistance towards the antifungal agents and contributes to the increasing disease incidence which is supported by the fact that sessile cells within the biofilm are less susceptible to antifungal agents than the planktonic cells (Silva et al 2017). The treatment of biofilm-based infection remains an escalating clinical problem because of lack in biofilm specific drugs for *C. albicans* (Nobile and Johnson 2015).

Antibiotic susceptibility testing is one of the ways to determine the resistance of the organism towards the antimicrobial agents and determination of minimum inhibitory concentration is the best choice to understand the actual degree of susceptibility or resistance towards the antimicrobial agents. Some species of the *Candida* like *C. glabrata* and *C. krusei* are intrinsically resistant to Fluconazole (Izquierdo et al 2015). It has also been reported that these Fluconazole resistant *C. albicans* strains appear to be cross-resistant to other azoles (Richardson and Warnock2012). Similarly, rare but reported case of Amphotericin B resistance of *Candida* has been attributed to the alterations in the cell membrane, including reduced amounts of ergosterol, and were isolated following prolonged treatment (Richardson and Warnock 2012). With the increasing number of clinical isolates' resistance towards the commonly used antifungal agents, more specifically, by the production of biofilm in case of *C. albicans*, there is a growing need for antifungal susceptibility testing of the biofilm-producers which can contribute towards the pool for therapeutic approaches. In this regard, this study aims at determining the hospital-based prevalence of *C. albicans,* assess their biofilm formation and standard (planktonic) and sessile susceptibility against some commonly used antifungals.

METHODS

This was a qualitative, laboratory-based cross-sectional study carried out from April to October 2018 in KIST Medical College and Teaching Hospital, Lalitpur. A total of 58 *Candida* isolates were collected from different clinical samples (sputum, high vaginal swabs, associated catheter devices and wound swabs) from the patients visiting both inpatient and outpatient departments. Clinical samples were grown on three different media: Sabouraud Dextrose agar (SDA) supplemented with 0.05 mg/L chloramphenicol, Cystein Lactose Electrolyte Deficient (CLED), and Hichrom agar for 24-48 hours at 35-37°C. All the media used were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Identification of isolates was done based on colony characteristics, microscopic observation, germ tube formation test, and biochemical tests (Shrestha et al 2020).

A yeast suspension was prepared from the pure culture of yeast as follows: First, a small amount of stock culture was inoculated on Sabouraud Dextrose Agar (SDA) containing Chloramphenicol by using a sterile loop and incubated at 37°C for 24–48 h. The yeast was then harvested and suspended in RPMI-1640 medium at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1×10^7 yeast cells/mL (Subramanya

et al 2017).

Qualitative biofilm measurement was done by inoculating the fresh yeast culture in nutrient broth at 37° C for 48 hours. After 48 hours, the liquid was pipetted out, washed with 2 mL phosphate buffer solution (PBS) and again pipetted out slowly to avoid disruption of the biofilm. It was then air-dried, stained with 1% crystal violet (CV) for 25 mins and washed with distilled water solubilized with 2 ml of 30% glacial acetic acid resulting in visible biofilm. Quantitative biofilm measurement was performed by crystal violet staining method (staining for biomass) in 96 well microtiter plates (Inci et al 2010) and metabolic activity of the biofilm was further determined by a well-established enzymatic method using XTT (2, 3-bis (2-methoxy-4-nitro-5-sulffophenyl)-2H-tetrazolium-5-carboxanilide) as previously described (Pierce et al 2010).

Antifungal susceptibility testing (AST) was performed and interpreted for all the *C. albicans* isolates by modified Kirby-Bauer disk diffusion method. Antifungal discs of Fluconazole (10 µg), Ketoconazole (10 µg), Clotrimazole (10 µg), and Amphotericin B (10 µg) were used for susceptibility testing on Muller Hinton Agar (MHA) supplemented with 2% glucose and 5 µg/mL methylene blue. Results of AST were interpreted following Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) and susceptibility criteria were determined as per the recommendation guidelines provided by the company (HiMedia, Mumbai, India). Planktonic MICs and Sessile MICs (SMICs) were determined against the drugs-Amphotericin B, Clotrimazole and Fluconazole using broth micro-dilution methods (Ramage et al 2001b) following guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) documents (CLSI 2012).

RESULTS

Out of 58 candida species, 21 (36.2%) were reported as *Candida albicans* whereas 37 (63.8%) belonged to non-albicans *Candida* species (NCAC) (Figure 1).

The majority of *Candida* isolates were recovered from urine 29 (50%) followed by sputum 15 (25.9%), high vaginal swab (HVS) 7 (12.1%), catheter tip 6 (10.3%) and wound swab 1 (1.7%). Majority of *Candida* species (n=29) were isolated from urine among which 9 (31.0%) were *Candida albicans*. The prevalence of *Candida albicans* was seen higher in high vaginal swabs (57.1%) followed by sputum (46.7%) and urine (31.0%). Only 1 isolate out of 6 from catheter tip culture (16.66%) was *C. albicans*; whereas the only one Candida recovered from wound swab was non-albicans *Candida* species (Table 1).

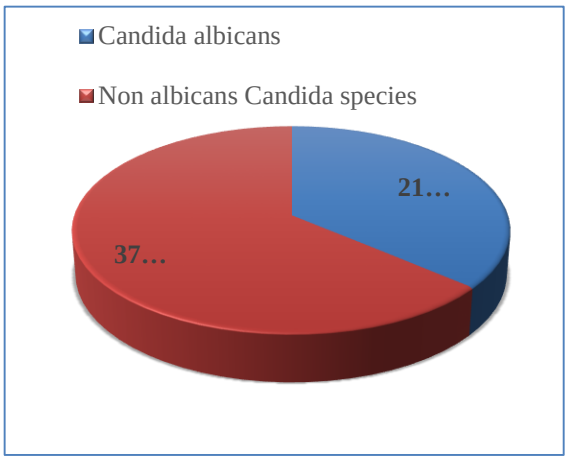

Figure 1: Distribution of *Candida* isolates

Qualitative study of biofilm formation showed that 4 (19.1%) *Candida albicans* were strong biofilm producers. 11 (52.3%) *Candida albicans* isolates were moderate and 6 (28.6%) of total *C. albicans* produced weak or none biofilms. A majority (85.7%, 18/21) of *C. albicans* isolates gave biofilm positive test in microtiter plate method as specified by crystal violet staining. The OD was intensely positive in 5 (23.8%) isolates, while moderate and mild biofilm formation was shown by 6 and 7 *C. albicans* isolates respectively. Three OD values were observed below the cut-off values representing negative or no biofilm formation. The optical densities of all the isolates are given in figure 2. The optical density value for each isolate represents the average of 2 replicate values. The mean OD value was 0.0549 with standard deviation of 0.319. The maximum reading was 0.112 corresponding to the strongest biofilm forming isolates while lowest was 0.011.

The metabolic activity of the biofilm revealed that the average absorbance reading following the metabolic reduction of tetrazolium salt was 0.577. The maximum optical density value corresponding to the highest metabolic activity in biofilm cells was 1.127; while the lowest OD reading was 0.090. All the value represents the metabolic activity of the biofilm cell (Figure 3).

Table 1: Distribution of *Candida* species in different clinical specimens

Sample	*C. albicans* n (%)	NCAC n (%)	Total n (%)
Urine	9 (31.0%)	20 (68.1%)	29 (50.0)
Sputum	7 (46.7%)	8 (53.3%)	15 (25.9)
HVS	4 (57.1%)	3 (42.9%)	7 (12.1)
Catheter tip	1 (16.7%)	5 (83.3%)	6 (10.3)
Wound swab	0	1 (100%)	1 (1.7)
Total	21 (36.21%)	37 (63.79%)	58 (100)

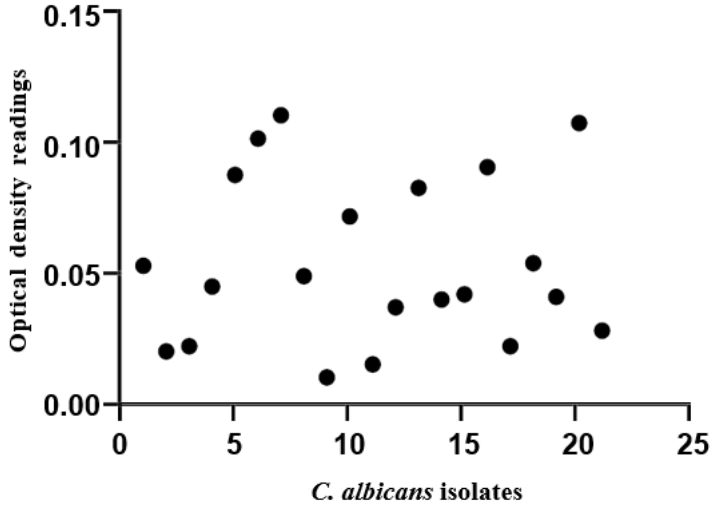

Figure 2: Optical density of biofilm production by crystal violet method by *C. albicans*

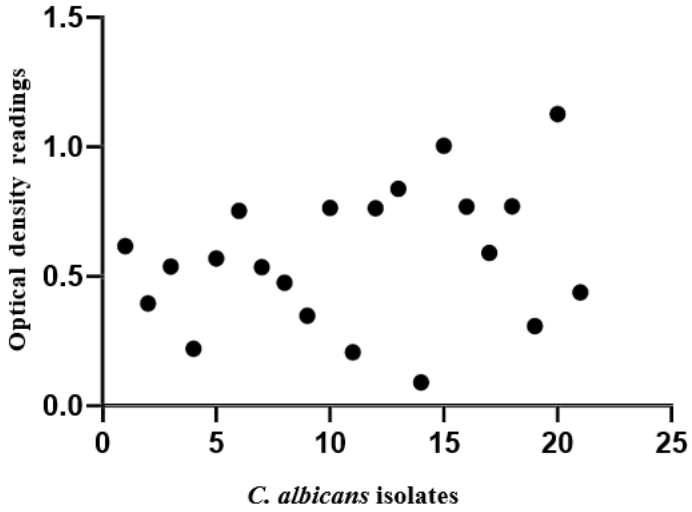

Figure 3: Optical density of biofilm formation by XTT metabolic reduction by *C. albicans*

Spearman correlation was used to assess the consistency of the two semi-quantitative methods of biofilm production used in this study. Interestingly these two methods correlated well with correlation coefficient (r) 0.569 and p=0.007. However, the maximum and minimum reading belonged to different isolates in two methods. For CV maximum reading represented isolate no.7 while isolate no. 20 exhibited maximum OD value in XTT assay. Similarly, the lowest colorimetric value among these two methods corresponds to two different isolates (Figure 4).

Most of the *C. albicans* were fairly susceptible to Amphotericin B, Fluconazole and Clotrimazole with 76.2%, 71.4% and 66.7% susceptibility respectively. Highest level of resistance was observed with Ketoconazole (52.4%) followed by Amphotericin B (23.8%) (Table 2).

The Fluconazole MIC value of different *C. albicans* isolates were in the range 0.06 to >64 µg/mL after 24 hours incubation at 37ºC. Most of *C. albicans* isolates (80.9%) were fairly susceptible (≤8µg/mL) to Fluconazole, 4.8% were susceptible dose dependent while 14.3% were resistant (≥64 µg/mL). The Amphotericin B MIC value of different *C. albicans* isolates were in the range 0.12 to ≥16 µg/mL after 24 hours incubation at 37ºC. Majority of *C. albicans* weresusceptible to Amphotericin B. 76.2 % of *C. albicans* had an MIC of ≤1 µg/mL while the rest 23.8% had MIC greater than 1µg/mL. Moreover, the Clotrimazole MIC value of different *C. albicans* isolates were in the range of 0.12 to ≥16µg/mL after 24 hours incubation at 37ºC. Most of the *C. albicans* isolates (76.2%) were fairly susceptible to Clotrimazole, whereas 21.8% isolates had MIC greater than 1µg/mL (Table 3).

Majority of *C. albicans* biofilm (71.5%) were resistant to Fluconazole with sessile minimum inhibitory concentration greater than 1024 µg/mL. Biofilms of 6 (28.5%) isolates were inhibited by Fluconazole; however, the minimum inhibitory concentration at 50% inhibition was 2 to 4 folds higher than the maximum concentration used for the planktonic susceptibility testing. Amphotericin B showed a variable minimum inhibitory concentration on the biofilm between different isolates. As indicated by SMIC50, 61.9% of *Candida* isolates biofilms were fairly susceptible to Amphotericin B and remaining 39.1% were comparatively resistant with SMIC50 1µg/mL and above. In addition, Clotrimazole was relatively less effective towards *C. albicans* biofilms. Only 3 individual biofilms of *C. albicans* had SMIC50 and SMIC80 below 1µg/mL. A total of 6 (28.6%) *C. albicans* biofilms were totally resistant to Clotrimazole with SMIC50 and SMIC80 greater than the maximum used concentration (16µg/mL). Two to eight fold increase in minimum inhibitory concentration was observed between planktonic and sessile cells (Table 4).

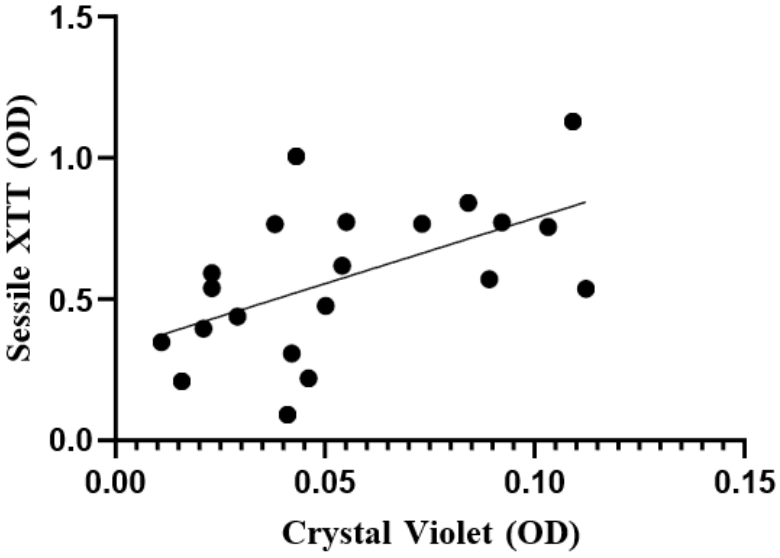

Figure 4: Correlation of methods of biofilm formation (Crystal violet correlated well withsessile metabolic activity).

Table 2: Antifungal susceptibility test of *C. albicans* against commonly used antifungalagents by disk-diffusion method

Antifungal drugs	Antifungal susceptibility pattern (n=21)		
	Resistant, n (%)	SDD, n (%)	Susceptible, n (%)
Amphotericin B	5 (23.8%)	0	16 (76.2%)
Clotrimazole	2 (9.5%)	5 (23.8%)	14 (66.7%)
Fluconazole	4 (19.1%)	2 (9.5%)	15 (71.4%)
Ketoconazole	11 (52.4%)	2 (9.5%)	8 (38.1%)

Table 3: Response of *C. albicans* isolates to different concentrations of antifungal drugs

Candida Isolates	CLSI breakpoints (µg/mL)							
	Amphotericin B			Fluconazole			Clotrimazole	
	S (≤ 4)	SDD (8–16)	R (≥16)	S (≤8)	SDD (16-32)	R (≥64)	S (≤1)	R (>1)
Candida albicans	16 (76.2%)	3 (14.3%)	2 (9.5%)	17 (81.0%)	1 (4.7%)	3 (14.3%)	16 (76.2%)	5 (21.8%)

Table 4: Sessile MICs of different *C. albicans* isolates against three different antifungals

***C. albicans* isolate**	**Sample**	**Amphotericin B**		**Fluconazole**		**Clotrimazole**	
		SMIC50	SMIC80	SMIC50	SMIC80	SMIC50	SMIC80
1	Urine	1	2	512	1024	4	8
2		2	4	>1024	>1024	2	4
3		1	1	>1024	>1024	1	2
4		<0.12	<0.12	>1024	>1024	0.5	0.5
5		0.5	1	>1024	>1024	>16	>16
6		0.5	1	1024	>1024	2	4
7		>16	>16	>1024	>1024	2	4
8		0.5	1	128	256	2	4
9		2	4	32	64	0.12	0.25

10	Sputum	0.25	0.5	1024	1024	1	2
11		<0.12	<0.12	>1024	>1024	>16	>16
12		0.5	1	>1024	>1024	>16	>16
13		0.5	1	>1024	>1024	4	8
14		0.25	0.5	>1024	>1024	>16	>16
15		0.5	1	>1024	>1024	2	4
16		1	2	>1024	>1024	>16	>16
17	HVS	<0.12	<0.12	512	1024	1	2
18		0.5	1	>1024	>1024	0.25	0.5
19		0.5	1	256	512	2	4
20		1	2	>1024	>1024	2	4
21	Catheter tip	1	2	>1024	>1024	>16	>16

DISCUSSION

Candida species remain to be a predominant cause of fungal infection ranking higher among the yeasts. This study reported *C. albicans* as a significant fungal pathogen accounting for 36.2% among total yeasts isolates which was inconsistent with the previous studies (Kandel et al 2017; Khadka et al 2017; Subramanya et al 2017). This relative lower figure can be attributed to different factors such as demographics of the patients, their immune status and exposure to pathogen as well as site of infection (Gnat et al 2019). The changing epidemiology of candidiasis and an increasing trend of non-albicans *Candida* over the last three decades certainly must be taken into account and health personnel must be aware of the importance and implication of the non-albicans *Candida* while diagnosing and especially during the selection of the antifungal drugs although no significant differences have been found regarding their susceptibility (Sobel et al 2011; Chow et al 2008).

In this study majority of *Candida* species were isolated from urine of which 31.0% were *C. albicans*. On the other hand, the prevalence of *C. albicans* was seen higher in high vaginal swab as 57.1% of the *Candida* species were *Candida albicans*-an indication that the vaginal candidiasis by *C. albicans* was common. *Candida* species can be isolated from oral samples like sputum or throat swab in both commensal state and in cases of the oral candidiasis (Raju and Rajjapa 2011). In this study one fourth of the *Candida* species (25.9%) were isolated form sputum samples among which *Candida albicans* accounted for 46.7%. There exists a wide variation in the prevalence of *C. albicans* in sputum in different researches ranging from 24% to 70% (Khadka et al 2017, Jha et al 2006). This indicated that species are more likely to be isolated from sputum samples either it be as commensal or as pathogenic which highlights the importance of clinical symptoms represented as thrush or oral candidiasis. None of the isolates were obtained from blood and CSF probably because these are normally sterile

body fluids and candidemia and *Candida* meningitis is not commonly reported and carry grave prognosis (Reef and Mayer 1995).

The qualitative method of biofilm formation among *C. albicans* showed that 71.4% of *C. albicans* isolates were biofilm producers while remaining 28.6 % were either weak biofilm producers or non-biofilm producers. Similar study performed in India reported 61% biofilm producers among a total of 26 *C. albicans* isolates (Sida et al 2016). The biofilm-producing *C. albicans* isolates were further identified by microtiter plate method using crystal violet assay and reported that 85.7% of *C. albicans* isolates were biofilm producers. A comparable study done in Nepal documented 75% of *C. albicans* isolates were biofilm positive (Subramanya et al 2017). A high degree of biofilm forming ability represents the potential of the pathogen to cause the invasive disease (Hasan et al 2009).

In this study XTT results were interpreted directly under the absorbance value as the ability to form the biofilms. The average OD value was 0.577 with highest and lowest value 1.127 and 0.090 respectively. The range of optical density value in similar study was observed in between 1.0 and 1.5 which indicated a higher biofilm forming capacity of the test isolates (Pierce et al 2010). Biofilm quantification as determined by crystal violet and XTT assay correlated well with each other. This correlation can be because of the increased number of living and dividing cells than metabolically inactive ECM as the biofilm in early phase was used for the study. The obvious difference in absorbance value can be attributed to difference in the enzymatic action, dye intensity and also because the experiment was carried in different days under different laboratory environments for two different methods. Based on the strength and weakness of both the tested methods along with the correlation date, it is reasonable to support the use of both methods for biofilm quantification and alongside it is safe to suggest that neither of the methods be used alone for the biofilm quantification.Together and in parallel these two methods are good indicators of biofilm production by clinical isolates.

Antifungal susceptibility profile revealed that 76.2% of *C. albicans* were susceptible to Amphotericin while the highest level of resistance was observed with Ketoconazole (52.4%). This finding is similar to the previous study done in Nepal (Khadka et al 2017). Most *C. albicans* (80.9%) were susceptible to Fluconazole with an MIC of ≤8 µg/mL while 14.29% were resistant (≥64 µg/mL). Similarly, majority of the *C. albicans* isolates were susceptible to Clotrimazole; only 9.525% of *C. albicans* isolates had MIC ≥16 µg/mL. The susceptibility pattern obtained in this study against azole antifungals are in agreement with a previous study where higher susceptibility rates were observed against azole antifungal drugs (Rathod et al 2012). However, some investigations reported higher resistance rate of Fluconazole and Clotrimazole and other azole drugs (El houssaini et al 2018; Zhang et al 2015). The development of resistance against azole antifungals can be due to the alteration of the lanosterol 14 α demethylase target enzyme because of either overexpression or mutation in the ERG11 gene encoding the enzyme (Henry et al 2000). Another reason can be because of the overexpression of efflux pumps mediated by the activation of expression of ATP binding cassette or major facilitator superfamily transporters (Paul et al 2014). In addition, Majority of the *C. albicans* isolates were susceptible to Amphotericin B with 76.19 % of *C. albicans* having an MIC of ≤1 µg/mL while the rest 23.81% had an MIC > 1 µg/mL. Amphotericin B is still widely used drug of choice for most fatal and disseminated fungal infection; however, its high cost makes it difficult to afford by majority of the patients especially in the developing countries.

Susceptibility of clinical *C. albicans* biofilms to Amphotericin B is often reported to be higher than azoles antifungal agents. Azole antifungal agents are often known to have decreased susceptibility against *Candida* biofilms (Tobudic et al 2012). Sessile MIC value for Fluconazole was found to be 2 to 4 times higher than the maximum concentration used for the plankton susceptibility testing. However, 8-fold increase in sessile MIC for Clotrimazole was observed. The finding in this study is in line with the literatures as higher MICs were seen in biofilm-associated cells as compared to planktonic cells (Pierce et al 2008; Shuford et al 2007). Obtaining the standard MIC results from clinical microbiology laboratory might be insufficient when it comes to initiate appropriate dosing level to completely eradicate an infection.

CONCLUSION

Candida albicans is still the most prevalent fungal pathogenic yeast but the prevalence rate was found to be less than the expected range. Higher proportion of non-albicans *Candida* was found among the clinical isolates. It was apparent form the study that species has higher prevalence in urine sample and vaginal candidiasis is common among Nepalese women caused by *C. albicans*. Majority of the *C. albicans* isolates were biofilm-producing drug resistant which remain as a challenge for therapeutic world with decreased susceptibility against common

antifungals. Proper administration of antifungal drugs should be prioritized only with susceptibility result testing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Susceptibility to Fluoroquinolones among *Salmonella enterica* Serovars in Blood Culture

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ABSTRACT

Objectives: The study was designed to analyze the antibiotic susceptibility pattern of fluoroquinolones among *Salmonella enterica*.

Methods: A cross-sectional study was carried out at National Public Health Laboratory, Kathmandu. Blood samples were collected from suspected enteric fever patients and cultured in BACTEC standard/10 Aerobic/F culture vials. Isolates obtained from the vials with bacterial growth were identified by standard procedure. Serotyping of the identified isolates *Salmonella enterica* was done. An antibiotic susceptibility test was done by Kirby-Bauer disc diffusion method and results were interpreted according to Clinical Laboratory Standards Institute (CLSI 2014) guidelines.

Results: Among 404 samples, 17 (4.2%) were positive for *Salmonella enterica* in which 9 (52.9%) were *Salmonella* Typhi and 8 (47.1%) were *Salmonella* Paratyphi A. All the *Salmonella* isolates showed resistance to nalidixic acid and ampicillin and showed sensitivity to ceftriaxone and chloramphenicol. No multi-drug resistant isolates were identified in this study. All isolates of *Salmonella* Typhi and *Salmonella* Paratyphi A showed the reduced susceptibility to ciprofloxacin and ofloxacin.

Conclusion: It is concluded that fluoroquinolones cannot be considered as the drug of choice for the treatment of *Salmonella* infections due to their high level of reduced susceptibility and resistance to fluoroquinolones and third generation cephalosporin antibiotics like ceftriaxone remains better choice of drugs against fluoroquinolone-resistant *Salmonella* Typhi and Paratyphi.

Key words: Enteric fever, *Salmonella enterica*, fluoroquinolones, nalidixic acid resistant

INTRODUCTION

Enteric fever is a serious bloodstream infection caused by *Salmonella enterica* serovars Typhi and Paratyphi A and is an important cause of morbidity and mortality (Britto et al 2020; Maes et al 2020). The global estimated cases of enteric fever and deaths due to enteric fever in 2017 are 14.3 million and 135.9 thousand respectively, whereas *Salmonella enterica* serotype Typhi caused 76·3% of cases of enteric fever (GBD 2017). Enteric fever has been a public health concern in Nepal, with *S.* Typhi and *S.* Paratyphi A consistently being regularly isolated from the blood of febrile patients in Kathmandu Valley since the early 1990s (Maskey et al 2008; Murdoch et al 2004). In developing countries like Nepal, the mainstay therapy is antibiotics to prevent the complications associated with enteric fever illness and death of the patients. With the introduction of chloramphenicol for the treatment of typhoid fever in 1948, often fatal disease was transformed into a readily treatable condition and the cases has been reduced to less than 1% from about 30%, however a major setback occurred with the emergence of resistance to chloramphenicol and other antimicrobial agents (Parry et al 2002).

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Antibiotics recommended by World Health Organization (WHO) for enteric fever treatment are chloramphenicol, ampicillin and cotrimoxazole (trimethoprim-sulfamethoxazole), fluoroquinolones, third-generation cephalosporins (ceftriaxone, cefixime) and azithromycin for the treatment of enteric fever (WHO 2003). However, the reduced susceptibility of *Salmonella enterica* isolates to commonly used antibiotics continues to be a major problem for effective therapy of enteric fever, prolonging the duration of fever and leaving patients at risk of further complications (Bhetwal et al 2017; Zellweger et al 2017). Due to the emergence of multidrug resistance (MDR) strains of *Salmonella*, the antibiotic treatment of enteric fever with the first line antibiotics chloramphenicol, ampicillin, and cotrimoxazole has been affected (Eng et al. 2015), which led to the use of fluoroquinolones, mainly ciprofloxacin, and third generation cephalosporin (Bhutta 2006; Bhan et al 2005; Pokharel et al 2009)

Fluoroquinolones, such as ciprofloxacin and ofloxacin, have become a mainstay for treating severe *Salmonella* infections (Sjölund-Karlsson et al 2014). However, strains of *Salmonella* with increased levels of resistance to fluoroquinolones have been reported in South Asia (Browne et al. 2020) and in Nepal (Acharya et al 2012; Bhetwal et al 2017; Maskey at al 2008). Nalidixic acid resistance (NAR) is a marker for predicting decreased susceptibility (low-level of resistance) to ciprofloxacin among *S. enterica* serovar Typhi and Paratyphi, and also an indicator of treatment failure to ciprofloxacin (Acharya et al 2012; Khademi et al 2020; Rudresh et al 2015).

In the developing countries like Nepal where minimum inhibitory concentration of antibiotics is not routinely determined in laboratories, fluoroquinolones especially ciprofloxacin is still used for the treatment. There would be the possibility of treatment failure of infections with *S.* Typhi and *S.* Paratyphi A strains with reduced fluoroquinolone susceptibility (Crump et al 2004; Woods et al 2006). This study was thus designed with the objectives to determine the reduced susceptibility pattern of *Salmonella* isolates towards fluoroquinolones and to find out the minimum inhibitory concentration (MIC) value of the ciprofloxacin and ofloxacin which might help to know the effective drug dose to be used for the treatment of the typhoid fever.

METHODS

The cross-sectional study was carried out at National Public Health Laboratory (NPHL), Kathmandu, Nepal from June 2016 to November 2016. This study was conducted on clinically defined enteric fever patients of all age groups of both sexes who visited NPHL requesting for blood culture and susceptibility testing. The ethical approval for this study was obtained from Nepal Health Research Council, Kathmandu, Nepal (Approval no. 363/2016).

A total of 404 blood samples from patients suspected of enteric fever were included in the study after obtaining the consent and details on clinical history, age and sex of the individual were recorded. The exclusion criteria for samples were improper labeling, insufficient blood volume, inappropriate collection and transport, and samples from patients with prior antibiotic therapy within 1 week. About 3-5 mL of blood from patients was collected and aseptically inoculated into BACTEC standard/10 Aerobic/F culture vials. The inoculated culture vials were immediately transported to the laboratory and incubated in BACTEC fluorescent series instruments. Incubation was continued for 7 days until growth indication was obtained in BACTEC. The culture bottles were observed daily for indication of microbial growth. The growth was indicated by the red alarm in the BACTEC machine. The aliquot from vials with growth of bacteria were subcultured on MacConkey agar (MA) and blood agar (BA) plates. The final subculture for visually negative culture bottles was done after 7 days of incubation.

The isolated colonies of bacteria obtained on MA and BA were analysed for the identification as *Salmonella* spp by Gram staining and biochemical tests. Various biochemical tests- catalase test, oxidase test, sulphide indole motility (SIM) test, methyl red test, Voges Proskauer test, triple sugar iron (TSI) test, citrate test and urease test were performed for Gram negative rods (Cheesbrough 2012; WHO 2003). Serotyping of bacteria identified as *Salmonella enterica* was also done to confirm the isolates with antisera by observing the agglutination reaction between antigen and antibodies. For serotyping, O, H and Vi antigen of Denka – Seiken company Ltd, Japan was used. (add few details of serotyping). After complete identification, isolates were preserved in tryptic soy broth with 25% glycerol at -70 ^{0}C.

Antibiotic susceptibility tests (AST) of the identified bacteria were performed by modified Kirby Bauer disc diffusion method on Mueller Hinton Agar (MHA) plate following CLSI guideline 2014. Minimum inhibitory concentration (MIC) of ciprofloxacin and ofloxacin were determined by broth dilution method the concentrations of 0.125 to 512 µg/mL for ciprofloxacin and 0.125 to 512 µg/mL for ofloxacin, and following the guidelines of CLSI

(2014). *Escherichia coli* ATCC 25922 was used as the quality control strain.

RESULTS

Out of 404 cases, only 17 (4.2%) cases were found to be culture positive for *S. enterica*. Among the *Salmonella* isolates, 9(52.9%) were *S*. Typhi and 8 (47.1%) were *S*. Paratyphi.

Growth of *Salmonella enterica* was not obtained in the samples of patients of age greater than 40 years. The highest percentage of growth was seen in the age group 11-20 and 21-30 years (29.4%). Greater number of male patients were infected with *Salmonella* than females as shown in Table 1.

Ceftriaxone and chloramphenicol were 100% effective to all isolates followed by cotrimoxazole. However, organisms showed resistance towards quinolone group antibiotics as nalidixic acid was 100% ineffective followed by ciprofloxacin (47.1%) as shown in Table 2.

The MIC breakpoint values of ciprofloxacin used for interpretation were ≤0.06 µg/mL, 0.125-0.5 µg/mL and ≥1 µg/mL as sensitive, intermediate and resistant respectively according to CLSI guideline 2014. In this study, the highest MIC value of ciprofloxacin for Nalidixic Acid Resistant isolates was 1 µg/mL and the lowest was 0.25 µg/mL as shown in Table 3, whereas the highest MIC value of ofloxacin was 1 µg/mL and the lowest was 0.5 µg/mL as shown in Table 4.

Table 1. Distribution of the serotypes of *Salmonella* in different age group and gender

Age Group (years)	Number of enteric fever cases with growth of *Salmonella enterica*				Total
	Male		Female		
	S. Typhi	*S*. Paratyphi	*S*. Typhi	*S*. Paratyphi	
0-10	2	2	-	-	4
11-20	1	2	1	1	5
21-30	3	1	1	-	5
31-40	-	2	1	-	3
Total	6	7	3	1	17

Table 2: Antibiotic susceptibility pattern of *Salmonella* serotypes (concentration of antibiotics)

Antibiotic Used	Antibiotic Susceptibility Pattern					
	Susceptible		Intermediate		Resistant	
	Number	Percent (%)	Number	Percent (%)	Number	Percent (%)
Nalidixic Acid (30 mcg)	-	-	-	-	17	100
Ciprofloxacin (5 mcg)	1	5.9	8	47.1	8	47.1
Ceftriaxone (30 mcg)	17	100	-	-	-	-
Ampicillin (10 mcg)	-	-	-	-	17	100
Cotrimoxazole (25 mcg)	15	88.3	2	11.8	-	-
Azithromycin (15 mcg)	8	47.1	4	23.5	5	29.4
Chloramphenicol (30 mcg)	17	100	-	-	-	-

Ofloxacin (5 mcg)	11	64.7	6	35.3	-	-
Cefixime (5 mcg)	14	82.4	3	17.6	-	-
Amikacin (30 mcg)	16	94.1	1	5.9	-	-

Table 3: MIC of Ciprofloxacin susceptibility pattern of Nalidixic Acid resistant *Salmonella* serovars for *S.* Typhi and *S.* Paratyphi.

MIC (µg/mL)	*S.* Typhi (N=9)	Sensitivity pattern towards Ciprofloxacin	*S.* Paratyphi A (N=8)	Sensitivity pattern towards Ciprofloxacin	MIC Breakpoints
≤0.015	-	Sensitive (N=0)	-	sensitive (N=0)	Sensitive ≤0.06 µg/mL
0.03	-		-		
0.06	-		-		
0.125	-	Intermediate (N=8) 88.9%	-	Intermediate (N=7) 87.5%	Intermediate 0.125-0.5 µg/mL
0.25	6		5		
0.5	2		2		
1	1	Resistant (N=1) 11.1%	1	Resistant (N=1) 12.5%	Resistant ≥1 µg/mL
2	-		-		

Table 4: MIC of Ofloxacin susceptibility pattern of nalidixic acid resistant *Salmonella* serovars for *S.* Typhi and *S.* Paratyphi.

MIC (µg/mL)	*S.* Typhi (N=9)	Sensitivity pattern towards Ofloxacin	*S.* Paratyphi A (N=8)	Sensitivity pattern towards Ofloxacin	MIC Breakpoints
≤0.015	-	Sensitive (N=0)	-	Sensitive (N=0)	Sensitive ≤0.12 µg/mL
0.03	-		-		
0.06	-		-		
0.125	-				
0.25	5	Intermediate (N=9) 100%	-	Intermediate (N=8) 100%	Intermediate 0.25-1 µg/mL
0.5	3		6		
1	1		2		
2	-	Resistant (N=0)	-	Resistant (N=0)	Resistant ≥2 µg/mL

DISCUSSION

Salmonella enterica in the blood culture of suspected patients was found only 4.2% and similar culture positivity results have also been reported in other studies from Nepal (Bhetwal et al 2017; Shrestha et al 2016). However, the relatively higher growth rate was reported by Sharma et al (2006) and Khanal et al (2007) as 6.9% and 5.1% respectively. Rewrite these sentences. Growth rate of the present study was low but even lower growth rate has been reported as 2% in Raza et el 2012 and 2.3% among children in Kathmandu by Maskey et al 2008. Decrease in growth might be the result of antibiotic therapy even in milder cases of fever (Malla et al 2005; Khanal et al 2007).

The positive cases were higher (76.5%) in male patients than in female (23.5%). This study result was comparable to the study done in Lalitpur by Pandey et al (2015), which reported 70.2% male and 29.7% female positive cases. Previous studies from Nepal also have shown higher prevalence of enteric fever in males than in females (Sharma et al 2003; Shakya et al 2008; Prajapati et al 2008). This study showed a higher prevalence of enteric fever among males as the number of samples was higher in male. This gender wise difference in the prevalence of enteric fever may be due to sample size (male:female = 1.23:1) and their relatively more outdoor activities exposing them to the source of infection. Majority of the cases were of the age group between 11-20 years and 21-30 years, followed by 0-10 years and 31 -40 years. Similar types of the result have been reported in the study carried out by Agrawal et al 2014 with majority 14% of cases in the age group 5-18 years. These age groups include school and college going children. The possible causes for enteric fever being common in these age groups include their mobility, consumption of unhygienic food and water in street vendors, schools and colleges (Walson et al 2001).(references)

Two serotypes i.e. *S*. Typhi (52.9%) and *S*. Paratyphi A (47.1%) were identified in this study. This result was comparatively similar with the study result of Raza et al (2012) as *S*. Typhi (66.7%) and *S*. Paratyphi A (33.3%) and in the study done by Gurung et al (2017), 54% *S*. Typhi and 46% *S*. Paratyphi A.

The isolates were tested against ten antibiotic discs for performing the antibiotic susceptibility testing. Among the isolated *S*. Typhi showed 100% sensitivity towards chloramphenicol and cotrimoxazole while *S.* Paratyphi A showed 100% sensitivity to chloramphenicol and 75% to cotrimoxazole which was similar to the study done by Amatya et al (2007) and Joshi et al (2011). In this study, though chloramphenicol was found susceptible to all the isolates, it is not recommended as a drug of choice due to its side effects. Ceftriaxone should be recommended only if the first and second line antibiotics failed to evoke a satisfactory response or if the isolate is resistant to nalidixic acid. So, it should be a last line drug during empirical therapy and also shows the high sensitivity to chloramphenicol and cotrimoxazole (Manchanda et al 2006; Neupane et al 2008; Prajapati et al 2008; Sharma et al 2007; Acharya et al 2012). Ofloxacin and cefixime was shown to be 77.8% sensitive to *S*.Typhi followed by azithromycin (55.6%), whereas in *S*. Paratyphi, cefixime shows 87.5% sensitivity followed by ofloxacin (50%) and azithromycin (37.5%). As in this study ampicillin was 100% resistant to all the isolates, and similar report of high percentage of ampicillin-resistant isolates (70.6% S. Typhi and 78.3% S. Paratyphi A) was shown in a study done in Chitwan by Acharya et al (2012).

In this study, all isolates were found to be 100% nalidixic acid resistant (NAR), which was higher in comparison to other studies. This trend of higher nalidixic acid resistance was also found in a study conducted in Kathmandu by Shirakawa et al (2006) and Agrawal et al (2014) in which nalidixic acid resistant in *S*. Typhi were 73.3% and 90.2% and *S*. Paratyphi were 94.9% and 81.8% respectively. In developing countries, the high resistance of nalidixic acid is often due to self- medication (Mincey and Parkulo 2001), the suboptimal quality of antimicrobial drugs, and poor community and patient hygiene (Walson et al 2001).

This study also showed high frequency of ciprofloxacin resistant isolates with 55.6% in *S*. Typhi, 37.5% in *S*. Paratyphi A. High resistance to ciprofloxacin was also observed in study of Poudel et al 2014 with 31.3% in *S*. Typhi, 4% in *S*. Paratyphi A. This increased resistance reflects the overuse of ciprofloxacin in the treatment of typhoid, as well as in other unrelated infections. Incomplete treatment may also be a factor contributing to development of resistance. Third generation cephalosporin, ceftriaxone showed 100% susceptibility for both *S*. Typhi and *S*. Paratyphi A strains in present study. Similarly, a study conducted by Sharma et al 2003 in Dhulikhel hospital also reported 100% efficiency of ceftriaxone to both strains. Ceftriaxone remains as the last line of drug against infections with ciprofloxacin resistant *Salmonella* when it is resistant to other first line drugs (Bhatia et al 2007; Raza et al 2012).

According to CLSI (2014), susceptible, intermediate and resistant breakpoints for ciprofloxacin among *Salmonella*

spp. are ≤0.06 µg/mL, 0.125-0.5 µg/mL and ≥1 µg/mL (respective inhibition zone diameter to 5 µg ciprofloxacin are ≥31 mm, 21-30 mm and ≤20 mm). Similarly, for ofloxacin are ≤0.12 µg/mL, 0.25-1 µg/mL and ≥2 µg/mL (respective inhibition zone diameter to 5 µg ofloxacin are ≥16 mm, 13-15 mm and ≤12 mm) (CLSI 2014). In this study, only one NAS strain was ciprofloxacin sensitive by disc diffusion method but none of the strain was susceptible in MIC (MIC ≤0.06 µg/mL). Among 17 NAR isolates, 8 (47.1%) isolates were resistant by disc diffusion but only 2 (11.8%) were found to be resistant by MIC test (MIC 1 µg/mL). However, 15 (88.2%) showed the reduced susceptibility towards ciprofloxacin (MIC value 0.125-0.5 µg/mL). The reduced susceptibility to ciprofloxacin in *S*. Typhi and *S*. Paratyphi A was strongly correlated with resistance to nalidixic acid. Similarly, by performing MIC test towards ofloxacin to all NAS isolates, 11(64.71%) isolates were sensitive by disc diffusion but none of the isolate was sensitive as MIC value ≤0.12 µg/mL. All 17(100%) showed the reduced susceptibility towards ofloxacin (MIC value 0.25-1 µg/mL). Similarly, in the study done by Acharya et al 2012, it was reported that nalidixic acid disc diffusion recommended by CLSI (2014) to screen reduced susceptibility to fluoroquinolones was well correlated with reduced fluoroquinolones susceptibility in the *Salmonella* isolates.

Many studies done in Kathmandu have also reported the cases of enteric fever treated with fluoroquinolones with prolonged time or treatment failure. The MIC of ciprofloxacin and ofloxacin of such strains is steadily increasing, although the MIC values were still below CLSI (2014) recommended breakpoint (≤1 and ≥4 µg/mL) (Adhikari et al 2012; Nagshetty et al 2010; Rudresh et al 2015). However, it is not clear whether fluoroquinolones can still be used as first-line drugs for the treatment of typhoid fever, and if used whether this has any adverse impact on clinical outcomes other than treatment failure such as development of complications and morbidity assessed in terms of total duration of illness. In such a scenario, this present study was carried out to determine the infection of NARST isolates and the effectiveness of fluoroquinolones against the isolates. Because of the rising rates of quinolone resistance, there is a clear need to identify improved strategies for treating typhoid fever as highly resistant organisms may be isolated in near future (WHO 2003). The drug of choice for the treatment of enteric fever is ceftriaxone, Chloramphinicol, Amikacin however Cefixime and Cotrimoxazole can be used for the treatment with antibiotic susceptibility test.

CONCLUSION

The prevalence of *Salmonella enterica* serovar Typhi was found to be relatively higher than *Salmonella enterica* serovar Paratyphi A among significant growth obtained from blood culture. Though fluoroquinolones are the first choice for the treatment of enteric fever, high level of reduced susceptibility and resistance to fluoroquinolones (*S*. Typhi and *S*. Paratyphi - resistant to nalidixic acid) were observed, raising question in the efficacy of fluoroquinolones used for the treatment of enteric fever. Therefore, the third generation cephalosporin antibiotics like ceftriaxone might be a better choice for treatment against fluoroquinolone resistant *Salmonella* Typhi and Paratyphi. Hence, this study suggests that nalidixic acid susceptibility test by disc diffusion method can be used as the screening test to determine decreased susceptibility of *Salmonella* strains to fluoroquinolones and MIC determination becomes mandatory for NAR *Salmonella* strains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Prevalence of Methicillin Resistant *Staphylococcus aureus* among Dumpsite Workers in Kathmandu Valley

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ABSTRACT

Objectives: The main objective of this study was to determine prevalence of methicillin resistance *Staphylococcus aureus* among dumpsite workers.

Methods: Total 60 nasal swab samples were collected. Conventional microbiological methods were used to isolate and identify *S. aureus.* Antibiotic susceptibility test was performed by Kriby Bauer disc diffusion method. MRSA was confirmed by using Cefoxitin disc. The organism showing resistance against three or more class were considered as MDRSA.

Results: The prevalence rate of *S. aureus* and MRSA was found to be 46.67% (28/60) and 6.67% (4/60) in total population. The nasal carriage rate of *S. aureus* was found to be higher in age group 28-37 (70.06%, 12/17), gender male (47.5%, 19/40), district Kathmandu (70%, 14/20) and dumpsite workers (50%, 15/30). The isolated *S. aureus* were resistant to Penicillin (35.7%), Erythromycin (35.7%), Cefoxitin (14.3%) and Oxacillin (10.7%). *S. aureus* was susceptible (100%) to Tetracycline, Gentamycin, Ciprofloxacin, Co-trimoxazole, Chloramphenicol, and Vancomycin. Multidrug resistant *S. aureus* was not found in community of Kathmandu Valley.

Conclusion: The study shows prevalence of MRSA strains of *S. aureus* in Kathmandu Valley. The occurrence of MRSA indicates development of Community acquired-antibiotic resistant bacteria.

Keywords: *S. aureus,* MRSA, dumpsite workers, multidrug resistant *S. aureus* (MDRSA)

INTRODUCTION

S. aureus, a commensal normal flora of skin and anterior nares of human body, has been arising as a potentially serious pathogen. Over a period, it became able to adapt to the selective pressure of antibiotics such as Penicillin followed by Methicillin resulting in the emergence and spread of (MRSA) Methicillin Resistant *Staphylococcus aureus* (Deurenberg et al 2008). Its resistivity has been seen in different classes of antibiotic including various other β-lactam antibiotics, aminoglycosides, tetracycline etc. harboring a multi-drug resistant (MDR) strains (Chen and Huang 2014).

S. aureus is alleged for inducing human infections in both the hospital as well as community setting (Uhlemann et al2014; Bonesso et al 2014) with a plethora of infections ranging from simple soft skin tissue infection to invasive life scaring bacteremia and septicemia (Bhatta et al 2015). In present days, MRSA strains are found to be responsible for causing infection such as minor skin and soft tissue infections(SSTIs) in community setting, so called Community acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) (Kawada-Matsuo et al 2012).

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About 5-10% of CA-MRSA infection are invasive and live scaring (Uhlemann et al 2014). The origination of CA-MRSA is still a subject of debate and are presumed to be feral descendant of hospital isolates and horizontal transfer of methicillin resistant determinant. Besides, different studies also showed that contamination in the environment could be the source of CA-MRSA infections (Boyce 2007)

Recent studies have shown that the prevalence rate of MRSA in different parts of Nepal ranges from 39% to 69% and the burden of MRSA infection is high in Asia (Bhatta et al 2015; Subedi and Brahmadathan 2005). The study on prevalence of MRSA colonization in the community, in case of low-income countries, are missing. Dumpsite workers are continuously exposed to different type of wastes, so are more prone to *S. aureus* colonization causing CA-MRSA infection and can serve as asymptomatic carriers disseminating infections in the community. Recent studies have reported the increasing infection by CA-MRSA and the emergence of MDR strains leading to a serious public health threat (Boswihi and Udo 2018). Therefore, the prevalence of CA-MRSA colonization and infection needs to be better explained. Thus, this type of study would be helpful for promoting the public health by increasing awareness in maintaining personal and public hygiene, reducing the over exploitation of antimicrobials and the incidence of resistant gene.

METHODS

Study design, site and period

The study was conducted at Microbiology laboratory of St. Xavier's College (Maitighar, Kathmandu) to gain knowledge regarding the prevalence of methicillin resistance *S. aureus* among dumpsite workers in Kathmandu Valley and covered the total period of five months from August 2018 to January 2019.

Study population and sample size

Total 60 nasal swab samples were collected from different places of Kathmandu valley after receiving the ethical approval from Nepal Health Research Council (NHRC) **(Reg. no.:584/2018)**. Among 60 samples, 30 samples were obtained from dumpsite workers (10 samples each from Kathmandu, Lalitpur and Bhaktapur district) and 30 samples from public were taken as control. Informed consent was taken before the sample collection. The samples of dumpsite workers were collected from their attendance office i.e. Kathmandu (Ward no. 11 office, Thapathali), Lalitpur (Lalitpur metropolitan office, Balkumari) and Bhaktapur (Suryabinayak Municipality Office). Similarly, for control group, the samples of residents visiting the laboratory area from each districts were collected.

Isolation

Nasal swabs were collected and transported to laboratory in peptone broth within 2 hours by maintaining 4°C in the icebox (Higgins 2008). The sample was cultured on Mannitol Salt Agar (MSA) and *S. aureus* were identified colonies showing yellow colonies on MSA, Gram positive cocci in cluster, catalase positive, oxidase negative, coagulase positive and fermentative on O/F test. All the reagents and media were used of HiMedia Company.

Susceptibility testing

All the *S. aureus* isolates obtained were subjected to in vitro antibiotics susceptibility testing by Kirby- Bauer disc diffusion method as recommended by CLSI (2014). The screening of Methicillin Resistant *Staphylococcus aureus* (MRSA) was done by using Cefoxitin disc (30µg). The organism showing resistance against three or more class were considered as MDRSA (Magiorakos et al 2011).

Data analysis

Raw data obtained from laboratory investigation were tabulated and presented in defined tables and graphs to explore the findings. The data were analyzed using Excel and IBM SPSS statistics 21.0 version software.

RESULTS

Growth profile analysis

Out of 60 nasal swab sample received, 44/60 (73.33%) samples showed growth in MSA plates while 16 /60 (26.67%) samples showed no growth. 63.63% (28/44) were found to be *S. aureus* and 36.36% (16/44) were found to be other bacteria. (Figure 1).

Antibiotic sensitivity pattern for *Staphylococcus aureus*

Among 28 *S. aureus* isolates, 35.4%, 14.3%, 10.7% and 35.7%, were resistant to Penicillin, Cefoxitin, Oxacillin and Erythromycin respectively while all the isolates were sensitive towards Tetracycline, Gentamicin, Ciprofloxacin and Cotrimoxazole. Out of 28 isolates, four isolates were resistant to Cefoxitin and were assumed MRSA and 3 isolates were resistant to Oxacillin and hence, were

assumed to be ORSA (Oxacillin Resistant *Staphylococcus aureus*). (Table 1)

Distribution of *Staphylococcus aureus* and MRSA on different basis

S. aureus was found in 47.5% [19/40] male and 45% [9/20] female participants. Similarly, 7.5% [3/40] and 5% [1/20] of male and female subjects respectively harbor MRSA strains.

This study includes participants of the age between 18-58 years old. Colonization with MRSA was highest 11.76% [2/17] among the age group 28-38 years, followed by the age group of 18-37 years [10.53%, 2/19].

This study includes participants from three districts i.e. Kathmandu, Bhaktapur and Lalitpur. The colonization MRSA was higher in Lalitpur [15%, 3/20], followed by Kathmandu [5%, 1/20] and no colonization in Bhaktapur.

The study showed that the colonization of MRSA was higher in Dumpsite worker [10%, 3/30] than in community people [3.33%, 1/30]. However, there was no significant association between the bacterial load (MRSA) and Population type. (p= 0.557) (Table 2).

Antibiotic sensitivity pattern of MRSA isolates

About 25% (1/4), MRSA isolate was found to be resistant against Erythromycin and all 4 isolates were susceptible to Tetracycline, Gentamicin, Ciprofloxacin, Vancomycin and Chloramphenicol. There was no prevalence of MDR strains of *S. aureus*. (Table 3)

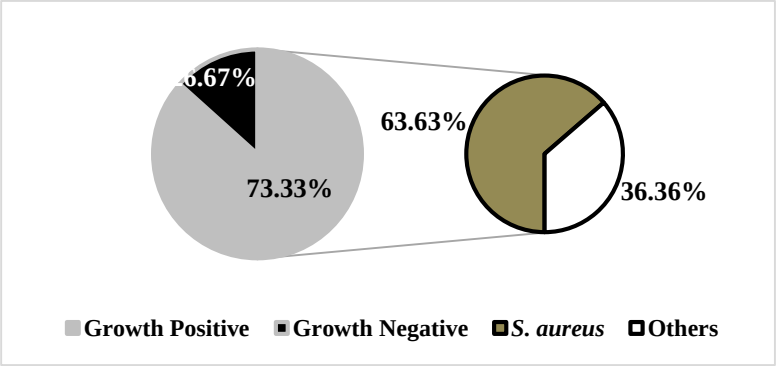

Figure 1: Pie Chart showing growth profile in nasal swab sample

Table 1: Antibiotic Sensitivity Pattern for *Staphylococcus aureus*

Antibiotic Class	Antibiotic (mcg)	Total *S. aureus* isolates(N= 28)		
		Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
β-lactam	Penicillin(10)	18(64.3)	-	10(35.7)
	Cefoxitin(30)	24(85.7)	-	4(14.3)
	Oxacillin(1)	25(89.2)	-	3(10.7)
Macrolide	Erythromycin(15)	16(57.1)	2(7.14)	10(35.7)
Tetracycline	Tetracycline(30)	28(100)	-	-
Aminoglycoside	Gentamicin(30)	28(100)	-	-
Floroquinolones	Ciprofloxacin(5)	28(100)	-	-
Sulphonamide	Cotrimoxazole(25)	28(100)	-	-
Miscellaneous	Chloramphenicol(30)	28(100)	-	-

Table 2: Distribution of *Staphylococcus aureus* and MRSA into different categories.

Category	Division	No. of Samples	*Staphylococcus aureus*		MRSA	
			Number	Percentage (%)	Number	Percentage (%)
Gender	Male	40	19	47.5	3	7.5
	Female	20	9	45	1	5
Age group	18-28	19	8	42.11	2	10.53
	28-38	17	12	70.06	2	11.76
	38-48	16	7	43.75	0	0
	48-58	8	1	12.5	0	0
Districts	Lalitpur	20	10	50	3	15
	Kathmandu	20	14	70	1	5
	Bhaktapur	20	4	20	0	0
Population Type	Dump site workers (DSW)	30	15	50	3	10
	Community Peoples (CPs)	30	13	43.33	1	3.33

Table 3: Antibiotic Sensitivity Pattern of MRSA isolates (Determination of MDR strains)

Antibiotic Class	Antibiotics (mcg)	Total MRSA isolates	
		Sensitive No. (%)	Resistant No. (%)
Macrolide	Erythromycin(15)	3(75)	1(25)
Tetracycline	Tetracycline(30)	4(100)	-
Aminoglycoside	Gentamicin(30)	4(100)	-
Floroquinolones	Ciprofloxacin(5)	4(100)	-
Sulphonamide	Cotrimoxazole(25)	4(100)	-
Glycopeptides	Vancomycin(30)	4(100)	-
Miscellaneous	Chloramphenicol(30)	4(100)	-

DISCUSSION

S. aureus, a commensal of the human microbiota, can act as an opportunistic pathogen causing infections. With the emergence of resistance against various antibiotic, *S. aureus* infection in both the hospital and community setting, has become a global health threat (Yilmaz and Aslantas 2017). This study was conducted with the concern to archive the status of prevalence of methicillin resistant *S. aureus* among dumpsite workers in Kathmandu Valley.

The prevalence rate *S. aureus* was 46.67% (28/60) in the community setting of Kathmandu Valley. The nasal carriage was higher than studies carried out by Shrestha et al (2010), Bhatt et al (2014) and Khatri et al (2017) which was 27.1%, 30.4% and 18.3% receptively. This indicates nasal carrier rate of *S. aureus* is higher in community setting as compared to previous studies conducted in a hospital setting of Nepal. Similarly, our results of colonization of *S. aureus* is comparable to study of Joachim et al (2018), Kejela & Bacha (2013), and Akerele et al (2015) having prevalence rate 41.1%, 47.74%, and 49.5% respectively. In contrary, our result showed low prevalence than the studies of Bonesso et al (2014), Hasan et al (2016) and Kumar et al 2011 having prevalence 56.9%, 60.6% and 72.5% respectively. The nasal colonization of *S. aureus* was found to be higher in age group 28-37 (70.06%, 12/17), gender male (47.5%, 19/40), district Kathmandu (70%, 14/20) and dumpsite workers (50%, 15/30). The prevalence rate also depends upon the type of study population, geographical locations and socio-economic conditions. The variation might be due to frequent exposure to polluted environment, poor hygienic condition etc. impairing the immune status of individual and making them (DSWs and CPs) more susceptible to be colonized by *S. aureus.* (Sangvik et al 2011).

The isolated *S. aureus* were found to be sensitive against Tetracycline, Gentamicin, Ciprofloxacin, Co-trimoxazole, and Chloramphenicol making it effective for treatment. Whereas, about 35.7%, 14.3%, 10.7%, 35.7% of *S. aureus* isolates were resistant to Penicillin, Cefoxitin, Oxacillin, and Erythromycin respectively. Similar results were observed in the studies of Naimi et al (2003), Wang et al (2018) and Baggett et al (2003) showing Vancomycin 100% susceptible. Yilmaz and Aslantas (2017) and Early et al (2012) also showed 100% susceptible to chloramphenicol. Resistant pattern of Erythromycin was comparable to studies of Wang et al (2018) (46.2%), and Naimi et al (2003) (44%). Ciprofloxacin was also 100% susceptible in study of Baggett et al (2003).

MRSA predominance rate was found to be 6.67% (4/60) in Kathmandu Valley, which was higher than Shrestha et al (2010) i.e. 2.3%.

The MRSA colonization rate was 15.4%, 19%, 34.75%, 21.1% in hospital based study conducted by Subedi and Brahmadathan (2005); Bhatt et al (2014); Bhatta et al (2015) and Khanal et al (2018) in context of Nepal that is higher than our study. However, studies in community acquired MRSA are less reported and on correlating with the above studies, it suggests that MRSA prevalence in community setting is lower than in hospital setting of Nepal. Similarly, Joachim et al (2018) noted MRSA predominance to be 37.6% in Tanzania and Bonesso et al (2014) stated it to be 10.6% in Brazil. This indicates that MRSA prevalence in Nepal is lower in contrast to Tanzania and higher as compared to Brazil. The variable result is due to the variable distribution of MRSA strains depending upon geographical location, study population and different laboratory technique used. (Sollid et al 2014).

The study showed the MRSA nasal carrier rate to be higher among early (18-28) and mid age (28-38) group population i.e. 3.33% (2/60) in each group and was absent as age increases (<40). Similar reports were highlighted by McMullen et al (2009) i.e. 56% (high) MRSA in mid age group people. The higher MRSA ubiquity among mid and early age group people may be due to improper intake of antibiotics (Sollid et al 2014). The prevalence rate of MRSA was found to be higher in male (5%, 3/60) in this study. Similar results were seen in investigations by McMullen et al (2009) (52.2%), Shrestha et al (2010) (28.6%) and Khanal and Jha (2010) (75%) showing high prevalence in male than in female. This suggests that male are more prone to MRSA colonization. This might be due to differences in hygienic habits, hormonal difference (estrogen level affects the expression of virulence factor of *S. aureus)* and other factors like occupation, frequent exposure to contact supports, socio-economic status, obesity may also trigger the MRSA carrier rate among genders whose further research need to be done. (Humphreys et al 2015). In this study, the male: female ratio was 2:1 and MRSA isolated from 3 males were DSWs, who are more frequently exposed to polluted sites and belongs to low socioeconomic background while 1 MRSA isolated from female belong to community people as a result of which MRSA carrier rate was found higher in male than in female.

The higher prevalence of MRSA was among DSWs i.e. 5% (3/60). According to CDC (2006), CA-MRSA colonization and infection is more frequently occurring in individual with poor hygiene and cleanliness, adults from low socio-economic condition, participation in activities resulting in compromised skin surface, limited access to health, frequent antibiotic exposure etc. The high prevalence of MRSA in DSWs may be due to the repeated exposure to different types of wastage and pollution, contact with the contaminated

inanimate materials, lack of hygienic condition, low socio economic status etc. making them more susceptible to get colonized by CA-MRS. (McMullen et al 2009). Hence, serve as a carrier and reservoir of *S. aureus infection* in the community.

The study on ubiquity of MRSA in community setting of Kathmandu valley has not been described, although studies on hospital setting has been recorded. This study showed the prevalence rate of CA-MRSA to be higher in Lalitpur districts (5.00%). Distribution pattern of CA-MRSA varies with geographical location, genotypic strains of species organism and type of sample population (Sollid et al 2014). None of the *S. aureus* isolates showed resistivity against at least one agents of any three antimicrobial class indicating absence of multi drug resistant *S. aureus* in the community. However, according to Magiorakos et al, 2011; in case of *S. aureus*, all MRSA isolates are defined as MDR because resistance to oxacillin or cefoxitin predicts non susceptible to all categories of beta- lactam with exception of anti-MRSA cephalosporins. This considers that all the MRSA strain to be MDR strains of *S. aureus*. This warrants an attention about threat and presence of MDR strains *S. aureus.* These findings do not signify the complete absence of CA-MRSA or MDRSA infection. Nevertheless, it widens the proficiency of the status on prevalence of *S. aureus,* CA-MRSA and MDRSA in community setting of Kathmandu Valley of Nepal.

CONCLUSION

The prevalence of MRSA strains of *S. aureus* in Kathmandu Valley was observed. Resistant to Cefoxitin predicts non susceptible to all categories of beta- lactam indicating development of Community acquired Multidrug resistant organism. This alarms the presence of increasing trends of antibiotic resistant *S. aureus.* Future spread and outbreak of strains can be prevented by routine surveillance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Seroprevalence of Brucellosis among Pigs of Commercial Farms in Chitwan District of Nepal

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ABSTRACT

Objectives: This study aimed to determine the seroprevalence of brucellosis among pigs of commercial farms in Chitwan district of Nepal.

Methods: This cross sectional prospective study was conducted among 100 pigs of commercial farms located in western region of Chitwan district of Nepal. Blood sample was collected from each pig by the trained veterinarians and serum was extracted. Each serum sample was processed for Rose Bengal plate test (RBPT) and ELISA for the detection of *Brucella* spp. Data was analyzed using SPSS software version 21.0 and a p-value of less than 0.05 was considered as significant.

Results: Prevalence of brucellosis in pigs was found to be 15% (15/100) by RBPT and 10% (10/100) by ELISA. There was statistically insignificant difference (p=0.98) in gender wise prevalence of brucellosis among pigs. Younger pigs below one year of age were more susceptible to brucellosis than the older pigs. Landrace breed showed more positive test results compared to other breeds.

Conclusion: Pigs can be the potential source of transmission of brucellosis to humans. Considering the high economic loss on livestock sector and possible transmission to humans, awareness program and appropriate control strategies is warranted. Breed and age factors should be considered while adopting the control measures of brucellosis among pigs.

Keywords: *Brucella,* pigs, commercial farms, Nepal

INTRODUCTION

Brucellosis is an infectious bacterial disease that primarily affects livestock and humans (Pappas et al., 2006). The etiological agent of brucellosis is a Gram negative bacterium of the genus *Brucella.* Of the nine recognized species of *Brucella*, three species namely *B. melitensis, B. abortus* and *B. suis* are of economic importance. Porcine brucellosis is an infection caused by biovar 1, 2 or 3 of *Brucella suis* (CDC 2021). The disease affects a wide range of animals including ruminants in which it is characterized by abortion (Corbel 1997). Brucellosis in pigs is also characterized by stillbirths or weak piglets. It occurs in many countries where pigs are raised. The mode of transmission among animals is through exposure of mucous membranes, inhalation of aerosols or direct contact with infected materials (Kolo et al. 2019). Humans contract brucellosis from animals through ingestion of contaminated, unboiled or unpasteurized milk and by direct contact with infected animals, animal carcasses and aborted materials (CDC 2021).

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Brucellosis occurs worldwide but is much controlled in developed countries due to routine screening of domestic animals and animal vaccination program. Though some European and Asian countries have been declared free of Brucella, it is still endemic in Asian countries, such as Sri Lanka , India , China, Pakistan , Mongolia and Nepal (Acharya 2016). Brucellosis in animals has already been reported from different districts of Nepal (Jackson et al. 2014). Most of the people are engaged in agriculture and livestock sector is the major contributor for livelihood in Nepal. Considering the animal and human health disorders, occupational risks, and the economic burden it imparts, knowledge on the status of brucellosis infection in animals and establishing the epidemiology could be valuable for farmers, veterinarians, researchers, consumers, disease prevention and control program planners and any others concerned with better animal and human health. The epidemiology of brucellosis varies markedly with region and over time. Most of the published studies from Nepal have focused on bovine brucellosis with sparse information on brucellosis among small ruminants. Hence, this study aimed to determine the seroprevalence of brucellosis among pigs of commercial farms in Chitwan district of Nepal.

METHODOLOGY

Study population

This cross-sectional prospective study was conducted among pigs of commercial farms located at western region of Chitwan district, Nepal. Altogether 100 pigs were selected randomly. Blood samples from age 1 month to 5 years were taken from different pig farms. The males and females of Landrace, Tamworth, Hampshire and Pakhribas black breeds of pigs were noted.

Sample collection and processing

Five ml of blood sample was collected from each pig from ear vein at the respective farms by the trained veterinarian. The samples were transported immediately to the Theriogenology laboratory of Agriculture and Forestry University (AFU), Rampur, Chitwan, Nepal. Serum sample was extracted from each blood sample by letting it to clot and centrifugation at 5000 rpm for 5-10 minutes (Cheesbrough 2009).

Rose Bengal plate test (RBPT)

Each serum sample was processed for RBPT following manufacturer's instruction (prioCheck Rose Bengal Test®). Briefly, 30µl of test serum was mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter. The mixture was agitated gently for four minutes at ambient temperature, and then observed for agglutination. Any visible reaction was considered to be positive.

ELISA

Each sample was also processed for ELISA following manufacturer's instruction (IDVet®) Briefly, 10 µl of each of the negative control, positive control and test serum were added to 190 µl of dilution buffer in the respective wells and incubated at 21°C (± 5°C) for 45 min (± 4 min). After washing with wash solution, 100 µl of the conjugate 1x was added to each well and incubated at 21°C (± 5°C) for 30 min ± 3 min. After washing, 100 µl of the substrate solution was added to each well and again incubated at 21°C (± 5°C) for 15 min ± 2 min in the dark. Then, 100 µl of the stop solution was added to each well and the absorbance was read at 450 nm. The test was considered to be valid if the mean value of the absorbance of positive control is greater than 0.350 and the ratio of the mean values of the positive and negative controls is greater than 3.

Data analysis

Data were analyzed using SPSS software version 21.0 and a p-value of less than 0.05 was considered significant for the statistical analysis of gender wise, age wise prevalence, breed wise prevalence, prevalence of brucellosis on the basis of vaccination.

RESULTS

Demographic characteristics of pigs tested for brucellosis

A total of 100 pig serum samples were collected from different commercial farms of western Chitwan, Nepal. Of the total samples, 67 were from females and 33 were from male pigs (Table 1).

About 63% of pigs were vaccinated with routine vaccines. Blood samples from age 1 month to 5 years were taken from different pig farms. Among total samples, 74% samples were collected from pigs below one year of age.

RBPT and ELISA results

Prevalence of brucellosis in pigs was found to be 15% (15/100) by RBPT and 10% (10/100) by indirect ELISA. Out of the 15 samples positive for RBPT, only 5 samples showed positivity also for ELISA. Remaining 5 samples were positive by ELISA but negative by RBPT. Correlation between the RBPT and ELISA of the same sample was 0.327 (Spearman's rho correlation coefficient).

Out of total samples, 14.92% (10/67) of females and 15.15% (5/33) of males were positive for *Brucella*. However, there was statistically insignificant difference (p=0.98) in gender wise prevalence of brucellosis in pig. Younger pigs below one year of age 26.08% (6/23) were more susceptible to brucellosis than the older pigs

11.68% (9/77). Landrace breed showed more positive test results compared to other breeds.

Table 1. Demographic characteristics of pigs tested for brucellosis

Breeds of pigs	Male (No.)	Female (No.)	Total (No.) tested
Landrace	20	40	60
Tamworth	6	10	16
Hampshire	5	15	20
Pakhribas black	2	2	4
Total	**33**	**67**	**100**

DISCUSSION

The seropositivity for brucellosis was 21.58% among 190 samples of pigs in Kathmandu valley which is higher than the present finding (Rana 2005). The discrepancy may be due to the use of different diagnostic techniques as the later study used. The seropositivity of 7.18% for brucellosis using the Brewer Diagnostic Card (BDC) was found in 153 samples of pigs from Itahari, Nepal (Shrestha, 2008). The present study result (15%) shows that it is quite convincing to a similar study by Shrestha et al. (2008). A much higher seroprevalence to *Brucella* has recently been found in France: overall, 31.6% of 2313 wild boars were positive between 1994 and 2000 (Garin-Bastuji et al. 2014). The overall seroprevalence rate found in the present study was lower as compared with the findings of 6.7% from Bangladesh (Islam et al. 2013). The variations in the seroprevalence might have been due to the disparity in geographical locations, climatic conditions and management practices in the different study areas. Other studies have also indicated that the rate of brucellosis infection varies among pig herds, from farm to farm or by country by origin of tested pigs (wild or domesticated) and by testing method used (Godfroid and Kasbohrer 2002). Furthermore, contact with other animals was also reported to be major risk factors that were influencing the occurrence brucellosis (Yang et al. 2021).

Among the total samples, females showed high prevalence 14.92% (10/67) than that of males 15.15% (5/33) with insignificant association (P< 0.005).This finding was in disagreement with a study done by Kebeta et al. (2015) showed that higher seroprevalence of brucellosis in female 8.2% than male 1.6% with significant association (P< 0.005) (Kebeta 2015).This finding was also in disagreement with the observation of Rahman et al. (2012) who found a high prevalence of brucellosis (7%) in female and 5.9% in male pigs in Bangladesh (Rahman 2012). Similar observation was also recorded by other investigator in wild boar (*Susscrofa*) from Republic of Croatia (Cvetnic et al. 2009). The higher rate of infection in females might be due to infection within the female reproductive tract providing a potential reservoir for the organism to propagate. In the study of Shrestha et al. (2008), samples from female showed high prevalence 9.23% (6/65) than that of males 5.7% (5/88) which is less than the present study of which samples of the ages below 1 year showed 26.08% (6/23) and ages above 1 year showed 11.68% (9/77) positive reaction for RBPT.

With regard to the age of the animal higher seroprevalence was observed in young (< 12months) (5.9%) as compared to adult (≥ 12months) pigs (3.6%) by Kebetaet al. (2015). This result was in disagreement with the findings of Rahman et al. (2012) who found higher prevalence of brucellosis in aged animal (8.1%) than young (0.0%). Previous study by Leite et al. (2014) identified that young age of the animals as risk factor that was influencing the occurrence of brucellosis (Leite 2014).

The *B. suis* are usually transmitted between animals by contact with the placenta, fetus, fetal fluids, aerosol route, ingestion of contaminated raw meat through mucus membrane, broken skin and vaginal discharges from an infected animal (Ngbede 2013). Brucellosis is a major public and animal health problem in areas like Nepal with intensive mixed types of farming and where owners cohabit with their animals during night (Acharya 2016).

Studies have shown that inclusion of different tests could increase the detection rate of infectious disease other than brucellosis as well (Sharma 2021). The major limitations of our study lies in the fact that our detection technique was based on serological methods. Hence, the seroprevalence might have been underestimated. Inclusion of molecular methods like PCR might increase

the detection rate.

CONCLUSION

Pigs can be the potential source transmission of brucellosis to humans. Considering the high economic loss on livestock sector and possible transmission to humans, awareness program and appropriate control strategies is warranted. Breed and age factors should be considered while adopting the control measures of brucellosis among pigs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antibiogram and Biofilm Development among *Klebsiella pneumoniae* from Clinical Isolates

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ABSTRACT

Objectives: This study was aimed to evaluate antibiotic resistance pattern and biofilm formation in *K. pneumoniae* strains isolated from different clinical specimens and to study on association of drug resistance pattern with biofilm formation.

Methods: A total of 944 clinical samples from patients attending Sahid Gangalal National Heart Center were processed from September 2019 to March 2020 to identify possible bacterial pathogens following standard microbiological procedures. *K. pneumonaie* isolates were further subjected to antibiotic susceptibility testing using modified Kirby Bauer disc diffusion technique. Biofilm formation was evaluated by tissue culture plate technique.

Results: Of the total 944 samples, 15.47% (146) samples showed bacterial growth, among which 23.97% (35) were *K. pneumoniae*. Out of 35 *K. pneumoniae* isolates, 45.71% (16) were multidrug-resistant and 42.86% (15) were extensively drug-resistant. Sixty percent (21) of *K. pneumoniae* feebly produced biofilm. Significant association was observed between biofilm production and exhibition of multidrug resistance (p value<0.05).

Conclusion: Prevalence of antibiotics resistant *K. pneumoniae*in hospital setting is high and alarming. Significant association between drug resistance pattern and biofilm production implicates need of an immediate response to limit growth and spread of drug resistant microbes in clinical settings.

Keywords: *Kleibsella pneumoniae*, multidrug resistance, biofilm, antibiotic susceptibility test, Nepal

INTRODUCTION

Pregnant women are one of the most vulnerable groupsAntimicrobials are substances or drugs such as antibacterial, antivirals, antifungals and antiparasitics used to prevent or treat infections caused by microorganisms. Antimicrobial resistance (AMR) occurs when microorganism undergo the alteration in their genetic constitution and resist the antimicrobial agents making the treatments ineffective (Adegbite et al., 2022). AMR has become a matter of global concern. All the countries across globe have been affected by antimicrobial resistance but the burden is higher in low-income and middle-income countries. Every year 7,00,000 people lose their life because of antimicrobial resistance and it is estimated that if no any prompt actions are taken against it, by 2050, AMR will cause loss of life of 10 million peoples and $US100 trillion (Pokharel et al., 2019). WHO has declared AMR as one of the top 10 global public health threats to humanity (WHO 2021).

AMR problem is more frequent in a developing country like Nepal where indiscriminate, inadequate and inappropriate use of antimicrobials and self-medication are quite common. Studies have reported high burden of drug resistant/ bacteria in Nepal (Gurung et al., 2020; Raut et al., 2020).

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Klebsiella pneumonia is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium.It is an important nosocomial pathogen involved in urinary tract infections, hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), surgical-wound infection, bacteremia, and septicemia (Li et al., 2014).The most important virulence factors contributing to ***K. pneumoniae*** pathogenesis are capsular polysaccharides, type 1 and type 3 pili, which can contribute to biofilm formation (Lie et al., 2014: Murphy and Clegg 2012 and Podschun and Ullmann 1998).These bacteria can produce a thick layer of extracellular biofilm as a virulence factor that helps the organism in attaching to living or abiotic surfaces, preventing the effects of antimicrobial agents (Vutto et al., 2019). The first biofilm-forming ***K. pneumoniae*** strain was described at the end of the 1988s (Le Chevallier et al., 1998). WHO has listed *K.pneumoniae* in critical category, which includes multidrug resistant bacteria that pose a particular threat in hospitals, nursing homes, and among patients, whose care requires devices such as ventilators and blood catheters (WHO 2017).

METHODS

Study type, site and sample population

This prospective cross sectional study was conducted from September 2019 to March 2020. All the samples were received and processed in Microbiology Department of Sahid Gangalal National Heart Center, (SGNHC), Bansbari, Kathmandu while the biofilm detection was done at Med-Micro Research Laboratory, (MMRL), Babarmahal, Kathmandu. Patient who provided their socio-demographic information and expressed their interest to be a part of study by providing samples were included in the study. Only properly collected and well labeled samples were included in the study. A total of 944 samples were collected and processed for the study.

Isolation and identification:

The collected specimens were cultured simultaneously on Mac Conkey agar (MA), Blood agar (BA) and Michrom UTI agar by quadrant streaking method and incubating them at 37°C for 24 hours as per standard protocol (Cheesebrough 2006). The identification of growth of bacterial colonies from respective media was carried out on the basis of conventional microbiological procedures which includes colony characterstics, gram staining, standard biochemical tests. *K. pneumoniae* isolates obtained from the clinical specimens were considered for further study.

Antibiotic susceptibility test

Antibiotic susceptibility test (AST) was performed in-vitro using modified Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) as per CLSI guidelines (2019). Following antibiotic discs (Hi Media Laboratories, Pvt. Limited, India) were used: Ampicillin (AMP, 10 mcg), Piperacillin/tazobactum (PIT, 100/10 mcg), Ampicillin/sulbactum (AMS, 10/10 mcg), Ciprofloxacin (CIP, 5 mcg) Norfloxacin (NX, 10mcg), Cefalexin (CN, 30 mcg), Gentamycin (GEN, 10 mcg), Amikacin (AK, 30 mcg), Imipenem (IMP, 10mcg) ,Meropenem (10 mcg), Amoxycillin/clavulanic acid (AMC, 20/10 mcg), Cefotaxim (CTX, 30mcg), Cefepime (CPM, 30mcg), Nalixidic acid (NA, 30mcg), Nitrofurantoin (300 mcg), Cotrimoxazole (COT, 25mcg), Ceftazidime (CTZ, 30 mcg), Ceftriaxone (CTR, 30 mcg), Polymyxin-B (PB, 300 units) and Colistin (CL 10mcg). Results were interpreted on the basis of CLSI guidelines (2019).

MDR and XDR classification

Isolates showing non-susceptibility (either resistant or intermediate) to at least one agent in three or more antimicrobial categories were identified as multi drug resistant(MDR) and those isolates which acquired non-susceptibility to ≥3 classes of antibiotics of antimicrobial class were identified as XDR (Magiorakos et al. 2012). Confirmed *K. pneumoniae* isolates were preserved by using 20% glycerol in TSB for further analysis.

Detection of Biofilm formation

Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth (TSB) with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh TSB medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times.

Purity plate

The purity plate was used to make sure that the inoculums used for biochemical tests was pure culture or not. Thus, while performing biochemical tests; the same inoculums were sub-cultured on one half of NA before inoculation (pre purity) and other half ot that NA after inoculation (post purity). The maintenance of aseptic condition is indicated by the growth of same organism in pure form in both pre and post purity halves of the NA medium.

Data analysis

Data analysis was done using computer based software program Statistical Package for the Social Package version 21.0 and the p-value was calculated using Chi-square test.

Ethical approval

The study was reviewed and approved by Ethical Review Board (ERB), Nepal Health Research Council (NHRC) on 17th January 2020 (Reg No: 834/2019).

RESULTS

All-together 944 non-duplicative samples were processed, in which bacterial growth was observed only in 146(16%) samples, while 798 (84%) were culture negative. Out of 146 isolates, *K pneumoniae*were predominant isolates with highest frequency 35 (23.97%) followed by *E coli* 33 (22.60%), *Staphylococcus aureus* 28 (19.17%), *Acinetobacter*spp 19 (13.01%), *CONS* 10 (6.8%), *Serratia*spp 7 (4.7%), *Pseudomonas aeruginosa* 6 (4.1%), *Citrobacterfreundii* 2 (1.36%), *Proteus mirabilis* 2 (1.36%), *Streptococcus pneumonia* 2 (1.36%), *Providencia* spp 1 (0.68%), *Enterobacter*spp 1 (0.68%) (Table 1).

Out of 35 *K. pneumoniae* isolates maximum number of isolates were isolated from urine samples 18 (51.43%), followed by sputum 7 (20%), pus/wound 4 (11.43%), CVP tips 3 (8.57%), blood 2 (5.71%), pleural fluid 1 (2.86%) (Figure 1).

Antibiotic susceptibility testing revealed that all the isolates i.e. n=35 were found to be sensitive to colistin (100%). Other antibiotics to which majority of bacterial isolates were sensitive to polymyxin B 63 (89%) followed by meropenem 21 (60%).All these isolates were resistant to ampicillin (100%). Besides this, only 9% of isolates were found to be sensitive towards ciprofloxacin, cephalexin (Table 2).

Out of 35(100%) isolates, 15 (43%) were found to be XDR, 16(46%) were found to be MDR, and 4 (11%) were found to be susceptible isolates. Detection of biofilm was carried out by Tissue culture method, in which 60% (n=21) *K. pneumoniae* were found to be weak biofilm producers and 40% (n=14) were found to be non-biofilm producers (Figure 2).

Analyzing antibiotic resistance pattern of all 35 isolates of *K. pneumoniae* 15(43%) of them were found to be XDR, 16(46) of them were MDR and 4(11%) were susceptible isolates against antibiotics. Statistical association (chi square) calculated between MDR and Biofilm producers was also found significant (p=0.000) (Table 3).

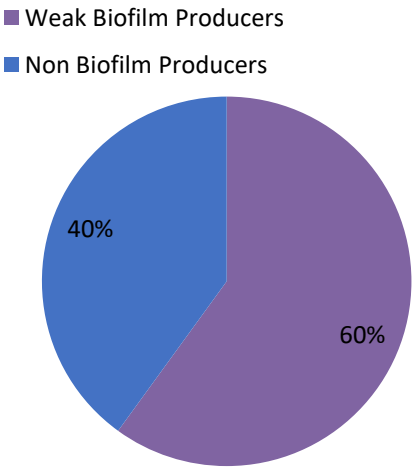
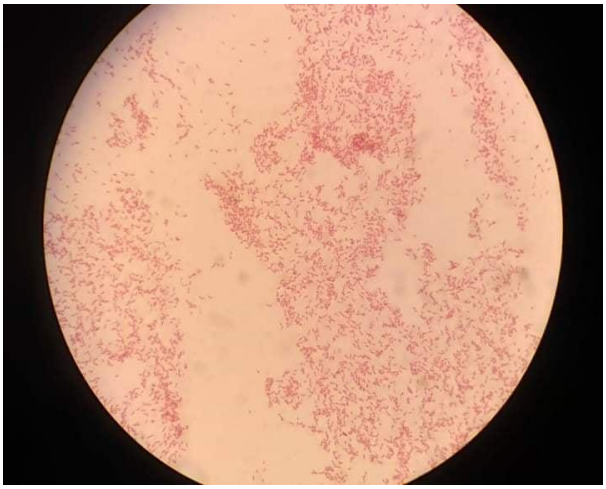


Figure 2: Biofilm Prodution among *K. pneumoniae* isolates



Photograph 1: Gram staining for identification of Gram negative bacilli under 100x magnification

Table 1: Distribution of organisms among different clinical sample

Organism	Number of Samples						
	Blood	**Urine**	**Sputum**	**Pus**	***Body fluids**	****Others**	**Total**
Klebsiella pneumoniae	2	18	7	4	1	3	35 (23.97%)
Escherichia coli	-	31	1	-	-	1	33 (22.60%)
Staphylococcus aureus	7	10	1	6	1	3	28 (19.17%)
*Acinetobacter*spp	5	2	3	3	-	6	19 (13.01%)
*CONS***	3	1	2	3	-	1	10 (6.8%)
*Serratia*spp	-	-	-	6	-	1	7 (4.7%)
Pseudomonas aeruginosa	2	1	2	1	-	-	6 (4.1%)
Proteus mirabilis	-	2	-	-	-	-	2 (1.36%)
Citrobacter freundii	-	1	-	1	-	-	2 (1.36%)
Streptococcus pneumoniae	1	-	1	-	-	-	2 (1.36%)
*Enterobacter*spp	-	-	-	1	-	-	1 (0.68%)
*Providencia*spp	-	1	-	-	-	-	1 (0.68%)
Total	**20**	**67**	**17**	**25**	**2**	**15**	**146**

*Body fluids: Pericardial fluid, Pleural fluid

**Others: Endotracheal tube tips Mitral valve tissue, Aortic valve tissue, Foleys tip, CVP tip

***CONS: Coagulase negative *Staphylococci*

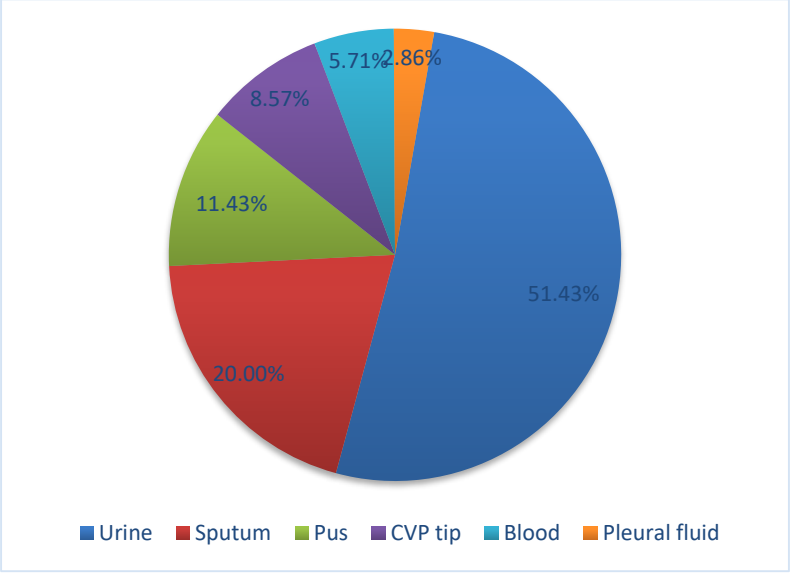


Figure 1: Frequency of *K. pneumoniae* among clinical sample

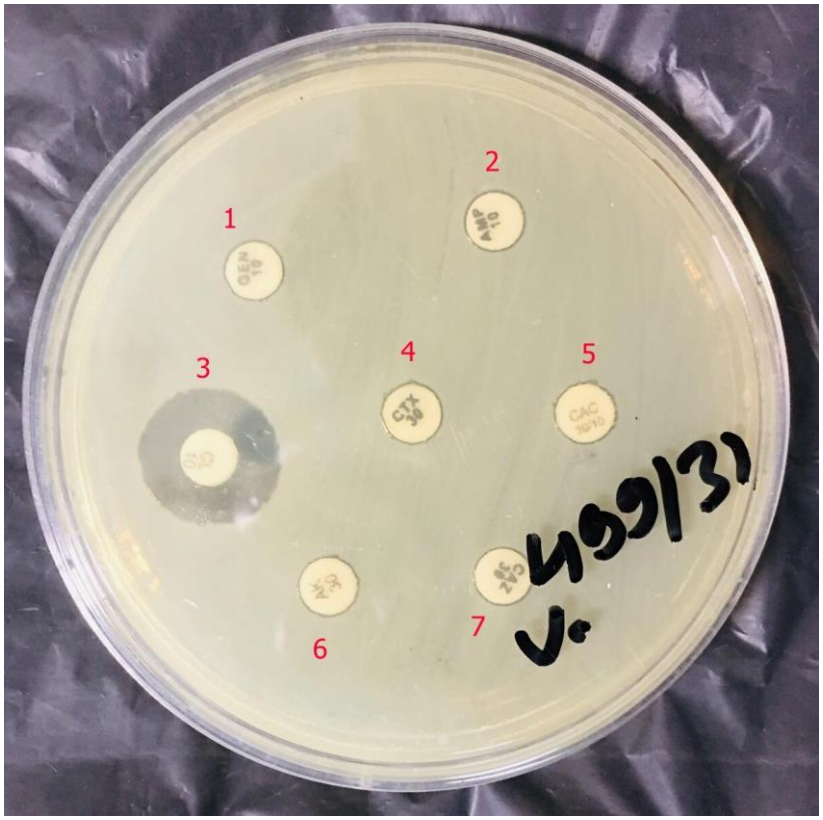
Table 2: Antibiotic Susceptibility Pattern of *K. pneumoniae*

S.N.	Categories	Antibiotics	Frequency of *K. peumoniae* isolates	
			Sensitive No. (%)	Resistance No. (%)
A	Aminoglycosides	Amikacin	14 (40.0)	21 (60.0)
		Gentamicin	13 (37.0)	22 (63.0)
B	Antipseudomonal penicillin+ ß-lactamase inhibitors	Piperacillin/tazobactam	12 (34.0)	23 (66.0)
C	Carbapenems	Meropenem	21 (60.0)	14 (40.0)
		Imipenem	18 (51.0)	17 (49.0)
D	Extended spectrum cephalosporins 3rd and 4th generation cephalosporins	Ceftriaxone	10 (29.0)	25 (71.0)
		Ceftazidime	8 (23.0)	27 (77.0)
		Cefepime	7(20.0)	28 (80.0)
		Cefotaxime	6 (17.0)	29 (83.0)
E	Fluroquinolones	Ciprofloxacin	3 (9.0)	32 (91.0)
F	Folate pathway inhibitor	Cotrimoxazole	17 (49.0)	18 (51.0)
G	Penicillin	Ampicillin	0 (0.0)	35 (100.0)
H	Penicillin +ß-lactamase inhibitors	Amoxycillin/clavulanic acid	11 (31.0)	24 (69.0)
		Ampicillin/sulbactam	10 (29.0)	25 (71.0)
I	Polymyxin	Polymyxin B	31(89.0)	4 (11.0)
		Colistin	100 (100.0)	0 (0.0)

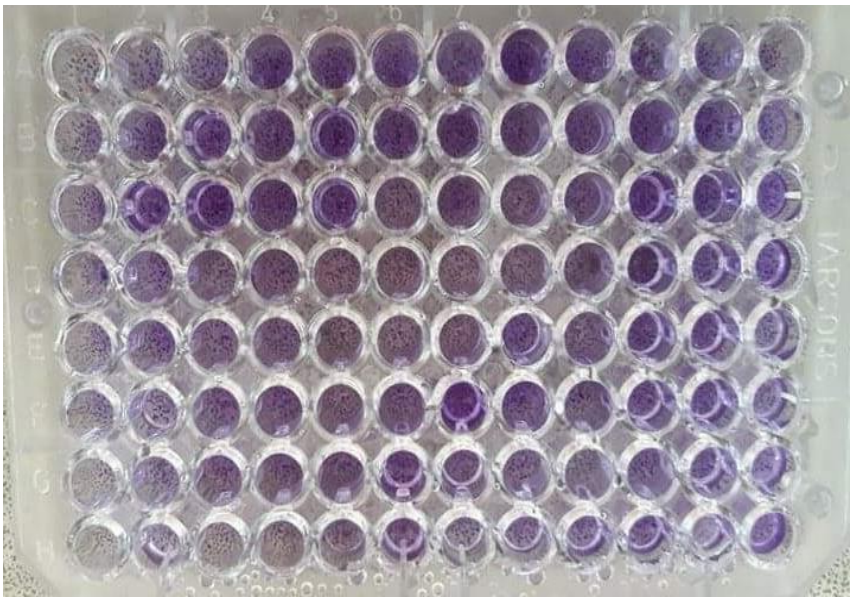
Table 3: Distribution of biofilm among multi drug resistance *K. pneumoniae*

Variable	Total	Weak biofilm	Non biofilm	P-value
Susceptible strain	4(11%)	1(4.8%)	3(21.4%)	<0.05
MDR	16(46%)	5(23.8%)	11(78.6%)	
XDR	15(43%)	15(71.4%)	0	
Total	**35 (100%)**	**21 (100%)**	**14 (100%)**	

Photograph 2: Biochemical test for confirmation of *K. pneumoniae* (From left to right) (Fermentative, Motility –ve, Indole –ve, MR –ve, VP +ve, Citrate +ve, TSI A/A $^{gas+/H2S\text{-}ve}$, Urease +ve)



Photograph 3: Antibiotic susceptibility test for *K. pneumoniae* (1: GEN=R, **2:** AMP=R, **3:** CL=S, **4:** CTX=R, **5:** CAC=R, **6:** AK=R, **7:** CAZ=R) *S= Sensitive and **R= Resistant



Photograph 4: **Biofilm detection by Microtitre plate method.**

DISCUSSION

Even though *K. pneumonia* can be frequently found in the mouth, on the skin, and in the intestine of an individual, it can be detrimental on immunocompromised patients causing urinary tract infections, respiratory tract infections, and septicemia. Multidrug Resistant *K. pneumoniae* leaves the therapeutic options limited than the susceptible one. The prevalence of multidrug resistance has risen remarkably since the introduction and unrestricted use of new generation antibiotics. High pathogenic capacity, ability to produce extended spectrum-lactamase (ESBLs), carbapenemase and biofilms enables this organism to develop antibiotic resistance faster than other bacteria (Munita JM and Arias CA 2016). This study was performed to detect the biofilm formation *K. pneumoniae* isolates and their relation to the antibiotic resistance.

Of the total 944 clinical samples analyzed, 16% (146) showed growth on different culture media. This finding is in harmony with the finding of Parajuli et al., (2017), Adhikari et al., (2018) and Shrestha et al., (2019) who reported similar bacterial growth of 19.68%, 17.79%, 16% respectively during their study. Out of 146 isolated organisms, *K. pneumoniae* were predominant isolates with the number of 35 (23.97%) followed by the *E. coli* 33 (23.60%) which is in concordance with the study conducted in the Department of Microbiology, B.P. Koirala Institute of Health Sciences (BPKIHS), Dharan, Nepal by Shrestha et al., (2019). High percentage of *K. pneumoniae* was isolated from urine 51%, followed by sputum (20%), pus (11%), CVP tip (8%), blood (6), pleural fluid (3%). These finding identify *K. pneumoniae*as an important cause of hospital acquired urinary tract infection. Beirao et al., (2011) and Singh et al., (2015) also reported that during his study, highest number of *K pneumoniae* were obtained from urine. Central venous catheter is frequently used in ICU settings however, their colonization with different types of organism increases the hospital stay and mortality in these patients. It is usually done in an emergency procedure patient with prolonged hospital stays, critical conditions, especially those on ventilators and patients with multiple invasive devices are most likely to have a greater risk of infections. In a study conducted by Sapkota et al., (2017), they found prevalence of isolation rate of *K. pneumoniae*in CVC tip was 3 %. Colonization of *K. pneumoniae* on CVP tip occurs at the time of catheter insertion at this time skin commensal as well as iatrogenic organisms have chances to colonize in the insertion-site later these organisms reached to the deep portion of skin and colonized in catheter which results in blood stream infection. It has been stated that central venous catheterization longer than five to seven days was associated with a higher risk of catheter-related infection (Moro et al., 1994).

Antibiotic susceptibility test of all the *K. pneumoniae* isolates were performed against 16 different antibiotics of the different chemical classes. In terms of sensitivity most of the bacterial strains were found to be sensitive against colistin (100.0%), followed by polymyxin B (89%). The bacterial isolates exhibited 100% resistant towards ampicillin which is in harmony to the finding of Adhikari et al (2018); Beyene et al (2019): Livermore and Woodford (2006). Aminoglycosides usually have better activity against clinically important Gram-negative bacteria, but in this study, we observed that the isolates exhibit higher resistance rates of 63% to gentamycin and 60% to amikacin respectively. This result resembles to the finding of Khanal et al., (2013) who reported similar resistance rate of 69% and 54% exhibited by *K. pneumoniae* against gentamycin and amikacin respectively. Carbapenems are an important class of β- lactams antibiotic, this study showed higher percentage of resistance towards imipenem 49% followed by meropenem 40%. Meropenem was reported more effective than imipenem in this study which differ with earlier studies conducted by Piller et al., (2008); Mishra et al (2012); Shrestha et al., (2014). This might be due to the reason that meropenem are more active against Gram negative bacilli, while imipenem are more active against Gram positive cocci. Hence, the activity of carbapenem depends on types and species of organism as well as meropenem is a new drug in comparison to imipenem (Zhanel et al., 1998). The increasing trend of carbapenem resistance recorded in different studies of Nepal might be due to overuses/inappropriate uses of carbapenem antibiotics or might be due to prevalence of ESBL producers, which causes extensive use of carbapenem antibiotics as an empirical option (Hawkey and Jones 2009). Biofilm production test in this study was conducted by Tissue culture plate assay. Since, tissue culture method is the quantitative and termed as gold standard method (Christensen et al., 1985). Using the method 60% of total isolates were found to be biofilm producers. It has been reported that more than 50% of total human infection are associated with biofilm production (Costerton et al., 1987). Biofilm producing strains showed relatively high drug resistance against all antibiotics tested as compared to non-biofilm producing strains. In the present study, strong correlation was noted between biofilm production and resistance to multiple antibiotics at significance level 0.05, where almost biofilm producing strains were MDR phenotypes. A higher proportion of antibiotic resistance in biofilm producers in comparison to non-producers has been documented in many studies (Dumaru et al. 2019; Shrestha et al. 2017). This was in accordance with study carried out by Stewart (2002).

CONCLUSION

Irrational use of antibiotics has resulted in dramatic increase of multi drug resistant K. pneumonia. It has become a matter of challenges to today's world. Periodic study on status of antimicrobial resistance is crucial to assess its spread. Different innate factor or mutational changes in organism can make it adaptive against antimicrobial agents. In this study, we aim to explore whether biofilm produced by organism plays crucial role in impairing resistance against antimicrobials.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Determination of Inhibitory Effects of *Allium sativum* Extract on Biofilm Production by Clinical *Staphylococcus aureus* Isolates

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ABSTRACT

Objectives: To determine anti-biofilm effect of fresh garlic extract (FGE) on *Staphylococcus aureus* biofilm production and the relationship between methicillin resistance and biofilm production intensity.

Methods: Clinical *S. aureus* isolates were identified methicillin resistant *S. aureus* (MRSA) by cefoxitin disc diffusion method. The anti-biofilm effects of FGE on *S. aureus* biofilm biomass determination was done using crystal violet assay.

Results: Among 71 *S. aureus* isolates, MRSA were 37 (52.1%). Among biofilm producers, comparison of biofilm biomass (absorbance at 492 nm) showed no significant differences in biofilm formation ability between MRSA and MSSA ($p=0.325$). Use of 10% FGE decreased biofilm production in MRSA and MSSA by 40.4% ($p<0.001$) and 48.1% ($p<0.001$) respectively. Detachment assay using sodium dodecyl sulfate showed that control group biofilm biomass was decreased by 41.2%, while test group was decreased by 61.7% ($p<0.001$).

Conclusion: Garlic extracts has potency as an anti-biofilm agent and could be developed and used to manage different *S. aureus* biofilm related infections.

Key words: Fresh garlic extract, biofilms, MRSA, *Staphylococcus aureus,* Nepal

INTRODUCTION

Staphylcoccus aureus, is a pathogen with its natural reservoir in humans**.** This pathogen causes skin, wound and burn infections, septicaemia and endocarditis (Tong et al 2015)**.** Between 63-65% of hospital acquired infections (HAI) are due to antibiotics resistant bacteria (Cassini et al 2018), antibiotics resistant strains of *S. aureus* is one of the major causative agents (Wu et al 2021). Vancomycin resistant *S. aureus* (VRSA) has been reported from various parts of the world (Rossi et al 2014; Hasan et al 2016; Azhar et al 2017; Shekarabi et al 2017; ElSayed et al 2018; Wu et al 2021).

Unlike in nutrient rich conditions of laboratory where bacteria grow planktonically, bacteria growing naturally form complex aggregated structures called biofilms (Costerton et al 2005). The ability to form a biofilm, gives the pathogen's ability to produce chronic diseases such as chronic osteomyelitis, chronic cystitis, chronic prostatitis, chronic otitis media, chronic pneumonia in patients with cystic fibrosis and dental plaques (Lebeaux et al 2013). In addition, biofilm producing microorganisms also cause infections of various organs by producing biofilms on implanted biomedical devices (Mack et al 2004). Various microbial pathogens growing as biofilms are resistant to most antimicrobial agents, whereas same pathogens growing planktonically are sensitive to virtually all antibiotics tested (Olson et al 2002).

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Usually, the compounds that kill or inhibit bacterial growth are used routinely to reduce production of biofilm, but application of these compounds at sub-inhibitory levels may stimulate biofilm formation (Nucleo et al 2009). Because of these reasons, compounds that inhibit biofilm formation without affecting bacterial growth are getting attentions.

Garlic is being used as therapeutic and prophylactic agent for a long time. The principal organosulphur compound in intact garlic cloves is alliin (+ S-allyl-L-cysteine sulfoxide). Upon chopping or crushing garlic, allinase enzymes activates and converts alliin to form 2-propenesulfenic acid, which self-condenses to form allicin (diallyl thiosulfinate). Allicin is only present in fresh, raw garlic and raw garlic preparation contains about 3.1 mg/g of allicin (Block 1992). Allicin readily diffuses across both artificial and natural phospholipid membranes (Miron et al 2000). The antimicrobial effects of allicin is due to interaction with thiol- containing enzymes and at slightly higher concentrations other enzymes, such as dehydrogenases or thioredoxin reductases, might be affected which could be lethal to microorganisms (Ankri and Mirelman 1999).

Garlic extracts showed excellent antibacterial activity against wound pathogens such as *S. aureus* and *S. epidermidis* (Nidadavolu et al 2012). Likewise, application of fresh garlic ointment lead to more organized and rapid wound healing due to activation of fibroblasts by allicin (Alhashim and Lombardo 2018). Ratthawongjirakul and Thongkerd (2016) reported significant reduction in biofilm formation of *S. aureus* under chemopreventive and chemotherapeutic conditions.

Natural products are used as an alternative medicine for treatment of various diseases because of less side effects, inexpensiveness and better patient tolerance. In this study we determined the biofilm production intensity of *S. aureus* isolates, identified the inhibitory effect of fresh garlic extract (FGE) on *S. aureus* biofilm production and assessed the effects of FGE on biofilm detachment of *S. aureus*.

METHODS

Research design

This was a cross-sectional quantitative study and primary data were collected from May to December 2018. Garlic bulbs were collected and tested against biofilm of *S. aureus*.

Collection of garlic bulbs and test bacteria

The garlic samples were collected from the local market of Lalitpur and disinfected at the laboratory. Seventy-one *S. aureus* isolates from the clinical samples collected during May to August 2018 were kindly provided by Clinical Microbiology Laboratory of the Alka hospital, Lalitpur. Amies transport medium was used for bacterial isolates transportation and stored at -20°C for further processing.

Preparation of garlic extract

A 100 gram of the bulb of garlic was squeezed using mortar and pestle. The squeezed sample was sucked at 200ml distilled water for overnight with shaking at 30°C. Then the extractions were filtered through muslin cloth and through Whatman no.1 filter paper. The aqueous extract was kept in sterile bottle in refrigerator at -20°C until use (Suleria et al 2012).

Re-confirmation and characterization of clinical isolates of *S. aureus*

S. aureus isolates first streaked on blood agar (BA) and incubated at 37°C for 24 hours. Round, raised, opaque and β- hemolytic colonies of size 1-2 mm growing on BA was grown on nutrient broth (NB) for about 3 hours at 37°C. Then organisms from NB were streaked on freshly prepared mannitol salt agar (MSA) and incubated at 37°C for 24 hours. Primary characterization of isolates was done on the basis of fermentation of mannitol, catalase, oxidase, coagulase (slide and tube) and DNase tests.

Phenotypic detection of MRSA

Identified *S. aureus* isolates were subjected to modified Kirby-Bauer's disc diffusion test as recommended by CLSI guidelines (CLSI 2014). The cefoxitin (30 mcg) disc (Hi-media) was used to detect MRSA. The inoculums were prepared by transferring 2-3 identical colonies from nutrient agar to sterile normal saline. The turbidity of the inoculums were made equivalent to 0.5 McFarland standard. The lawn culture of the test inoculums was prepared by swabbing MHA with a sterile cotton swab dipped into inoculums. Cefoxitin (30 mcg) disc was applied to the inoculated MHA plate and incubated at 35ºC for 18 hours. After incubation, the zone of inhibition of ≤21 mm around the disc was identified as MRSA. The MRSA COL strain was used as positive control and *S. aureus* ATCC 25923 as negative control.

Screening of biofilm producing *S. aureus* strains

Tube method, a qualitative method was applied for biofilm detection. A loopful of test organisms were inoculated with 2 ml of tryptone soya broth (TSB) with 1% glucose in test tubes. The tubes were incubated at 37°C for 48 hours. After incubation, tubes were decanted and washed with phosphate buffered saline (pH 7.4) and air dried (for 30 minutes) in inverted position. Tubes were stained with

crystal violet (1%) and washed to remove excess stain with distilled water. Tubes were dried in inverted position for 18-24 hours. Biofilm formation was considered positive when a visible thick film lined the bottom of the tube.

Static biofilm formation assay and determination of inhibitory effects of fresh garlic extract on biofilm formation of *S. aureus*

S. aureus was grown on TSB for overnight at 37°C. The culture was diluted (1:20) with fresh TSB. The diluted cultures (150 µl) without garlic extract were aliquoted into 96-well microtiter plate as controls. The diluted cultures (150 µl) with FGE (10%) were aliquoted into 96-well microtiter plates as tests. Along with controls and tests, un-inoculated and FGE free TSB and un-inoculated and FGE-supplemented TSB was applied on 96 microtiter plates adjacently. Three replicate wells for each treatment were performed. The plate's surface was sealed by applying paraffin tape and incubated at 37°C for 24 hours. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with phosphate buffered saline (PBS) (pH=7.4). The plates were subsequently dried at 60°C for 30 minutes. The adherent biofilms in each well were stained with 175 µl of a 0.1% (w/v) solution of crystal violet in water at room temperature for 15 minutes. The plates were rinsed three times with water by submerging in a tub of water and tapping vigorously on a paper towel to completely remove all excess cells and dye. The plates were dried overnight at room temperature. Approximately 175 µl of ethanol (99.9%) was added to each well to solubilize the crystal violet. Then the absorbance at 492 nm was measured using an ELISA plate reader (O'Neill et al 2007).

The mean OD_{492} values for the control and tested wells were subtracted from the mean OD_{492} values obtained from the un-inoculated FGE-free and un- inoculated FGE-supplemented wells, respectively to calculate biofilm inhibitory effect of FGE.

Determination of effect of fresh garlic extract on biofilm detachment of *S. aureus*

S. aureus was grown on TSB overnight at 37°C. Culture was diluted (1:20) with fresh TSB. The diluted cultures (150 µl) without garlic extract were aliquoted into 96-well microtiter plate as controls. The diluted cultures (150 µl) with FGE (10%) were aliquoted into 96-well microtiter plates as tests. Along with controls and tests, un-inoculated-Sodium dodecyl sulfate (SDS) (5 µl) present-FGE free TSB and un-inoculated-SDS (5 µl) present-FGE supplemented TSB was applied on 96 microtiter plates adjacently. Three replicate wells for each treatment were performed. The plate's surface was sealed by applying paraffin tape and incubated at 37°C for 24 hours. Then 5 µl of 10% SDS was added to each well, and the mixture was incubated for 30 minutes. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with phosphate buffered saline (PBS) (pH=7.4). The plates were subsequently dried at 60°C for 30 minutes. The adherent biofilms in each well were stained with 175 µl of a 0.1% (w/v) solution of crystal violet in water at room temperature for 15 minutes. The plates were rinsed three times with water by submerging in a tub of water and tapping vigorously on a paper towel to completely remove all excess cells and dye. The plates were dried at room temperature overnight. Approximately 175 µl of ethanol (99.9%) was added to each well to solubilize the crystal violet. Absorbance at 492 nm was measured using an ELISA plate reader (O' Neill et al 2007).

The mean OD_{492} values of the control and tested wells were subtracted from the mean OD_{492} values of un-inoculated-SDS (5 µl) present-FGE free TSB and un-inoculated-SDS (5 µl) present-FGE supplemented TSB wells, respectively to calculate biofilm detachment capacity of FGE.

Statistical analysis

The data were analyzed using SPSS software version 25.0. The biofilm absorbance data were expressed as a mean± standard deviation (S.D.). In order to determine p-value for association, Chi-square test and Mann-Whitney U-test were used.

RESULTS

Rate of MRSA and MSSA and biofilm production

Of 71 *S. aureus* isolates, 37 (52.1%) were MRSA and 34 (47.8%) were MSSA. Out of 71 *S. aureus* strains, 87.3% (n=62) were biofilm producers and 12.6% (n=9) were biofilm non-producers.

Among 37 MRSA strains examined, 59.4% (n=22) were moderate biofilm producers. The weak biofilms were produced by 32.4% (n=12) of MRSA strains, while 8.1% (n=3) of strains were strong biofilm producers. Similarly, out of 34 MSSA strains, 41.1% (n=14) were moderate biofilm producers, followed by 29.4% (n=10) weak biofilm producers, 26.4% (n=9) no biofilm producers and 2.9% (n=1) strong biofilm producers (Table 1).

The average absorbance of biofilm biomass of MRSA and MSSA groups were 0.4329±0.2566 and 0.3696±0.1925, respectively (Figure 1). Comparison of average absorbance of biofilm biomass showed that there was no statistically

significant difference in biofilm forming ability between MRSA and MSSA strains (p=0.325).

Effect of fresh garlic extract on biofilm formation of *S. aureus* isolates

The average absorbance of biofilm biomass of FGE-untreated and FGE-treated groups were 0.4074±0.2333 and 0.2315±0.1428, respectively (Figure 2). The average absorbance of biofilm biomass in FGE treated isolates were significantly lower than those in the control FGE untreated group (p<0.001). The biofilm formation for the experimental group (i.e. cultures containing FGE) was 43.1% less than the amount of biofilm formation in the control group (Figure 2).

The average absorbance of biofilm biomass of FGE-untreated MRSA and FGE-treated MRSA groups were 0.4329±0.2566 and 0.2581±0.1571, respectively. The biofilm biomass in FGE-treated MRSA isolates were significantly lower than those in the control FGE-untreated MRSA group (p=0.001). Similarly, the average absorbance of biofilm biomass of FGE-untreated MSSA and FGE-treated MSSA group were 0.3696±0.1925 and 0.1920±0.1100, respectively. The biofilm biomass in FGE-treated MSSA isolates were significantly lower than those in the FGE-untreated MSSA group (p<0.001). The biofilm biomass was decreased by 40.3% and 48.1% in MRSA and MSSA test groups respectively (Figure 3).

Effect of fresh garlic extract on biofilm detachment of *S. aureus* isolates

The average absorbance of biofilm biomass of the control SDS treated-FGE untreated group was 0.2394±0.1482, while that of SDS treated-FGE treated group was 0.1559±0.1198, with statistically significant difference (p<0.001). The biofilm biomass in SDS-FGE-untreated groups were decreased by 41.2% and those in SDS-FGE-treated groups were decreased by 61.7% (Figure 4).

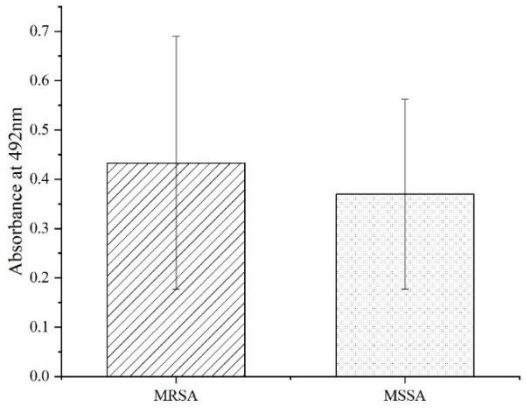

Figure 1: Comparison of average absorbance of biofilm formation by MRSA and MSSA

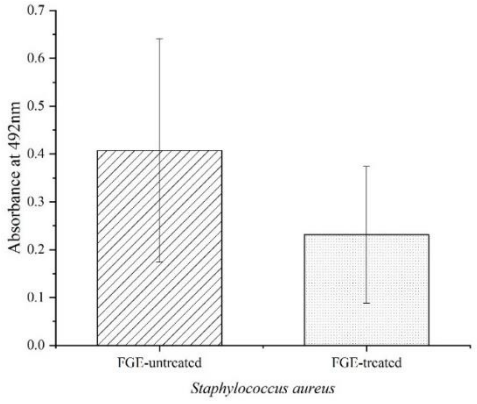

Figure 2: Comparison of average absorbance of values of biofilm biomass of FGE-untreated isolates and FGE-treated isolates

Table 1: Biofilm production among MRSA and MSSA

S. aureus Phenotypes	Strong biofilm producers (SBP) %	Moderate biofilm producers (MBP) %	Weak biofilm producers (WBP) %	No biofilm producers (NBP) %	Total (%)
MRSA	3 (4.2)	22 (30.9)	12 (16.9)	0 (0.0)	37 (52.1)
MSSA	1 (1.4)	14 (19.7)	10 (12.6)	9 (12.6)	34 (47.8)
Total	**4 (5.6)**	**36 (50.7)**	**22 (30.9)**	**9 (12.6)**	**71 (100.0)**

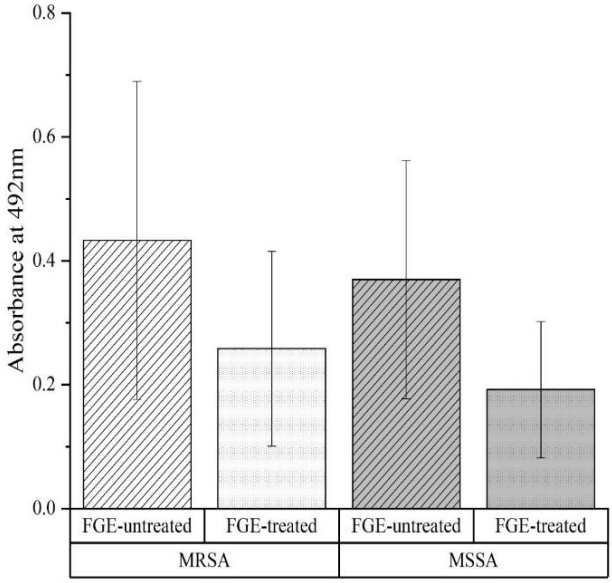

Figure 3: Comparison of an average values of biofilm biomass of FGE-untreated isolates of MRSA and MSSA with their FGE treated counterparts

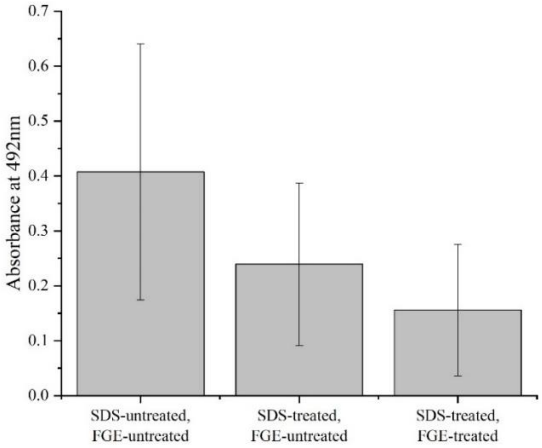

Figure 4: Comparison of average values of biofilm biomass of SDS untreated-FGE untreated isolates, SDS treated-FGE untreated isolates and SDS treated-FGE treated isolates

DISCUSSION

The MRSA in this study was found to be 52.1%. The reported prevalence of MRSA in Nepal was 21.1% to 68% in previous studies (Kumari et al 2008; Khanal and Jha 2010; Shrestha 2013; Shahi et al 2018; Khanal et al 2018). The rate of MRSA in hospitals of other countries was also similar (Wangai et al 2019; Hussein et al 2019) but different from some studies (Tariq and Javed 2019; Garoy et al 2019; Joshi et al 2013; Rajesh et al 2018; Dulon et al 2011; Adam and Abomughaid 2018; Omuse et al 2014). Hence, the prevalence of MRSA is variable among different countries and also between different regions of the same country. The various factors which affect intra- and intercountry variation in the prevalence of MRSA include differences in types of specimen, study population and study duration. Studies relying on genotypic detection by PCR tend to report the lower MRSA prevalence compared to phenotypic detection procedures such as cefoxitin disc diffusion test (Nwankwo and Nasiru 2011).

In this study, 100% MRSA isolates (37/37) produced biofilm whereas only 73.5% (25/34) MSSA possessed biofilm producing ability. The number of biofilm producers among MRSA is statistically significantly higher compared to MSSA strains (p= 0.001). There was no significant difference in biofilm production intensity between biofilm producing MRSA and MSSA isolates. The comparison of an average absorbance of biofilm biomass of MRSA and MSSA isolates showed no statistically significant differences. This result is broadly comparable to previous studies (Ghafourian et al 2013; Ghasemian et al 2016). The presence of large number of both strains of *S. aureus* as moderate biofilm producers (more than 55%) may help explain the high dissemination and the infection rate of *S. aureus* in healthcare facilities. Also the presence of large number (87.3%) of biofilm producing isolates in this study indicates the possibility of increased drug resistance in patients which may lead to treatment failures. However, other studies reported that biofilm production capacity is stronger in MRSA compared to MSSA (Manandhar et al 2018; Piechota et al 2018). MSSA strains produce NaCl induced biofilm whereas MRSA biofilms were glucose induced. A study of large collection of *S. aureus* isolates (114 MRSA and 98 MSSA) sampled from device-related infections containing 5 clonal complexes (CC5, CC8, CC22, CC30 and CC45) found that there is a significant relationship between *SarA* regulated PIA/PNAG and MSSA biofilm development. The biofilm development in MRSA is *ica* independent and involves a protein adhesion (s) regulated by *Sar A* and *Agr* (O'Neill et al 2007).

The FGE treatment significantly decreased the intensity of biofilm formation in both MRSA and MSSA isolates *in vitro*. There are also reports of antibacterial effects of garlic extract from the previous studies (Ratthawongjirakul and Thongkerd 2016; Ninyio et al 2016; Farrang et al 2019; Wu et al 2015), anti-biofilm effects (Ninyio et al 2016), used as remedy for cardiovascular diseases (Rahman and Lowe 2006), cancer (Roy et al 2016) and chemically induced hepatotoxicity (Ademilugi et al 2013). Raw FGE is used in this study because raw garlic contains large amount of allicin, which exhibits broad spectrum antimicrobial activity against Gram positive and Gram negative bacteria (Wallock-Richards et al 2014; Wu et al 2015; Reiter et al 2017) in addition to its anti-biofilm activity (Lihua et al 2013; Rasmussen et al 2005).

In this study, FGE (10%) inhibiting effects on biofilm was analyzed under chemo preventive conditions i.e. the *S. aureus* isolates were grown on microtiter plates in the presence of FGE. So, the reduced biofilm formation in the presence of FGE may be due to combination of killing of planktonic cells, reducing cell attachment to the surface and disturbing maturation of biofilms.

The toxic effects of garlic have been tested in a mouse model. The study suggested that garlic extract didn't exhibit toxicological effects at the hematological and the histological levels, but instead provided protective effects (Farrang et al 2019). Garlic extracts can be coated on biomedical devices, used as ointments for wound infections and used orally to combat pathogenic biofilm related bacteria.

The detachment assay performed in this study showed that detachment efficiency for *S. aureus* biofilm cells with FGE treatment was higher compared to biofilm cells without FGE. This result suggests that FGE treated biofilm cells are loosely attached to the surface than those untreated biofilm cells. It has industrial and commercial applications as garlic extracts can be used with surfactants in order to remove unwanted biofilms present in water pipeline, membrane filters used in water treatment plant, mixing tanks and vats in food industries etc.

This study possesses certain limitations. Due to limited resources molecular characteristics of *S. aureus* isolates could not be determined. This made the distinction of HA-MRSA and CA-MRSA impossible, making the source of infection uncertain.

CONCLUSION

There are no statistically significant differences between MRSA and MSSA in their biofilm producing ability. The study also shows that FGE inhibits biofilm production by both MRSA and MSSA isolates significantly, in addition to their ability to detach biofilms effectively.

This suggests that garlic extracts have potency as an anti-biofilm agent and could be developed and used to manage different *S. aureus* biofilm related infections.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Antifungal Susceptibility Testing of Pathogenic Aeromycoflora Isolated from Kathmandu

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ABSTRACT

Objective: To identify the predominant pathogenic aeromycoflora present in dense areas of Kathmandu, and perform their antifungal susceptibility test.

Methods: Aeromycoflora were isolated by the Gravity Plate method and identified by observing colony morphology and microscopic methods. For pathogenic mycoflora, MIC test was carried out following "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS document M38-A". The antifungal agents used were Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine available at pharmaceutical companies of Nepal during study period.

Results: *Penicillium spp* (43.8%) was found to be the most predominant aeromycoflora followed by *Cladosporium spp* (35.5%), and pathogenic *Aspergillus species* (21.4%). Among *Aspergillus species*, *Aspergillus niger* (71.7%) was predominant, followed by *A. flavus* (17.1%) and *A. fumigatus* (11.1%). *A. fumigatus* and *A. flavus* were found to be most sensitive towards Itraconazole (MIC range – <0.0625 - 1µg/ml) while *A. niger* to Ketoconazole (MIC- 0.0625 - >32µg/ml).

Conclusion: The finding of this study helped to identify the potent antifungal drugs available against the pathogenic aeromycoflora.

Key words: Aeromycoflora, *Aspergillus* spp, antifungal agents, MIC test, Nepal

INTRODUCTION

The occurrence of airborne mycobiota in outdoor and indoor environments as well as the fluctuations in their numbers and composition are receiving increasing attention within the framework of potential health hazards to both flora and fauna, including humans (Pyrri and Kapsanaki-Gotsi, 2007). Exposure to fungal spores occurs mostly indoor, but outdoor air is an important source of both aeroallergens and pathogens (O'Gorman and Fuller 2008). It is estimated that worldwide deaths attributed to fungal infections (>1 500 000) are as high as those of tuberculosis (1 500 000) (Denning, 2015).

The transmission of fungal spores to the human host is via inhalation. Among the myriad opportunistic fungal pathogens, *Candida albicans* and *Aspergillus fumigatus* cause the most well-known infections. Yet, the growing list of other opportunistic agents is of increasing importance. New and emerging fungal pathogens include opportunistic yeastlike fungi (e.g., *Trichosporon* species), the zygomycetes, hyaline molds (e.g., *Fusarium* and *Scedosporium* species), and a wide variety of dematiaceous fungi (Lass-Flör *et al.*, 2008).

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With the increasing number of immunocompromised individuals, mycoses have also increased in the last two decades, affecting millions of people worldwide. Invasive fungal infections are devastating and the mortality rates with the three most common species of human fungal pathogens are *Candida albicans*, 20%–40% ; *Aspergillus fumigatus*, 50%–90% ; and *Cryptococcus neoformans*, 20%–70% (Butts and Krysan, 2012). Depending on regional distinctions *Aspergillus flavus* and *Aspergillus terreus* are frequently reported as well and there is evidence that these non-fumigatus pathogens are increasingly common etiologic agents (Binder, 2012). In Asia, the Middle East and Africa, *A. flavus* is the cause of a broad spectrum human diseases due to its ability to survive in hot and arid climatic conditions compared to other *Aspergillus* spp. Worldwide, ~10% of cases of bronchopulmonary aspergillosis are caused by *A. flavus* (Rudramurthy et al., 2019).

Shrestha et al. (2010) reported that fungi are most prevalent in Kathmandu, Nepal and broad spectrum antifungal agents are widely used irrespective of antifungal susceptibility testing. With the increasing fungal infections, it has become a prerequisite to perform antifungal susceptibility testing so as to improve the health of patients with fungal infection.

In Nepal, plenty of studies were carried out in pathogenic bacteria with antibiotic susceptibility tests, but only a handful of studies were done in fungi having negligible antifungal susceptibility tests. Hence, the objective of this study is to survey aeromycoflora in a dense area of Kathmandu, identify predominant pathogenic fungi and perform their antifungal susceptibility test.

METHODS

This is a cross sectional study carried out in densely populated and core areas of Kathmandu city within the period of January to June 2012. The processing of the samples was carried out in the Microbiology laboratory of Amrit Science Campus and SANN International College.

Aeromycoflora were collected from four core locations (Ganesh Temple, Bhotahity, Mahaboudha and Bhedasing) of Kathmandu city. From each location, three different sites were selected representing both indoor and outdoor environments. Both indoor and outdoor samples were performed by Gravity Plate method (Colakoglu 1996; Hedayati et al. 2005) in Potato Dextrose Agar (PDA) medium with Chloramphenical. In each spot, two consecutive plates were kept and exposed for 10 minutes in the indoor environment and 5 minutes in the outdoor environment. A control plate (unexposed) was maintained at each site following the same condition as the sample. All the plates were transported to the laboratory as soon as possible and incubated at different temperatures i.e; one plate at 28^0C and another at 37^0C for 4-7 days till the fungal colonies developed. The number of colonies were counted and recorded every day. The isolated colonies were maintained as pure culture by transferring mycelium or spore into PDA medium for the further study. The identification of isolated fungi was done by studying colony morphology and microscopic methods using references [(Subramanian 1971; Barnett and Hunter 1972; Rapper et al. 1973; Barron 1977; Ellis 1985)]. The identified fungi were stored in sterile water at -20°C for further use.

The antifungal susceptibility test was performed for the predominant pathogenic fungi by following Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. NCCLS document M38-A (NCCLS, 2008). The medium used was RPMI-1640 medium and antifungal agents used were Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine.

RESULTS

The distribution of aeromycoflora in different collection sites showed more than ten different genus of fungi as *Penicillium spp, Cladosporium spp, Aspergillus spp, Alternaria spp, Mucor spp, Rhizopus spp, Trichoderma spp, Helminthosporium spp, Colletotrichum spp, Culvularia spp Fusarium spp* and few unidentified genera. Table (1) represented the predominant non-pathogenic aeromycoflora as *Penicillium* (43.8%) and *Cladosporium* (35.5%) and pathogenic as *Aspergillus* spp (21.4%). Table (1) also showed that indoor environments more polluted by aeromycoflora than outdoor environments.

Table (2) showed that among *Aspergillus* spp, Aspergillus niger (71.1%) was found most prevalent followed by *A. flavus* (17.1%) and *A. fumigatus* (11.1%). In case of resident and non-resident indoor environments, non-residents were more polluted.

Table (3) and (4) showed the MIC value of antifungal agents Ketoconazole and Itraconazole respectively against tested strains of *Aspergillus* spp considering the degree of pathogenicity. For other antibiotics also, MIC was determined following the same procedure.

Table (5) represented the MIC value of all antifungal agents against *Aspergillus* spp. The MIC range differ with *Aspergillus* spp and antifungal agents.

Table 1. Distribution of predominant and pathogenic mycoflora at different site in different environment

Collection site	Environment Type	*Penicillium spp*	*Cladosporium spp*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus sulphurous*
Ganesh Temple (G)	Outdoor (GO)	20(6%)	9(3%)	8(7%)	3(12%)	3(18%)	1(6%)
	Indoor (GI)	73(21%)	23(8%)	25(23%)	9(35%)	11(65%)	2(13%)
Bhotahity (B)	Outdoor(BO)	6(2%)	4(1%)	2(2%)	0(0%)	0(0%)	0(0%)
	Indoor (BI)	40(12%)	43(15%)	33(30%)	1(4%)	2(12%)	7(44%)
Mahaboudha (M)	Outdoor(MO)	14(4%)	9(3%)	7(6%)	3(12%)	1(6%)	0(0%)
	Indoor (MI)	69(20%)	92(33%)	13(12%)	3(12%)	0(0%)	4(25%)
Bhedasingh (D)	Outdoor(DO)	24(7%)	25(9%)	3(3%)	2(8%)	0(0%)	1(6%)
	Indoor (DI)	94(28%)	74(27%)	18(17%)	5(19%)	0(0%)	1(6%)
Total isolates		340(100%)	279(100%)	109(100%)	26(100 %)	17(100%)	16(100 %)
Grand total	**787**	**43.8%**	**35.5%**	**13.9%**	**3.3%**	**2.2%**	**2.0%**

Table2. Distribution of pathogenic fungi at different sites

Collection site	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*
A. Outdoor			
1. Ganesh Temple	8	3	3
2. Bhotahity	2	0	0
3. Mahaboudha	7	3	1
4. Bhedasingh	3	2	0
Total isolates	20 (18.3%)	8(30.8%)	4(23.5%)
B. Indoor (resident)			
House No. 1	18	1	5
House No 2	4	1	0
House No 3	12	4	0
Total isolates	34(31.1%)	6(23.1%)	5(29.4%)
C. Indoor (non-resident)			
Corporate house 1	1	1	0
Corporate house 2	6	1	0
Nepal Dairy	9	2	0
Subway	32	0	2
Seed shop	7	8	6
Total isolates	55(50.5%)	12(46.2%)	8(47.1%)
Grand Total (152)	**109(71.7%)**	**26(17.1%)**	**17(11.1%)**

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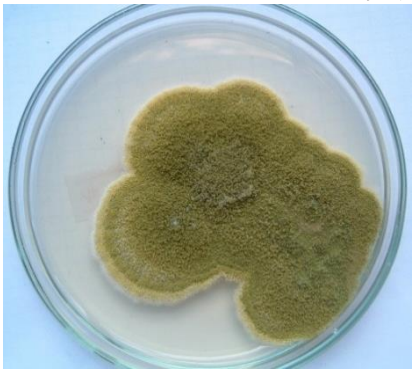
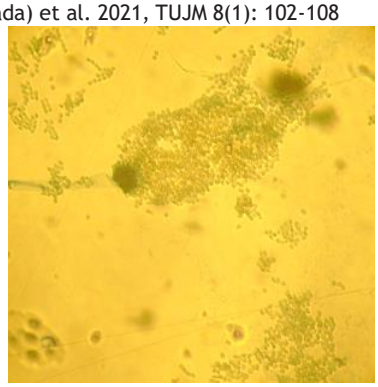
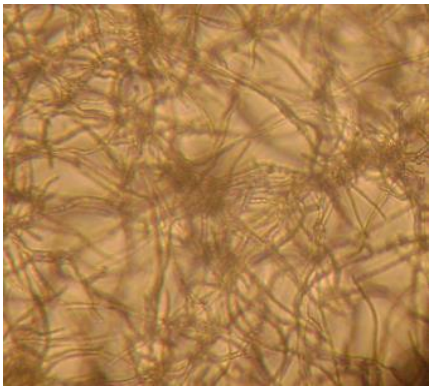
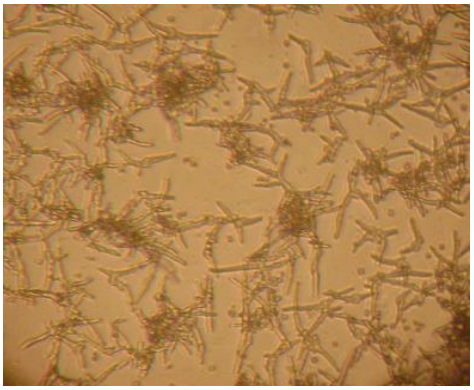



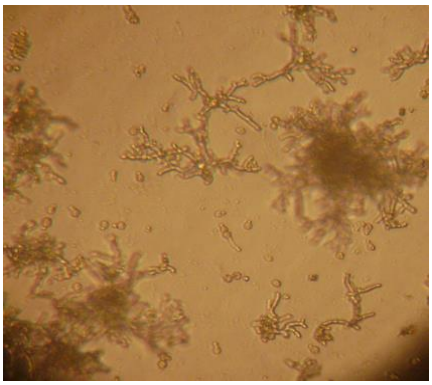
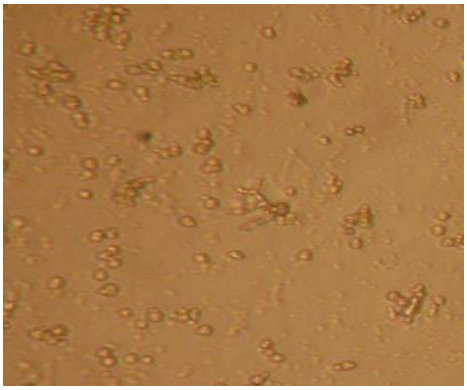
Ptotographs (1-3) of aeromicroflora: 1) Aeromycoflora isolated by gravity plate; 2) *Aspergillus fumigatus* (pure culture); and 3) *Aspergillus fumigatus* (microscopic view)

Table 3. MIC determination of Ketoconazole against *Aspergillus* spp

Ketoconazole concentration (µg/ml)	0 >32	1 32	2 16	3 8	4 4	5 2	6 1	7 0.5	8 0.25	9 0.125	10 0.0625	0 <0.0625	No growth	Total isolates
Aspergillus niger	1	1	2	2	8	2	1	2	2	2	1	0	0	24
Aspergillus flavus	0	1	0	7	6	1	0	0	0	0	0	0	0	15
Aspergillus fumigatus	0	2	8	0	1	1	1	1	0	0	0	0	0	14

A **B**

C **D**

Photograph 4 (A-D): Decrease fungal growth rate in successive higher concentration of antifungal agents in MIC test observed under inverted microscope.

Table 4. MIC determination of Itraconazole against *Aspergillus* spp

Itraconazole concentration (µg/ml)	0 >32	1 32	2 16	3 8	4 4	5 2	6 1	7 0.5	8 0.25	9 0.125	10 0.0625	0 <0.0625	No growth	Total isolate
Aspergillus niger	0	0	0	0	0	0	0	0	0	0	0	0	24	24
Aspergillus flavus	0	0	0	0	0	0	1	2	0	8	0	3	1	15
Aspergillus fumigatus	0	0	0	0	0	0	3	1	0	6	0	3	1	14

Table 5. AST pattern of antifungal agents against *Aspergillus* spp

Antifungal agent and MIC criterion (µg/ml)	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*
Ketoconazole			
MIC range	0.5 – 32.0	2.0 – 32.0	0.0625 - > 32.0
MIC (max.)	16.0	8.0	4.0
Itraconazole			
MIC range	<0.0625 – 1.0	<0.0625 – 1.0	-
MIC (max.)	0.125	0.125	-
Fluconazole			
MIC range	0.5 - >128	<0.25 - >128	-
MIC (max.)	8	>128	-
Cotrimazole			
MIC range	0.5 – 32.0	2.0 – 32.0	-
MIC (max.)	16.0	8.0	-
Terbinafine			
MIC range	<0.0625 – 0.5	0.125 – 4	-
MIC (max.)	0.25	0.25	-

DISCUSSION

four densely populated sites (Bhedasingh, Bhotahiti, Mahaboudha and Ganesh temple of Ason) were chosen and samples were collected from the outdoor and indoor environments of each site. In the indoor environment, residents and non-residents were considered.

More than ten different types of fungi were isolated in this study. *Penicillium spp* (43.8%) was found to be the most predominant aeromycoflora followed by *Cladosporium* spp (35.5%), and pathogenic *Aspergillus* species (24.1%) as shown by Table 1. Rajbhandari GS (2018) also stated that Aspergilli/ Penicilli group was recorded most predominant with 81.20% and *Cladosporium* as the second highest with its prevalence (8.72%) of the total isolates. The predominance of *Penicillium. Aspergillus* and *Cladosporium* spp were also mentioned by other studies, but differ in most predominant one. Studies by Herrero *et al.* (2006) in Spain and Pyrri and Kapsanaki-Gotsi (2007) in Greece observed *Cladosporium* spp (41% and 37.2% respectively) as most predominant while Shams-Ghahfarokhi *et al.* (2014) in Iran mentioned *Aspergillus* spp (31.3%) as most common one.

Air quality, both indoor and outdoor, is closely linked to a range of diseases, including respiratory, cardiovascular and vascular diseases. Therefore, indoor air pollution becomes a very important factor affecting the human health (Radwan and Abdel-Aziz, 2019). Table (1) showed that aeromycoflora content in indoor samples are higher than outdoor samples. Similar result was also mentioned in by Radwan and Abdel-Aziz (2019) in study of microbial content of air.

According to the pathogenicity, *A. fumigatus* is highly pathogenic followed by *A. flavus* and *A. niger.*

In this study, the distribution of *Aspergillus* spp showed *A. fumigatus* (11.1%) was found in low percent than *A. niger* (71.1%) and *A. flavus* (17.1%) which indicated less harm to human health. Considering the sampling site, highly pathogenic *A. fumigatus* found in higher percent at Ganesh Temple area in both indoor and outdoor environments indicating possible health hazard by fungal diseases residing in those areas than other sites.

Non-residental indoor environments were more polluted due to presence of higher percent of aeromycoflora (Table 2). This implies that working people are more vulnerable to fungal diseases. But this also represented that considering the most pathogenic species *A. fumigatus*, high percent (47.1%) was isolated from non-resident indoor followed by resident (29.4%) and by outdoor (23.5%). This indicates there is chances of increase in fungal diseases if ignored the possible outcomes.

The isolated *Aspergillus* Spp were further processed for antibiotic susceptibility test with the antifungal agents – Cotrimazole, Fluconazole, Ketoconazole, Itraconazole and Terbinafine which were collected from various pharmaceutical companies situated in Kathmandu Valley. Since *A. fumigatus* is the most pathogenic spp, every isolates were proceeded for test while for *A. flavus* and *A. niger*, selected strains representing all collected sites were tested. The MIC was determined as shown in Table 3 to 5. For all antifungal agents, drug concentration range was set as <0.0625 - >32 µg/ml so that it will cover number of concentration tested criteria as mentioned in NCCLS – M38-A guidelines. But in case of fluconazole, concentration upto >128 µg/ml was set as guideline mentioned higher MIC range for fluconazole. Table 3 and 4 showed the MIC value of antifungal agents Ketoconazole and Itraconazole respectively against tested strains of *Aspergillus* spp. For other antibiotics also, MIC was determined following the same procedure. Interpretation of MIC was done following NCCLS-M38-A guidelines. According to guidelines, for Fluconazole, resistant strains shows MIC >64 µg/ml, for Ketoconazole, MIC varies between 0.0313 and 16 µg/ml and for Itraconazole and new Triazoles, MICs >8 µg/ml are associated with clinical resistance to this agent. Espinel-Ingroff *et al.* (2001) mentioned MICs of Itraconazole as >8 µg/ml for resistant and 0.03 to 1 µg/ml for sensitive *Aspergillus* isolates.

Table 5 represented the MIC value of all antifungal agents. In the case of *A. fumigatus*, most sensitive was found to be Itraconazole (MIC – 0.125µg/ml) and least to be Ketoconazole and cotrimazole; in both case MIC - 16µg/ml which lied toward higher range indicating possibility of resistance development. Though Terbinafine also seemed to be sensitive (MIC-0.25 µg/ml), but no growth of isolates in more than 50% of the sample tested was observed. Denning *et al.* (1997) stated the resistant strain having MIC - >16µg/ml, which is alarming, but in our case no such result appeared. Howard *et al.* (2009) mentioned that the frequency of Itraconazole resistance was 5% in case of clinical isolates, but in this study the result differ as isolates were environmental. In case of *A. flavus,* Itraconazole (MIC – 0.125µg/ml) was found most effective followed by Terbinafin (0.25µg/ml) and resistant to Fluconazole (>128µg/ml). MIC value against *A. niger* was determined for Ketoconazole only (4µg/ml) and no growth was observed against all other antibiotics.

Pfaller *et al.* (2002) mentioned MIC range as 0.25 – 2 µg/ml for *A. fumigatus* and 0.25 – 1 µg/ml for *A. flavus* and Shi *et al.*(2010) determined the MIC as 0.064 - >32 µg/ml for *A. fumigatus* and 0.047 – 4 for *A. flavus* while Mosquera *et al.*(2002) mentioned MIC range for *Aspergillus* spp as 0.125 – 0.5 µg/ml for Itraconazole and 256 -512 µg/ml for Fluconazole. All above mentioned studies were carried with clinical isolates. So, their MIC range lied in higher range than environment isolates of this study.

This study reflected that available antifungal agents were effective for the pathogenic aeromycoflora and proper management of such agents in hospital can decrease resistance development.

CONCLUSION

The predominant mycoflora from different collection sites of Kathmandu city was found to be *Penicillium* spp and potent pathogen, *A. fumigatus*. From the antifungal susceptibility test, Itraconazole was found to be the drug of choice for *A. fumigatus* and *A. flavus* and Ketoconazole for *A. niger*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Antimicrobial Activity of Lactic Acid Bacteria Isolated from Traditional Fermented Food

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ABSTRACT

Objective: The main objective is to isolate Lactic acid bacteria from traditional fermented food of Kathmandu valley and to study their antimicrobial properties by agar well diffusion method.

Methods: A total of 30 samples of 4 different types of traditional fermented food (*Gundruk* and *sinki, Pickles* and *Dahi*) were obtained from Kathmandu valley and processed in Microbiology Laboratory of Padma Kanya Multiple Campus. For identification of Lactic acid bacteria (LAB), Gram staining, catalase and motile tests were done. In the carbohydrate fermentation test, all isolates were processed for fermentation of glucose, lactose, sucrose and fructose. Bacteriocin was extracted by precipitation method. Antibiotic susceptibility of the isolates was determined by using modified Kirby-Bauer disc diffusion method. The antimicrobial activity of Lactic acid bacterial (LAB) was done by agar well diffusion method.

Results: A total of 21 LAB isolates were identified which were 10 *Lactobacillus* spp (47.6%), 8 *Pediococcus* spp (38.0%) and 3 *Streptococcus* spp (14.3%). The antimicrobial activity of bacteriocin showed inhibitory activity against *Shigella* spp, *Escherichia coli* and *Bacillus* spp but didnot show inhibition to *Staphylococcus aureus*, *Salmonella* spp. and *Klebsiella pneumoniae*. For the quality control, the antimicrobial activity of bacteriocin was done on ATCC (25923) *Staphylococcus aureus.* All isolates were susceptible to ampicillin while resistant to nalidixic acid and Co-trimoxazole.

Conclusion: The present study showed the bacteriocin produced by LAB from traditional fermented food (*Gundruk* and *Sinki,* yogurt and *Pickle*) showed antimicrobial activity against different bacteria which underline its important role in improving food quality and increasing safety.

Keywords: Traditional fermented foods, lactic acid bacteria, bacteriocins, antimicrobial activity, Nepal

INTRODUCTION

Lactic acid bacteria (LAB) play an important role in food as their importance is associated mainly with their safe metabolic activity while growing in foods. They are utilizing available sugar for the production of organic acids and other metabolites which are natural acceptance for human consumption (Bourdichon et al. 2012). Fermented food products like curd and pickles had been an integral part of human diet from ancient time (Nuraida 2018). LAB are groups of Gram-positive bacteria. They are facultative anaerobes, catalase-negative, non-motile, do not produce spores, and create lactic acid as a metabolic end-product during carbohydrate fermentation. Based on their metabolic pathways, LAB is classified either homofermentative or heterofermentative. In homofermentative LAB, they ferment sugars to produce mainly lactic acid under anaerobic conditions. In heterofermentative LAB, sugars are fermented to produce ethanol, CO_2, and less lactic acid (Ayyash et al. 2018).

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Traditionally fermented food like *Pickles, Yogurt* and *Gundruk* and *Sinki* are usually homemade products obtained through spontaneous fermentation (Lee et al. 2004).

LAB are considered as a major group of probiotic bacteria which are known as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host (Salminen et al. 1999). So, LAB has a diverse range of health-promoting effects, including protection against infectious agents, immunomodulatory effects, anti-allergenic effects, anti-obesity effects, anti-oxidant effects, enhancing the bioavailability of vitamins/minerals, anti-anxiety effects (Mathur et al. 2020).

During fermentation, LAB produces compound like Bacteriocin which is ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum) or across genera (broad spectrum). It exerts a preservative effect on fermented food as a result of strong antagonistic activity against spoilage organisms as well as pathogenic bacteria like *E. coli, Bacillus* species, *Staphylococcus aureus* etc. (Gorya et al. 2013; Mobolaji & Wuraola 2011).

There are plenty of traditional fermented food in Nepali diet, among which *Pickles, Yogurt,* and *Gundruk* and *Sinki* are most common of Nepali kitchen. So, this study was done for identifying LAB in Nepali traditional fermented food and also analyzing its antimicrobial properties which can be used to increase self-life and quality of food as well as their application as bio preservatives.

METHODS

Sample collection

A convenience sampling technique was used for samples collection. A total of 30 samples (10 homemade *Yoghurt*, 10 *Pickles, 5 Sinki and 5 Gundruk* each 25 gms) were aseptically collected in sterilized zip lock plastic bags and transported to microbiology laboratory of Padma Kanya Multiple Campus. Samples were kept in a refrigerator (around 4^0C) until laboratory identification of Lactic acid bacteria.

Isolation of LAB

For isolation of LAB, 1gm of each sample (*Gundruk* and *Sinki*, *Yogurt* and *Pickle*) was added to 9ml of phosphate buffered saline to make 1/10 dilution and mixed the sample properly. The 1/10 dilution was then serially diluted up to 10^{-6} dilution. The samples from 10^{-2}, 10^{-4}, 10^{-6} dilution were pipetted out in the Petriplate. Then, De Man, Rogosa and Sharpe (MRS) media incorporated with 1% Calcium carbonate was poured properly and incubated at 35^0C for 48 hours. The isolated colony was sub-cultured again in MRS agar then the result was observed (Kazemipoor et al 2012).

Identifying LAB from sample

LAB was identified by Gram staining and biochemical tests. Further, carbohydrates fermentation for *Lactobacillus* species, *Streptococcus thermophillus* and *Pediococcus* species were done by observing the colour change and gas formation. In this study, all isolates were incubated at different temperature like 10˚C, 35˚C, 37˚C and 45˚C (Cheesbrough 2000).

Extraction of bacteriocin

The isolated LAB having inhibition property was propagated in 500ml MRS broth (pH 7.0) for 48 hours at 35^0c.The cultures were centrifuged at 10,000 rpm for 20 minutes at 4^0C to obtain a cell free suspension (CFS). Then pH of the CFS was adjusted to 7.0 by means of 1M NaOH. After adjusting pH, CFS was precipitated with ammonium sulphate (40% saturation). The bacteriocin was filtered through the Whatman's no.1 filter paper and eluted in potassium phosphate buffer (Yang et al 1992).

Determination of antimicrobial activity of bacteriocin

Fresh cultures of all test organisms were swabbed on the Muller Hinton agar. Wells were made on the media to load samples and control. Bacteriocin solutions and sterile water (control) were loaded on the respective well. The loaded samples were allowed to diffuse. Then the plates were incubated at 37^0C for 24 hours then the zone of inhibition were measured (Zhennai 2000).

Antibiotic susceptibility tests

A modified method of Kirby-Bauer disc diffusion method according to the CLSI (2006) was used for the study. The culture densities of isolated lactic acid bacteria were adjusted to 0.5McFarland. A suspension of the isolated LAB was spread over the MHA agar plates, antibiotic discs of ampicillin (10 µg), nalidixic acid (30 µg), azithromycin (15 µg), co-trimoxazole (30 µg) and cephalexin (30 µg) were placed on the surface of the agar plates. In an inverted position, the plates were incubated at 37^0C for 24 hours. The diameters of the inhibition zones around the discs were measured. The result was expressed as susceptible, intermediate, or resistant according to the standard chart.

RESULTS

In the present study, 21 LAB were isolated from 30 samples with 4 traditional fermented food samples. A total of 10 samples of *Gundruk and Sinki*, 10 samples of *Pickle* and 10

samples of *Yogurt* were included. The highest occurrence of LAB was found in *Yogurt* samples 11(36.6%) and lowest number of LAB was found in *Pickle* samples 4 (13.4%). The higher LAB isolates were *Lactobacillus* species (47.6%) and lower were *Streptococcus* species (14.3%).

Morphological characteristics of the LAB isolates were identified as Gram positive, catalase negative and non-motile. The cell morphology of all isolates was evaluated through microscopic observation. In the carbohydrate fermentation test, all isolates were able to ferment Sucrose and fructose. Among the isolates, only *Pediococcus* species were not able to ferment glucose and lactose. In this study, only one isolate (*Streptococcus thermophillus*) was able to grow at 45^{0}C. All the isolates of *Lactobacillus* species and *Pediococcus* species were able to grow both at 35^{0}C and 37^{0}C. In addition, the isolates of both *Pediococcus* species and *Lactobacillus* species show slow growth at higher temperature.

Isolated LAB (cell free filtrate) showed antimicrobial activity by zone of inhibition against *S. aureus*, *Shigella* species, *Salmonella* species, *E. coli*, *Klebsiella* species and *Bacillus* species. However, LAB did not show inhibition zone against *Staphylococcus aureus*, *Salmonella* species and *Klebsiella pnumoniae*.

This study showed, all the isolates were susceptible to ampicillin, but resistant to nalidixic acid and co-trimoxazole.

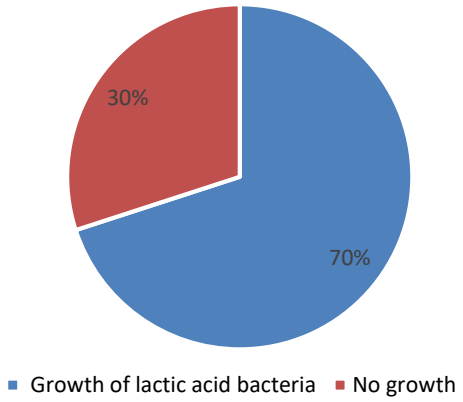

Figure 1: Growth profile of LAB

Table 1: Types of LAB isolated from fermented food samples

Fermented food samples	LAB isolates			Total N (%)
	Lactobacillus Species N (%)	*Pediococcus* *Species* N (%)	*Streptococcus thermophillus* N (%)	
Gundruk	0	6	0	6 (20.0)
Pickle	2	2	0	4 (13.4)
Yogurt	8	0	3	11(36.6)
Total	**10 (47.6)**	**8 (38.0)**	**3 (14.3)**	**21(70.0)**

Table 2: Morphology, physiological and biochemical characteristics of isolated genera of LAB

Characteristics	*Lactobacillus* species	*Pediococcus* species	*Streptococcus thermophillus*
Cell morphology	Rods	Cocci	Cocci/chain
Gram test	+	+	+
Motility test	-	-	-
Catalase	-	-	-

Fermentation of carbohydrates			
Glucose	+	-	+
Lactose	+	-	+
Sucrose	+	+	+
Fructose	+	+	+
Growth at different temperature			
10^0C	-	-	-
35^0C	+	+	+
37^0C	+	+	+
45^0C	-	-	+

+ = Positive, - = Negative

Table 3: Antimicrobial activity of test organism by LAB

Test organisms	**Zone of inhibition (mm)**		
	***Pediococcus* species**	***Lactobacillus* species**	***S. thermophillus***
Staphylococcus aureus ATCC (25923)	30	26	25
Staphylococcus aureus	-	-	-
Shigella species	26	27	29
Salmonella Typhi	-	-	-
Salmonella Paratyphi	-	-	-
Escherichia coli	25	26	30
Klebsiella pneumonia	-	-	-
Bacillus species	15	24	17

- = No zone of inhibition.

Table 4: Antibiotic susceptibility tests

LAB	**Inhibition zone (mm)**				
	NA	**COT**	**AMP**	**AZI**	**CFX**
Pediococcus species	R	R	S	S	R
Lactobacillus species	R	R	S	S	S
Streptococcus thermophillus	R	R	S	R	S

AMP=Ampicillin (10 µg), NA=Nalidixic acid (30 µg), AZI=Azithromycin (15 µg), COT=Co-trimoxazole (30 µg) and CFX= Cephalexin (30 µg)

DISCUSSION

In the present study, altogether 30 samples with 3 different types of fermented foods were included in which 21 LAB (70%) were isolated from traditional fermented food samples. By comparing the result of morphological, physiological and biochemical tests, the isolates were identified as 8 *Pediococcus* species (38.0%), 10 *Lactobacillus* species (47.6%) and 3 *Streptococcus thermophillus* (14.3%). Among total isolates, only one isolate (*Streptococcus thermophillus*) was able to grow at 45^{0}C whereas *Lactobacillus* and *Pediococcus* species were able to grow at 35^{0}C and 37^{0}C but not at 45^{0}C. The result of the present study is in accordance with the report of (Galvez et al. 2007) who revealed that members of LAB could be detected in a variety of habitats including fermented foods. LAB are able to ferment different carbohydrates like glucose, fructose, lactose etc. as they use the nutrients in the substrate for their own metabolism and cell growth and multiplies in food (from one million per millilitre to one billion per millilitre) (Robinson 1991). Lindquist (1998) reported that a medium that would support their growth must contain a fermentable carbohydrate and many growth factors.

In this study, only three bacteriocin producing 3 LAB genera were isolated from traditional Neplease fermented foods. Many papers have been published on isolation, characterization of LAB from traditional fermented foods. Fifty bacteriocin producing *Lactobacillus* species were isolated from traditional Nigerian fermented foods such as *Fufu, Garri, Nono* and *Ogi* (Adenike et al. 2007). Twelve bacteriocin producing LAB strains were isolated from Senegal fermented foods (Diop et al. 2007).

Antimicrobial substance (bacteriocin)of *Pediococcus* spp, *Lactobacillus* spp and *Streptococcus thermophillus* isolated from fermented food samples showed antimicrobial activity of against *S. aureus*, *Shigella* species, *Salmonella* species, *E. coli*, *Klebsiella* species and *Bacillus* species. Antimicrobial substance shown inhibited to *S. aureus* ATCC (25923) in 30 mm, 26 mm and 25 mm with respect to diameter of inhibition zone. Similarly, all of these isolates showed zone of inhibition of *Shigella* species in 26 mm, 27 mm and 29 mm while *E. coli* was inhibited by zone of inhibition of 25 mm, 26 mm and 30 mm respectively. Zone of inhibition also shown by *Bacillus* species in the range of 15 mm, 24 mm and 17 mm respectively. However, zone of inhibition did not show against *Staphylococcus aureus*, *Salmonella* species and *Klebsiella pnumoniae*.

Several studies have shown that pathogens such as enterotoxigenic *E. coli*, *Shigella flexneri, S. typhimurium* and *B. cereus* are adversely affected when present in traditional fermented foods (Kingamkono et al. 1995, Kunene et al. 2000, Obadina et al. 2006).

Some of the antimicrobial properties exhibited by these fermented foods may be as a result of the low pH of the food as well as metabolites produced by microorganisms such as LAB involved in the fermentation. Gilliland and Speck (1977) had earlier reported that Lactobacilli showed stronger antibacterial properties against Gram-positive bacteria (*S. aureus* and *Clostridium perfringens*) than Gram-negative bacteria (*E. coli*, *Salmonella* Typhi*, and Salmonella* Paratyphi).

Microbial food safety is an increasing public health concern worldwide and many Gram negative bacteria such as *E. coli, Klebsiella* spp together with Gram positive bacteria such as *S. aureus* have been implicated in food borne diseases (Mead et al. 1999). The LAB isolated from *Yoghurt, Fufu* and *Kunuzaki* in their study probably produced different antimicrobial compounds, the quantity of which might vary with time. Collins et al. (1983) also noted the inhibition of *Psuedomonas fragi* and *S. aureus* against other microorganisms by some LAB strains which contribute to their inhibitory activity.

Similarly, different studies were done for antimicrobial activity of LAB isolated by different fermented food. Kawahara et al. (2010) characterized the antibacterial LAB strain *L. curvatus* isolated from Nozawana-zuke pickles. The strain showed antibacterial activity against some putrefactive bacteria, such as *S. marcescens*, *L. monocytogenes*, and *S. aureus* subsp. *aureus*, without affecting the growth of all the LAB strains tested except for *L. curvatus* strain. In a study of Ozkalp et al. (2009), the lactic acid bacteria were isolated from caper pickle. Thus, bacteriocins produced by LAB are likely to have a much greater potential as preservative (Bromberg et al. 2004). Liasi et al. (2009) emphasized that research on antimicrobial substances produced by LAB, had led to their potential use as natural preservatives, which may be used to combat the growth of pathogenic microorganisms in the food industry. In this study, Antibiotic susceptibility disc test was also done for all LAB isolates which showed susceptible to Ampicillin, but resistant to Nalidixic acid and Co-trimoxazole.

CONCLUSION

The present study showed that the LAB were found to be high in traditional fermented yogurt samples while it was slightly less in two samples i.e. *Gundruk* and *Pickle*. The present study showed the bacteriocin produced by LAB showed antimicrobial activity against different bacteria which underline its important role in improving food quality and increasing safety.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Prevalence of *Candida* Carriage and *in Vitro* Evaluation of Phospholipase and Haemolysin Activity of Oral *Candida albicans* among Tobacco Users and Smokers in Dharan, Nepal

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ABSTRACT

Objectives: The main objective of this study was to determine the prevalence of *Candida* carriage among tobacco users and smokers along with in vitro evaluation of phospholipase and hemolysin activity of *Candida albicans.*

Methods: A laboratory based cross-sectional study was carried out in Dharan Sub-Metropolitan city, Eastern Nepal from June 2018 to November 2018. During the study 150 oral rinse samples were obtained from smokers (50), smokeless tobacco consumers (50) and non-tobacco users (50) as control group. The participants were provided 10 ml of normal saline and were asked to oral rinse for 1 minute. Oral rinse was collected in a sterile screw capped container and was transported to microbiology laboratory by maintaining the cold chain. The oral rinse sample was inoculated onto the Sabouraud dextrose agar with chloramphenicol and was incubated at 37°C for 3-4 days. The number of colonies of *Candida* was counted and *C. albicans* were identified by cultural characteristics, staining, germ tube test and chlamydospore formation test.

Results: The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group, 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The prevalence of Candida carriage was found to be significantly higher in the study group associated with tobacco chewers (P=0.008). However, the Candida carriage among smoker's group was not found to be statistically significant (P=0.059). Isolation of *Candida albicans* was higher among smokeless tobacco users 15 (30%), smokers 5 (10%) and non-users 6 (12%).

Conclusion: Colonization and carriage of *Candida* in the oral cavity of smokers and tobacco chewers were found to be higher than in controls. In addition, individuals with poor oral hygiene increase the risk of *Candida* colonization and infection under host debilitated condition.

Key words: *Candida*, Smokers, tobacco chewers, oral candidiasis, phospholipase, hemolysin

INTRODUCTION

Candida are almost universal on normal adult skin and Candida albicans is part of the normal flora of the mucous membranes of the respiratory, gastrointestinal, and female genital tracts (Spampinato and Leonardi, 2013). C. albicans lives in oral cavity of 40% of the healthy human population with no harmful effects (Jenkinson and Douglas, 2002).

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However, host becomes susceptible to infection in debilitated or immunocompromised condition (Lopez-Martínez 2010). In oral candidiasis the most commonly identified pathogen is *C. albicans* (Manfredi et al. 2004). *C. albicans* pathogenesis is described by its host defense mechanism, adherence, and production of tissue degrading hydrolytic enzymes like protease, phospholipase and haemolysin (Pandey et al. 2018).

The secretion of hemolysin is followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis (Tsang et al. 2007). Phospholipase enzyme digests the phospholipid in the host cell membrane, producing cell lysis and changes in the surface characteristics that aid adhesion and infection. As a result, phospholipase production might be utilized to discriminate virulent invasive strains from non-invasive colonizers (Sachin et al. 2012). Therefore, phospholipase and hemolysin tests are important to identify and study the pathogenic strains of *C. albicans* in respect to the invitro activity of their hydrolytic enzymes.

Oropharyngeal candidiasis is an infection in the mouth and throat area. Usually, it is characterized by the formation of white patches on top of the tongue and throughout the mouth, which is also known as 'thrush' (Patil et al. 2015). Clinically, oral candidiasis may present as pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, denture-associated erythematous candidiasis, angular cheilitis, median rhomboid glossitis and chronic mucocutaneous candidiasis (Napenas et al. 2009; Farah et al. 2010). Pseudomembranous candidiasis or thrush is the most common presentation of oral candidiasis (Akpan and Morgan, 2002). It presents clinically as confluent whitish-yellow creamy or yellow velvety plaques on the surfaces of the oral mucosa (Reichart et al. 2000; Farah et al. 2010).

Candida has known to be opportunistic pathogen under tobacco chewing conditions (Javed et al. 2014; Hsia et al. 1981). It is suggested that individuals chewing tobacco are susceptible to oral *Candida* infections than non-chewers (Abdulijabbar et al. 2017). Clinically, there are some factors that predispose to oral candidiasis including drug therapy, especially broad-spectrum antibiotics, immuno modulatory and xerogenic medications, blood dyscrasias and malignancy, dietary factors, endocrine disorders, immunologic disorders, and salivary changes (Ashman and Farah, 2005). Local factors that predispose to oral candidiasis include irritation from ill-fitting dentures and poor oral hygiene (Ashman and Farah, 2005).

Studies have shown that the innate and adaptive immune systems play a role in the clearing of fungal growth. T Helper 1 cells are known to produce cytokines that activate phagocytes to clear the pathogens (Romani 2000). There is a significant burden of serious oral and oropharyngeal infections in Nepal (Khwakhali and Denning, 2015). In immunocompromised people, oropharyngeal candidiasis is a prevalent opportunistic infection (Lamichhane et al. 2020). To best of my knowledge, this is the first study on assessment of oral *Candida* carriage among smokers and tobacco users from Dharan. Lack of epidemiological estimates can increase the risk of infections among susceptible groups of community. So, it was essence to evaluate the health of smokers and tobacco chewers in perspective to oral pathology. Therefore, this study was conducted to determine the prevalence of *Candida* carriage from tobacco users and smokers in Dharan, Nepal. In addition, this study aimed to characterize *C. albicans* on basis of Phospholipase and hemolysin activity.

METHODS

Study Design

This was the cross-sectional laboratory based study conducted from June 2018 to November 2018 in which 50 smokers, 50 smokeless tobacco consumers and 50 non-tobacco consumers (control) were included. During the study 150 oral rinse samples were analyzed in Microbiology laboratory of Central Campus of Technology, TU, Hattisar, Dharan. This research was conducted after receiving Ethical approval from Nepal Health Research Council (NHRC approval Registration no. 296/2018), Kathmandu, Nepal. Informed consent and socio-demographic information from participants were obtained through written form and questionnaire respectively.

Inclusion and Exclusion criteria

Inclusive criteria included active smokers, smokeless tobacco (*Paan and Gutka*) consumers and non-tobacco users as control group. Exclusion criteria included people with alcohol intake, on antibiotics, antifungals and steroids treatment for last 3 months, having a partial or complete dental prosthesis, and having diseases such as oral candidiasis, cancer, organ transplant patient, diabetes mellitus, hepatitis B and hepatitis C infections, HIV infection.

Sample collection

The participants were allowed to rinse 10 ml of sterile saline for 1 minute and oral rinse was collected by spitting in a broader capped sterile container. The oral rinse samples were transported to microbiology laboratory, maintaining cold chain on same day. All the collected samples were labeled with participant's identification number and processed within 2 hours of collection. In case of delay, the sample was usually stored at 4°C in the refrigerator.

Isolation and identification

A 50 µl of oral rinse sample was inoculated in Sabouraud dextrose agar (HiMedia, India) with chloramphenicol (0.05 gm/l) and incubated at 37°C for 3-4 days. Pure culture was identified by colony characteristics and simple staining. The number of colony was counted by colony counter and expressed as CFU/ml. For the identification of *C. albicans*, germ tube and Chlamydospore formation was evaluated as described by Deorukhkar and Roushani, (2018). For germ tube test the pure isolated colony of *C. albicans* was dispensed in 0.5 ml of serum and incubated at 37°C for 2 hours. After incubation the aliquot was taken in a clean slide and was observed under oil immersion for the formation of germ tube. In addition, Chlamydospore formation test was performed in which the pure isolated and suspected colony was cultured in corn meal agar (HiMedia, India). *Candida* that could form germ tube and Chlamydospore in corn agar was identified as *C. albicans*.

Phospholipase assay

The phospholipase test was done according to Samaranayakae et al. (1984). *C. albicans* growth suspension was maintained at 0.5 McFarland standards. Egg yolk agar media was inoculated by 10 µL of the inoculum and allowed to dry at room temperature. The plates were incubated at 37ºC for 48 hours. Phospholipase index (Pz) was be measured by dividing the diameter of the colony by the sum of diameter of the colony and the zone.

Hemolysin assay

Hemolysin activity was evaluated according to Tsang et al. (2007). Hemolysin production by *C. albicans* was performed by inoculation overnight culture of yeast on sugar-enriched sheep blood agar. The blood base agar media was prepared by adding 5-7 ml of fresh blood to 100 ml Sabouraud glucose agar with 3% glucose. *C. albicans* growth suspension was maintained at 0.5 McFarland standards. About 10 µL of the yeast inoculum was placed at the center of the plates. The plates were incubated at 37ºC in 5% CO_2 for 48 hours. Hemolytic Index (Hz value) was calculated by dividing the total diameter of the colony by the translucent halo zone.

Quality Control

Strains of *Candida albicans* ATCC 24433 was used as reference strain for the study. Reagents and culture media were regularly checked for their expiry date and performance. Equipment was standardized, optimized and its performance was checked through positive and negative controls.

Data analysis

The data was documented in MS-EXCEL 2010 was analyzed using statistical Package for Social Sciences (SPSS) version 16.0. Chisquare ($\chi 2$) was used for statistical analyses. The p value of equal or less than 0.05 at 95% confidence interval was used for statistical significance.

RESULTS

The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group, 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The prevalence of *Candida* carriage among tobacco chewers and non-chewers was found to be statistically significant (P= 0.008). However, the carriage of *Candida* among smokers and non-smoker was not found to be statistically significant in this study (P= 0.059). The incidence of *Candida* carriage among smokers and smokeless tobacco users were similar. Isolation of *Candida albicans* was higher among smokeless tobacco users 15 (30%), smokers 5 (10%) and control 6 (12%) (Table 1).

The highest colony forming unit of *Candida* carriage was reported among smokers (220-1350 CFU/ml) and smokeless tobacco users (456-1900 CFU/ml) than in control groups (124-800 CFU/ml) (Table 2).

The phospholipase activity was screened from 13 (26%) of *C. albicans* isolated from smokeless tobacco users with Pz values range of 0.65-0.77 and 11 (22%) *Candida albicans* were screened for haemolysin activity with Hz range from 0.64-0.85. The phospholipase activity was screened from 5 (10%) of *C. albicans* isolated from smokers with Pz values range of 0.66-0.68 and 4 (8%) *C. albicans* were screened for haemolysin activity with Hz range from 0.63-0.84. The phospholipase activity was screened from 4 (8%) of *C. albicans* isolated from control groups with Pz values range of 0.62-0.73 and 2 (4%) of *C. albicans* were screened for haemolysin activity with Hz range from 0.62-0.82 (Table 3). *C. albicans* isolates from smokeless tobacco users and smokers were found to express high degree of phospholipase and hemolytic activity compared to control.

Status of Oral Hygiene

In this study, 38 (76%) of smokers were reported to brush their teeth once a day, 5 (10%) of smokers were reported to brush their teeth twice a day, 5 (10%) smokers were reported to brush their teeth sometimes and rest 2 (4%) never brushed their teeth. Similarly, 28 (56%) of smokeless tobacco consumers were reported to brush their teeth once a day, 7 (14%) were reported to brush their teeth twice a day, 10 (20%) were reported to brush their teeth sometimes whereas 5 (10%) never brush their teeth. In case of control group, 28 (56%) were reported to brush their teeth once a day and rest 22 (44%) were reported to brush their teeth twice a day. The prevalence of *Candida* carriage among groups with good and poor oral hygiene was statistically significant (P=0.001).

Table 1: Prevalence of *Candida* carriage and *Candida albicans* in study groups.

Study Population (n)	*Candida* Carriage N (%)	*Candida albicans* N (%)
Smokeless tobacco consumer (50)	26 (52%)	15 (30%)
Smokers (50)	22 (44%)	5 (10%)
Control groups (50)	13 (26%)	6 (12%)

Table 2: CFU/ml of *Candida* carriage among study groups.

Study Population (n)	Minimum CFU/mL	Maximum CFU/mL	Mean	Standard deviation
Smokeless tobacco users (50)	456	1900	980.30	365.03
Smokers (50)	220	1350	814	353.83
Control groups (50)	124	800	460.76	228.41

Table 3: Phospholipase (Pz) and Haemolysin (Hz) Index of isolated *Candida albicans*.

Study population	No. of Phospholipase producing *C. albicans*	Pz Range	No. of Hemolysis producing *C. albicans*	Hz Range
Smokeless tobacco users (50)	13 (26%)	0.65-0.77	11 (22%)	0.64-0.85
Smokers (50)	5 (10%)	0.66-0.68	4 (8%)	0.63-0.84
Control groups (50)	4 (8%)	0.62-0.73	2 (4%)	0.62-0.82

DISCUSSION

Candida being a part of normal flora of oral cavity can cause opportunistic infections when host is compromised (Jayachandran et al. 2016). Oral candidiasis is associated with poor oral hygiene, diabetic conditions, immunosuppressive therapy in cancer disease, and intake of tobacco (Guggenheimer et al. 2000).

Chewing tobacco that includes *Gutka, Betel quid* (BQ) which is common habit in South Asian nations like in India, Pakistan, Bangladesh, Sri Lanka and Nepal. *Betel quid* is mixture of areca-nut, lime enveloped in piper betel leaf whereas *Gutka* is found in sachet (Javed et al. 2013). The possible explanation for greater *Candida* colonization in *Gutka* (smokeless tobacco) consumers could be due to the presence of nicotine and hydrocarbons such as polycyclic aromatic hydrocarbons acting as nutrient for oral yeast facilitating its growth (Abdulijabbar et al. 2017; Hsia et al. 1981). Tobacco usage leads to an increase in thickness of epithelial keratinized layer (Bancozy et al. 2001), decrease in levels of salivary IgA and supression in functions of polymorphonuclear leukocytes (Bennet and Reade 1982, Keten et al. 2015), thus facilitates the proliferation of *Candida* species. *Candida* has known to be opportunistic pathogen under immunosuppression and tobacco chewing conditions (Javed et al. 2014; Hsia et al. 1981).

In consistent to other studies even in this study, higher prevalence of *Candida* carriage was found in smokeless tobacco chewers. The prevalence of *Candida* carriage was reported to be 26 (52%) among smokeless tobacco users and 13 (26%) among control groups. The *Candida* colonization and tobacco chewing was statistically significant (P=0.008). However, Javed et al. (2014) showed no significant difference in oral *Candida* colonization among tobacco chewers and non-chewers (control). This contradiction in the study could be due to difference in sample collection techniques involved in complete enumeration of *Candida* from oral cavity.

In this study the *Candida* carriage among smokers was higher in comparison to non-smokers (controls). The prevalence of *Candida* carriage was reported to be 22 (44%) in smoker group and 13 (26%) among control groups.

Keten et al. (2015) reported highest oral *Candida* colonization among smokers and smokeless tobacco users. It is also hypothesized that cigarette smoke enhances adhesion,

growth and biofilm formation of *C. albicans* (Semlai et al. 2014). This study is consistent with the literature. In this study, no significant differences were observed between *Candida* carriage with age, gender, number and duration of smoking, duration of smokeless tobacco usage.

The phospholipase and haemolysin assay of isolated *C. albicans* from smokeless tobacco users and smokers were found to be higher in comparison to control groups. Hydrolytic enzymes confer microbial pathogenicity by mediating its adhesion, tissue damage, immune system evasion and dissemination. The Pz index was higher among test subjects than in controls which agreed to study by Maheronnaghsh et al. (2019).

In this study Hz index of oral *C. albicans* was higher among smokers and smokeless tobacco users. Hemolysin is another virulence factor that degrades the red blood cells of host and iron is released which is taken up by the yeast cells (Pandey et al. 2018). Tsang et al. (2007) reported all *C. albicans* isolates from oral cavity positive for hemolysin activity. The reason and mechanism behind increased phospholipase and hemolysin activity of *C. albicans* isolated from Smokers and smokeless tobacco users cannot be explained from this study.

Poor oral hygiene in smokers and smokeless tobacco users may contribute to higher oral *Candida* carriage rates in this study. The prevalence of oral *Candida* carriage in healthy control groups having good oral hygiene was low as compared to others. The lower frequency of *Candida* carriage in healthy control might be due to maintenance good oral hygiene and practice. This study can explain that being a part of normal flora the colonization and proliferation of *Candida* is indeed required before establishing infection.

CONCLUSION

This study concludes that prevalence of oral *Candida* carriage was significantly higher in smokers and smokeless tobacco users compared to non-users. Higher prevalence of *Candida* in poor oral condition alarms oral health warnings. *Candida* induces infection under immune-compromised and host predisposing conditions, the poor oral hygiene risks the host to oral candidiasis. Thus, strong preventive measures to abstain from tobacco products can reduce the risk of oral infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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