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# Tribhuvan University
# Journal of Microbiology

Central Department of Microbiology
Tribhuvan University, Kirtipur, Kathmandu, Nepal

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## INTRODUCTION

Tribhuvan University Journal of Microbiology (TUJM) is an official, peer reviewed, biomedical journal of the Central Department of Microbiology. It is published annually and publishes articles in the category of original article, review article, case report, letter to the editor.

The aim of the TUJM is to promote the publication of articles related to microbiology. Authors do not have to pay for submission, processing or publication of articles in TUJM.


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## 1. The preparation and presentation of manuscripts

Manuscripts should be drafted as concisely as possible. By submission of a manuscript to the journal, all authors warrant that they have the authority to publish the material and that the paper, or one substantially the same, has neither been published previously, nor is being considered for publication elsewhere.

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The manuscript must be typed double-spaced on A4 size white paper with Times New Roman font, size of 12 points (In hard printing-Book Antiqua). Individual papers have a limit of approximately 4000 words, including figures and tables. The pages should be numbered consecutively beginning with the title page. The first page should show: (a) the title; (b) name(s) of author(s) and place(s) where the work was done; (c) an abbreviated running headline not exceeding 35 letters and spaces; (d) the name, complete mailing address, email address, telephone and fax numbers of the author to whom all correspondence should be addressed and who will check the proofs. English language used in the manuscript should be of a publishable standard.

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The paper should have new concepts or the recording of facts. The manuscript should be prepared for a wide readership. As far as possible, the paper should present the results of an original scientific research. The paper will have the following sections:

**(a) ABSTRACT:** A brief summary of about 150-200 words, should give the major findings of the investigation under the following headings: Objectives; Methods; Results; Conclusion. A list of between four and six keywords should be added.

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Laverick MA, Wyn-Jones AP and Carter MJ (2004) Quantitative RT-PCR for the enumeration of noroviruses (Norwalk-like viruses) in water and sewage. *Lett Appl Microbiol* **39**: 127-135.

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The Latin binomial name of micro-organisms, plants and animals (other than farm animals) must be givenat first mention in the text; thereafter the generic name will be abbreviated in such a way that confusion isavoided when dealing with several genera all beginning with the same letter, viz. *Pseudomonas*, *Proteus*, *Pediococcus*, etc. (see list of abbreviations below). Subspecies are italicized (*Corynebacterium diphtheria* subsp. *mitis*; groups and types are printed in Roman and designated by capital letters or Arabic figures (e.g. *Staphylococcus aureus* group A).

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## Tables

Tables must be prepared using the same word processing package as the manuscript text. They should

not be embedded but be placed immediately following the main text. Do not submit tables separately. Tables must not include ruled vertical or horizontal lines with the exception of headers and a footer. The use of explanatory footnotes is permissible and they should be marked by the following (shown in order of preference): *, †, ‡, §, ,**, †† etc.

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Figures may be line drawings or photographs. They may be uploaded to the online submission site as separate files or included within the manuscript following the text and any tables. Do not embed figures in the text. All graphs, charts and diagrams must be submitted in a finished form and at their intended publication size. Authors are advised that poor quality figures may delay the publication of their paper. Symbols or keys representing data series in graphs and charts must not be shown on the figure itself but be included in the legend typed on a separate sheet.

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These must be of good quality and high contrast. The magnification must be indicated by adding a bar representing a stated length. Composite photographs can reduce the numbers that require publication. The Journal will not accept figures illustrating SDS-PAGE and agarose gels, with multiple lanes, where lane order has been rearranged using digital imaging software. The figure should also show sufficient of the gel to reveal reference markers (e.g. the sample origin and a tracker dye, or a lane of molecular mass markers). Captions should be set out in the same manner as that used for figures.

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Data that is integral to the paper must be made available in such a way as to enable readers to replicate, verify and build upon the conclusions published in the paper. Any restriction on the availability of this data must be disclosed at the time of submission.

Data may be included as part of the main article where practical. We recommend that data for which public repositories are widely used, and are accessible to all, should be deposited in such a repository prior to publication. The appropriate linking details and identifier(s) should then be included in the publication and where possible the repository, to facilitate linking between the journal article and the data. If such a repository does not exist, data should be included as supporting information to the published paper or authors should agree to make their data available upon reasonable request.

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## Letter of Conflict of Interest (If applicable)

## 3.2 Review Articles

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The review manuscript should not be simply a review of past work or be concentrated largely on unpublished results from the laboratory. There should be a distillation of early and present work within the field to show progress and explain the present interest and relevance. It is essential at the planning stage to realize that there is a limit to the number of pages available. The final manuscript must not exceed 4000 words with double-spaced typing, including references. The Tables and Figures must be considered as part of the text and the pages available for text reduced accordingly. References can make a heavy demand on the pages available to you, and it is suggested that you select key references only.

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The headings in these review articles are of the author's choice. The first page of the manuscript must give only (a) the title; (b) name(s) of author(s) and address; (c) an abbreviated title to be used for the running title

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# Tribhuvan University Journal of Microbiology

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EDITORIAL

# Contribution of Microbiologists in Nepal during COVID-19 Pandemic

Coronavirus disease (COVID-19) caused by novel coronavirus initiated from Wuhan China affected the global population and spread in 218 countries and territories. In Nepal, the first case of COVID-19 was reported in 23 January, 2020. Central Department of Microbiology, Tribhuvan University and microbiologists in the country contributed to strengthen laboratory diagnosis of COVID-19 in Nepal from the very beginning of the pandemic in the country. The department participated in COVID-19 control effort of the Nepal Government. Team of four microbiologists collected blood samples from the people in quarantine from Kailali and Kanchanpur districts to validate RDT kit for detection of SARS-CoV-2 infection. The team from the Central Department of Microbiology, Tribhuvan University established SARS-CoV-2 diagnostic laboratory through real time PCR in Karnali Province, Surkhet. Similarly, microbiologists contributed to establish real time PCR laboratories for SARS-CoV-2 diagnosis in province 5, Bhairahawa, TU Teaching Hospital, province 2 Janakpur and Mechi Kankai laboratory Surunga. Further, the microbiologists also trained laboratory personnel for detecting SARS-CoV-2 genes using real time PCR.

Microbiologists involved in creating awareness through series of media interviews to people on coronavirus infection and prevention methods. Microbiologists assessed the COVID-19 diagnostic laboratories of the country and submitted report with recommendations to improve the diagnostic capacities. Microbiologists are working in many diagnostic laboratories of the country to diagnose SARS-CoV-2. Realizing the contribution of microbiologists in COVID-19 pandemic, these molecular diagnostic laboratories should be converted to infectious disease diagnostic and research laboratories in post COVID and knowledge and skills of the microbiologists should be utilized in the country.

**Dr. Megha Raj Banjara**
Chief Editor
Tribhuvan University Journal of Microbiology (TUJM)

# Antifungal Susceptibility Pattern of *Candida* Isolates Causing Vulvovaginitis in Reproductive Age Group Women

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## ABSTRACT

**Objectives:** The study was designed to isolate and identify *Candida* species from high vaginal swab, and to determine the antifungal susceptibility pattern of *Candida* spp. among women of reproductive age group i.e. 15- 50 years old.

**Methods:** High vaginal swabs were processed to isolate *Candida* species and identified by Gram's stain, germ tube formation test, carbohydrate (glucose, sucrose, maltose, lactose) fermentation tests and antifungal susceptibility test were performed as recommended by Clinical Laboratory Standard Institute.

**Results:** Out of total 261 samples, 42.1% (110/261) were found to be culture positive for *Candida* spp. *Candida albicans* was the most common (56.4%) and among non-albicans, predominate species was *C. krusei* (19.1%) isolated from high vaginal swab specimens All the isolates of *Candida* species were sensitive to Nystatin and Miconazole and resistant to Itraconazole. Fluconazole, Itraconazole and Clotrimazole are widely used drug against vaginal candidiasis but showed high resistance which leads to treatment failure.

**Conclusion:** This study on the infection rate of *Candida* and its antifungal susceptibility pattern may help in the choice of appropriate therapy in the clinical setting.

**Keywords:** Vulvovaginal Candidiasis, Vaginal swab, Antifungal Susceptibility test, *Candida albicans*

## INTRODUCTION

Vulvovaginal candidiasis (VVC) or candida vaginitis is a common fungal infection among women of reproductive ages and is caused by overgrowth of *Candida* species as an opportunistic pathogen. Symptoms of VVC appear when the balance between the normal microorganisms of the vagina is lost and population of *C. albicans* or other species of *Candida* becomes larger in relation to the other microorganism populations (Saigal et al. 2011). The most important pathogenic species of *Candida* are *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei* (CDC 2010). Although a number of fungal species belonging to the genus *Candida* can cause acute VVC, *Candida albicans* is by far the most prevalent etiological agent, particularly for the most severe chronic condition known as recurrent vulvovaginal candidiasis (Cassone 2015).

If untreated they can lead to pelvic inflammatory disease (PID), which can cause long-term sequelae, such as tubal infertility, ectopic pregnancy, reproductive dysfunction and adverse pregnancy outcomes. The most commonly prescribed treatment for vaginal candidiasis has been the topical application of clotrimazole and imidazole antifungal agent. Fluconazole has emerged as the primary treatment option for virtually all forms of susceptible *Candida* infections in both immune competent and immune compromised hosts. Nystatin

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is known to be effective in vitro against a variety of *Candida* species (Cavelic et al 2005).

Nepalese people survive with comparatively very poor health status in South East Asia because of illiteracy, lack of personal hygiene and sanitary knowledge, malnutrition, low economic status and lack of proper techniques in using medical procedures. This justifies people being victimized by many infectious diseases in our country. In this scenario, vaginal candidiasis remains one of the leading causes of the health problem in the country. In this regard, this study aims to investigate the prevalence of vulvovaginal candidiasis and antifungal susceptibility pattern of isolated *Candida* species from women with the reproductive age group visiting the Department of Gynecology and Obstetrics at Bharatpur Hospital, Chitwan which may be helpful toevaluate the current situation of vaginal candidiasis, effect of the antifungal drugs used for treatment and its management throughout the country.

## MATERIALS AND METHODS

A hospital-based cross-sectional study among reproductive age group (15-49 years) (Mulu et al 2015) visiting Department of Gynaecology and Obstetrics at Bharatpur Hospital of Chitwan, Nepal was carried out. Two hundred and sixty one individuals (as calculated by Raosoft sample calculator with 5% of error margin, 95% confidence interval) were included in the study by convenient sampling method from those willing to give consent and without the condition of HIV/AIDS and/or diabetes, after the study being reviewed and approved by the ethical approval board of Nepal Health Research Council with approval number 167/2015.

Two high vaginal swabs from each of 261 patients collected after preliminary disease diagnosis as VVC by gynecologists were used as samples. The swab samples were then transported to microbiology laboratory and processed as soon as possible according to standard laboratory methods (Cheesbrough 2000).

One of the swabs from each patient, was used for direct microscopy test by Gram staining and another used for culture on two different media- Sabouraud Dextrose agar (HiMedia) supplemented with 0.05 mg/l chloramphenicol and Hichrom agar (HiMedia). Then plates of SDA/chloramphenicol and HiChrom agar were incubated at 35-37°C for 24- 48 hours. Identification of isolates was done by appearance of colonies, gram's stain reaction, germ tube formation test and biochemical characteristics (Cheesbrough 2000). *Candida* species were identified by the color of colonies in HiChrom agar.

Identification of *Candida* species was followed by antifungal susceptibility test was performed. The test was done by Kirby-Bauer disc diffusion technique according to the procedure described in the Clinical and Laboratory Standard Institute (CLSI-M44-A 2004); a standard dilution of the test isolate was prepared by matching it with 0.5 McFarland turbidity standard and was uniformly swabbed over the antifungal susceptibility assay agar (CLSI 2004). The antifungal discs (Hi-media, Mumbai, India) of fluconazole (25mcg), nystatin (50mcg), miconazole (30mcg), itraconazole (10mcg), amphotericin B (10mcg) and clotrimazole (10mcg) were placed on the medium and incubated at 37°C for 48 hours. After incubation period, the zone of inhibition were measured and results were interpreted as per the guidelines given by the Clinical Laboratory Standard Institute (CLSI).

Chi square test was used to evaluate apparent differences for significance at 95% confidence level. Associations of *Candida* isolates with different variables were tested. Results were considered significant if p-value was less than 0.05.. Statistical Package for the Social Sciences (SPSS version 21.0) was used for the Chi square test.

## RESULTS

From total of 261 high vaginal swab samples, 110 (42.15%) were found to be positive for vulvovaginal candidiasis. Out of 110 positive case, the patients of age group 30-35 years were mostly infected27/110 (24.5%) and minimum was of age group 15-20 years 5/110 (4.5%). However, the relation of infection with age group was not statistically significant (Table 1).

**Table 1: Age wise distribution of participants with growth positive case**

| Age Group | Frequency | Positive Number/Percentage (%, n=110) | p-value |
|---|---|---|---|
| 15-20 | 7 | 5 (4.5%) | p>0.05 |
| 20-25 | 37 | 22(2%) | |
| 25-30 | 42 | 22(20%) | |
| 30-35 | 67 | 27(24.5%) | |
| 35-40 | 63 | 17(15.5%) | |
| 40-45 | 28 | 11(10%) | |
| 45-49 | 17 | 6(5.5%) | |
| Total | 261 | 110(100%) | |

Among 72 women who used different types of contraceptives, 29 (26.4%) were found to be infected. Out of 189 contraceptive non-user women, 81(73.6%) were found to be infected. The relation between infection and use of contraceptives was not found to be statistically significant (Table 2).

**Table 2: Occurrence of VVC with the practice of contraceptive use**

| Contraceptive used | Growth positive | Growth negative | Total | p-value |
|---|---|---|---|---|
| | Number (%) | Number (%) | | |
| User | 29 (26.4%) | 43 (28.5%) | 72 | p>0.05 |
| Non-user | 81 (73.6%) | 108 (71.5%) | 189 | |
| Total | 110 | 151 | 261 | |

Five different species of *Candida (C. albicans, C. krusei, C. tropicalis, C. parapsilosis and C. dubliniensis)*were identified and few isolates were not identified so they were named as *Candida* species (Figure 1). *Candida albicans* was the most predominant isolated species. Among 110 infected samples, 82.72% contained isolates of only one species while the remaining samples were infected with more than species of *Candida* (Figure 2).

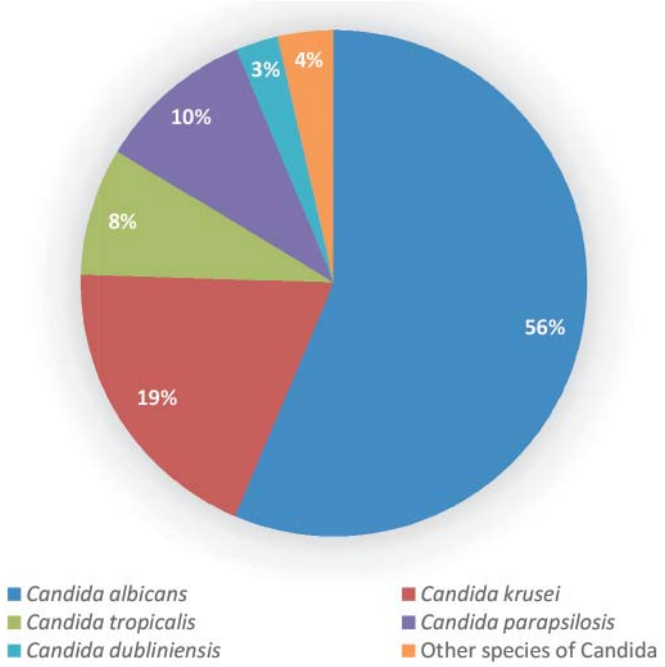


**Figure 1: Distribution of *Candida* species isolated from specimen**

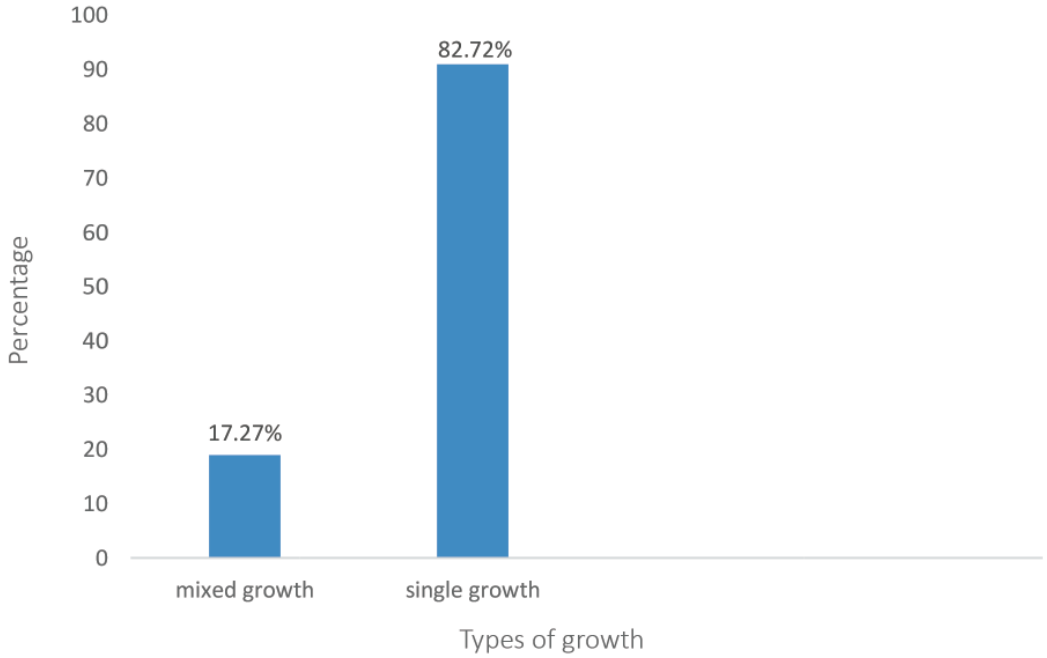


**Figure 2: Occurence of single and mixed growth**

Antifungal susceptibility test showed all the *Candida* isolates were susceptible to miconazole and nystatin. Besides, higher numbers of isolates (55%) were resistant to itraconazole as compared to other antifungals tested. In particular, *Candida albicans* showed least susceptibility to itraconazole.(Table 3).

**Table 3: Antifungal susceptibility pattern of isolated *Candida* species (n=? 110)**

| Antifungal agents | Susceptible (%) | Intermediate (%) | Resistance (%) |
|---|---|---|---|
| Fluconazole | 49 | 11 | 40 |
| Miconazole | 100 | - | - |
| Nystatin | 100 | - | - |
| Itraconazole | 11 | 34 | 55 |
| Amphotericin B | 54 | 45 | 1 |
| Clotrimazole | 53 | 32 | 15 |

**Table 4: Antifungal susceptibility pattern of *Candida albicans* isolates (n=? 62)**

| Antifungal agents | Susceptible (%) | Intermediate (%) | Resistance (%) |
|---|---|---|---|
| Fluconazole | 74.2 | 9.7 | 16.1 |
| Miconazole | 100 | - | - |
| Nystatin | 100 | - | - |
| Itraconazole | 16.1 | 50 | 33.9 |
| Amphotericin B | 67.7 | 30.6 | 1.6 |
| Clotrimazole | 75.1 | 22.6 | 1.6 |

## DISCUSSION

The study showed 42.1% prevalence of VVC among women with reproductive age which is similar with study carried out by Bello et al (2002) reported that 41% prevalence of VVC and other studies as 34.2% in Ghana and 65% in South Nigeria. Thus the results may offer a reasonable indication of the prevalence of defined yeast species in vaginitis. In contrast to this study, Rad et al (2012) concluded that 89.7% incidence of VVC in Iran whereas Jindal et al (2007) and Al-aKeel et al (2013) revealed only 23% and 22% prevalent of VVC among women with child bearing age respectively. The study

conducted by Bohara et al (2012) at Gynecological Outpatients Department of Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal concluded that out of 62% RTIs, vaginal candidiasis was found to be 25%.

The rate of infection of *Candida* species was highest among women of age group 30-35years (24.5%) and least for 15-20 years group (4.5%). Shrestha et al (2011) found the prevalence of vaginitis was common in the age group 20-29 and 30-39 years Manandhar et al (2005) observed the highest prevalence of vaginitis among the age group 25 to 29 years and least below 20 years age group. The highest prevalence in the age group 30-35 years might be due to the age being the most reproductively active age group and the high sexual exposure at this age and most of the women participated in this study were included from this age group. It is extremely rare before menarche, and its annual incidence increases dramatically towards the end of the second decades of life and peaks over the next two decades. Young women are particularly susceptible to infection because they have fewer antibodies to fight against pathogens (Prasad et al 2005). The prevalence of vaginitis is low among women with less than 20 years and after 40 years. This might be because during this period, vaginal dryness occurs or it may be because they have less sexual activity. The finding obtained by Crampin et al (2005) was somewhat different than this study in which the highest prevalent of RTIs among 16 to 22 years was reported.

From the study, it is clear that there is not significant relation in VVC infection and contraceptive use. This type of result is due to the involvement of contraceptive non-user women in higher number. However, Al-akeel et al (2013) reported that oral-contraceptive users were more likely to developed VVC than non-users.

One of the most important risks for positive *Candida* species from high vaginal swabs includes recent use of antibiotic/antifungal drugs. This study revealed that there is significant relation in VVC infection and recent drug use. The study carried out by Odysseas et al (2006) concluded that recent antibiotic use correlated positively with both *C. albicans* and non-albicans isolates.

The results of this study identified *Candida albicans* (56.36%) as the most common species of *Candida* causing vaginal candidiasis and among non-*albicans* species, *Candida krusei* was found to be common species (19.1%).This is probably due to the fact that in the general population, *C. albicans* predominates over other species. The results in this study are consistent with previous study by Akortha et al (2009) which reported that *Candida albicans* are the most prevalent with prevalent rates of 64%.

The current findings however contradict the earlier report by Devi and Mahehwari (2014) who reported among the non-albican species, *Candida tropicalis* was most predominant species followed by *C. krusei*. . Similar observations were found from other studies (Hollamdia and Young 2003; Spinillo et al 1994). An overall non albicans percentage were 24, 17 and 32 respectively reported by each of these researchers. Therefore, non-*albicans Candida* species are emerging significant pathogens (Moran et al 2003). This variation in reports may be attributed to the different sample sizes used in the studies.

In the study, Miconazole and Nystatin were found to be sensitive against almost all isolates of *Candida* species. Amphotericin B, Clotrimazole and Fluconazole have been reported as most active antifungal agents. Similar result was revealed by Salehei et al (2012), vaginal isolates of *Candida* were most sensitive to Miconazole, Clotrimazole, and Terbinafine, and least sensitive to Econazole, followed by Fluconazole and Ketoconazole. Another study by Vijaya et al (2011) found that 100% isolates were sensitive to Amphotericin B, Clotrimazole, Nystatin and Ketaconozole.

## CONCLUSION

Most commonly isolated species in this study was *Candida albicans* and among non *albicans* species, *Candida krusei* was most frequently isolated species. Routine speciation of *Candida* is becoming increasingly important because vaginal candidiasis due to non *albicans* species of *Candida* is increasing nowadays Antifungal susceptibility testing for isolated *Candida* species from high vaginal swab is important because resistance pattern of *Candida* species with routinely used antifungal agents is globally increasing. Almost all *Candida* species isolated in this study were susceptible to Nystatin and Miconazole. Fluconazole, Itraconazole and Clotrimazole are widely used drug against vaginal candidiasis but showed high resistance which may lead to treatment failure.

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# Antibacterial Activity of Common Spices Extracts on Bacterial Isolates found in *Kachhila*, a *Newari* Cuisine

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## ABSTRACT

**Objectives:** The objective of this study was to determine the antibacterial property of aqueous extracts of common spices used in the preparation of *Kachhila* such as garlic, ginger, and turmeric.

**Methods:** After washing and cleaning separately, aqueous extracts were extracted from each spice by crushed with mortar and pestle. Antibacterial activity of aqueous extracts of each spice was evaluated by using agar well diffusion assay and paper disc diffusion assay against test bacteria isolated from buff meat used in *Kachhila* preparation.

**Results:** Minimum inhibition concentration value of each spice was evaluated against all bacterial isolates. Total bacterial count (CFU/g) of prepared *Kachhila* was determined in each 30 mins interval up to 150 mins to reveal antibacterial activities of spices used in *Kachhila*. The antibacterial effect of aqueous extract of garlic extract was found to be the stronger in comparison, followed by turmeric and ginger against test bacterial isolates. The MIC of individual spice extract was found to be 125 to 4000 µl/ml against all the test bacteria. Spices used in *Kachhila* revealed that bacterial load decreased with time due to the antibacterial property of spices.

**Conclusion:** This study concluded that the antibacterial effect of aqueous extract of Garlic extract was stronger in comparison, followed by Turmeric and Ginger against four test bacteria isolated from buff meat namely; *E. coli, Salmonella* spp., *Pseudomonas* spp., *Staphylococcus aureus*. Therefore this study revealed that spices used in *Kachhila* have an antibacterial property and enhance the shelf life of *Kachhila.*

**Keywords**: Antibacterial property, Spices, Minimum Inhibition Concentration, *Kachhila*

## INTRODUCTION

*Kachhila*, one of the most popular snack items belonging to the *Newari* community, is made of raw meat. One among the two of the most popular cuisines, *Choila* and *Kachhila*, it is a cuisine especially unique to the *Newari* community. The *Newari* community is considered one of the richest communities in terms of culinary experience (Personal communication). The term meat refers to the flesh, skeletal muscle, and any attached connective tissue or fat excluding bone and bone marrow (Williams 2007). Meat is a good source of protein, essential fatty acids, minerals and vitamins but easily perishable because it provides a suitable medium for the growth of various microorganisms (Komba et al. 2012). Buffalo meat is the healthiest meat among red meats, known for human consumption because it is low in calories and cholesterol (Ranjhan 2013). It has almost 2-3 folds cost advantage over mutton and goat meat (APEDA 2008). The most important food-borne bacteria transmitted through meat include *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Clostridium perfringens*, *Yersinia enterocolitica,* and *Aeromonas hydrophila* (Zhao et al.2001; Bhandare et al. 2007).

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Spices have been used for many centuries by various cultures to enhance the flavor and aroma of our foods as our ancestors have recognized the usage of spices in food preservation and in the treatment of clinical ailments and there are several reports on the development of antibiotic resistance in diverse bacterial pathogens (Oli 2011). Spices are indispensable components of Nepalese cuisines since ancient times (Mahanrjan et al. 2019). The activity of herbs and spices is not only used to boosting flavor but also recognized for their preservative and medicinal value (Panpatil et al. 2013). The burgeoning concern about the safety of foods has recently led to the development of natural antimicrobials to control food-borne pathogens (Maharjan et al. 2019). The addition of spices in food imparts not only flavor and aroma of the foods but also provides antimicrobial properties (Nanasombat et al. 2002), due to the presence of their naturally derived components (Maharjan et al. 2019). Spices are parts of a plant that are used for providing the aroma, flavor, or piquancy to food and also for seasoning the food (Oli 2011). Spices have been defined as plant substances from the indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of foods (Germano and Germano 1998). Basically, spices are used for flavouring, masking the bad flavor of some foods, appetizer preservation, colouring of food, ayurvedic medicines, and even in cosmetics and perfumes. The composition of spices includes proteins, lipids, carbohydrates, vitamins, and various mineral compounds. The other constituents, however, are essential oil and oleoresins which give characteristic taste and flavor (Parry 1969).

Spices may contribute piquancy of foods and beverages (Praveen et al. 2006). Spices have been widely used in rituals and as flavorings and coloring agents since ancient times (Gottardi et al. 2016), but, various recent studies have increasingly reported on the antibacterial activity of spices against common Gram-positive and Gram-negative bacteria responsible for human infectious diseases and food safety problems (Irshad et al. 2017). Spices containing antimicrobial compounds are some of the most commonly used natural antimicrobial agents in foods (Indu et al. 2006). Therefore, actions must be taken to control this problem by using the plant extracts containing phytochemical having antimicrobial properties. (Agaoglu et al. 2007). Spices have been recognized for their value of preserving foods and medicinal values due to the presence of bioactive antimicrobial compounds (Papp et al. 2007). Being plants, the natural foodstuffs, spices appeal to consumers who tend to question the safety of synthetic additives (Sagdiç et al. 2003). The spices have a unique aroma and flavor which are derived from compounds known as phytochemicals or secondary metabolites (Avato et al. 2000). The phytochemicals are antimicrobial substances present in the spices which are capable of attracting benefits and repel harmful organisms; they also serve as photoprotectants and responds to environmental changes. Numerous classes of phytochemicals including the isoflavones, anthocyanins, and flavonoids are found associated with the spices (Feldberg et al. 1989).

The meat is a nutrient-dense medium ideal for many pathogens and spoilage microbes to colonize because of its high in moisture, rich in nitrogenous foods of various complexity, plentifully supplied with minerals and accessory growth factor, usually has some fermentable carbohydrate (glycogen), and is at favorable pH (Frazier and Westhoff 2009). The bacterial pathogens most frequently identified from illness associated with beef products are *Salmonella* spp., *Campylobacter, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Clostridium perfringens, Yersinia enterocolitica, Bacillus cereus*, and *Vibrio parahaemolyticus* (Gandhi and Chikinda 2007). Hence the main aim of this study is to isolate the bacteria found in the meat sample used for *Kachhila* and to evaluate the antimicrobial property of the extract from respective spices against the isolated bacteria.

## MATERIALS AND METHODS

### Collection of Spices

The ginger roots, fresh garlic, and turmeric were collected in plastic bags from the Dharan market in March 2019 and this study was carried out up to May 2019 months in the microbiology laboratory of Central Campus of Technology, Hattisar, Dharan.

For the study of antimicrobial activity of spices extracts, their different concentrations were tested on different test bacteria (*Staphylococcus aureus, E. coli*, *Salmonella* spp. and *Pseudomonas* spp.) isolated from buff meat ready for the preparation of *Kachhila.*

### Isolation of Bacteria from Buff Meat

About 5 g of buffalo meat sample was collected in a clean, dry, and sterile polythene bag from the local market of Dharan and transported to the laboratory and

immediately processed for isolation of bacteria within one hour or refrigerated at 4°C till further analysis were carried out and processed no later than 10 hours after purchase.

1 gram of the sample was first crushed and ground in a sterile mortar with the help of a sterile pestle and the 9 ml of sterile distilled water was added and further proceeded to serial dilution. Isolation of test bacteria was carried out from the various dilutions by using various selective medium and differential media by spread plate technique (Jarallah et al. 2014).

After gram staining, biochemical tests were performed for the identification of the genera and species of the organisms. The identification of significant isolates was done by using standard microbiological techniques as described in Bergey's Manual (Holt et al. 1994). After identification, the antibiotic susceptibility test of the isolates was done by the modified Kirby-Baur disc diffusion method as recommended by the Clinical Laboratory Standards Institute using Muellen-Hilton Agar (MHA) (CLSI 2006).

**Preparation of Spices Extract**

The collected spices were cleaned, descaled when necessary, and washed in sterile distilled water. In order to obtain the spice's extracts, about 100 g of each washed spice was crushed with mortar and pestle. The extracts were sieved through a fine mesh cloth and sterilized using a membrane filter (0.45-micron sterile filter). This extract was considered as the 100% concentration of the extract. The concentrations 75%, 50% and 25% were made diluting the concentrated extract with appropriate volumes of sterile distilled water (Joe et al., 2009).

Garlic extract was made differently due to the difficulty to filter the crushed material. One hundred grams of the descaled and cleaned garlic was taken and surface sterilized using ethanol. The ethanol was allowed to evaporate in a sterile laminar flow chamber, and the garlic was homogenized aseptically using a sterile mortar and pestle. The extract was aseptically squeezed out using sterile cheesecloth as done by Indu et al. (2006).

The aqueous extract (8 ml of 1000 µl/ml or 100%) was concentrated to 1 ml to make 8000 µl/ml as a stock solution by using heat at 40° C in a water bath. Then it was diluted by half fold dilutions (4000 µl/ml, 2000 µl/ml, 1000 µl/ml, 500 µl/ml, 250 µl/ml, 125 µl/ml, 62.5 µl/ml) for determination of MIC value of each spice extract against test bacteria.

**Antibacterial Activity Testing Using Agar Well Method (Cup Plate Method)**

The isolated bacteria from meat going to be prepared as *Kachhila* by using their selective media were inoculated into 10 ml of sterile nutrient broth and incubated at 37° C for 16-18 hours (0.5 Mc Far-land Standards).

Using a sterile cotton swab, the nutrient broth culture was swabbed on the surface of sterile Mueller-Hinton Agar (MHA) plates. Agar wells were prepared with the help of a sterilized cork borer with a 6 mm diameter. Using a micropipette, 100 microlitres of different concentrations of spices extracts (100%, 75%, 50%, and 25%) were added to different wells on the plate. The plates were incubated in an upright position at 37° C for 24 hours.

The diameter of inhibition zones was measured in mm and the r were recorded. The inhibition zones with a diameter of less than 6 mm were considered as having no antibacterial activity.

**Antibacterial Activity Testing Using the Filter Paper Disc Method**

For comparison with agar cup methods of antimicrobial assay, the filter paper disc method was also applied. Filter paper discs of 6 mm diameter were prepared and sterilized (Paper disc thickness taken was 0.05 mm). Using ethanol dipped and flamed forceps, these discs were aseptically placed over Mueller-Hinton Agar (MHA) plates seeded with the respective test microorganisms (Srinivasan et al., 2001). Ten microlitres of the various spices' extract from stock (10 times concentrated) was aseptically transferred to these discs. The plates were incubated in an upright position at 37° C for 24 hours. The diameter of inhibition zones were measured in mm and the results will be recorded.

- The concentration taken for filter paper disc assay was 10 times so that the amount of spice extract poured in agar cup was equivalent to the amount in paper disc.

**Antibiotic Susceptibility Testing**

The test microorganisms was also tested by modified Kirby Bauer Disc Diffusion methods for their sensitivity against the antibiotics (Hi-media, India) such as chloramphenicol (30 mcg), ciprofloxacin (5 mcg), nalidixic acid (30 mcg), streptomycin (10 mcg) and tetracycline (30 mcg) by the disc diffusion method.

The cultures were subcultured in sterile nutrient broth for 6-8 hours) at 37° C and turbidity was matched with that of 0.5 Mac Farland standard . Using sterile cotton swabs, the cultures were aseptically swabbed on the surface of sterile Mueller-Hinton Agar (MHA) plates. Using an ethanol dipped and flamed forceps, the antibiotic discs were aseptically placed over the seeded MHA plates sufficiently separated from each other to avoid overlapping of the inhibition zones. The plates were incubated at 37° C for 24 hours and the diameter of the inhibition zones were measured in mm. All the media used in the present investigation was obtained from Hi-media Laboratories Ltd., Mumbai, India.

**Preparation of Control Plate**

One control plate was prepared in each experiment by agar well diffusion to sterilized distilled water instead of ginger, garlic, and turmeric extract. Control plate was essential and very important to measure and determine whether the spice extract was the substance that will kill the bacteria and not cause by other elements and factors. The inhibitory effect of these spices was compared with that of 5 control antibiotics (chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin and tetracycline).

**Evaluation of Bacterial Load After Preparation of *Kachhila***

Buff Meat was minced into suitable small pieces that were used to prepare *Kachhila* according to a standard protocol. Bacterial load before and after use of spices in preparation of *Kachhila* were evaluated by performing serial dilution of 1 g sample followed by spread plate technique on Plate count agar. After preparation of *Kachhila* without adding salt, bacterial load was determined at various intervals of time viz. 0 mins, 30 mins, 60 mins, 90 mins, 120 mins and 150 mins respectively.

## RESULTS

A total of 3 different spices (Garlic, Ginger and Turmeric) were included in this study, collected from local market of Dharan. The crude extracts of spices were tested against altogether four test bacteria isolated from buffalo meat usually used for preparation of *Kachhila.*

**Antibacterial Activity Testing Using Agar Well Method (The Agar Cup Plate Method)**

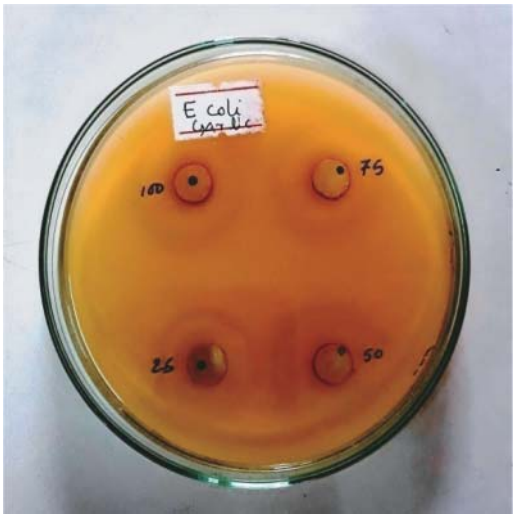
Overall, it was observed that among three spices, aqueous extracts of Garlic had inhibitory effect on both gram-positive and gram-negative bacteria.The different concentrations (100%, 75%, 50% and 25%) of aqueous extract of garlic showed least inhibition values (15 mm, 12 mm, 11 mm and 6 mm) against *E. coli* and highest inhibition values (21 mm, 20 mm, 17 mm and 11 mm) against *S. aureus.* Similarly, the different concentrations (100%, 75%, 50% and 25%) of aqueous extract of garlic showed zone of inhibition values such as 18 mm, 17 mm, 16 mm and 11 mm against *Salmonella* spp. and 19 mm, 18 mm, 16 mm and 7 mm against *Pseudomonas* spp. respectively (Table 1).

**Table 1: Antibacterial activity of spices extract in different percentage against test bacteria (the agar cup assay method)**

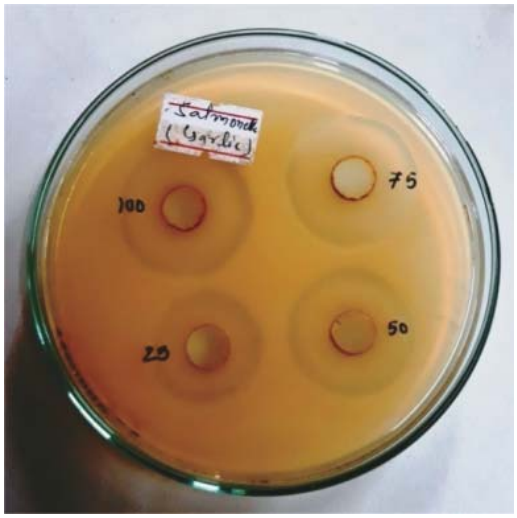
| Bacteria | Diameter of inhibition zone (in mm) against various concentrations of spices extract (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Garlic* | | | | *Turmeric* | | | | *Ginger* | | | |
| | 100 | 75 | 50 | 25 | 100 | 75 | 50 | 25 | 100 | 75 | 50 | 25 |
| E. coli | 15 | 12 | 11 | 6 | - | - | - | - | - | - | - | - |
| *Salmonella* spp. | 18 | 17 | 16 | 11 | - | - | - | - | - | - | - | - |
| *Pseudomonas* spp. | 19 | 18 | 16 | 7 | - | - | - | - | - | - | - | - |
| S. aureus | 21 | 20 | 17 | 11 | - | - | - | - | - | - | - | - |

**Antibacterial sensitivity testing using the filter paper disc diffusion method**

Discs containing 100% garlic extracts showed 9 mm against *E. coli*, 13 mm against *Salmonella* spp., 12 mm against *Pseudomonas* spp., and 15 mm against *S. aureus* (Table 2). Based on zone of inhibition, Antibacterial activity of Garlic was found to be less effective by paper disc diffusion method than agar well method.

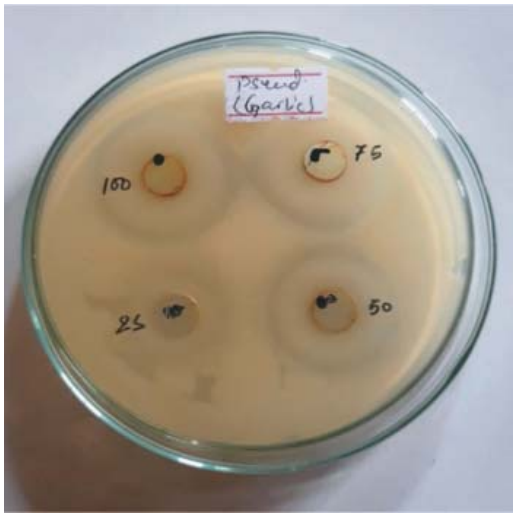



**Photograph 1: Zone of inhibition of garlic against *Escherichia coli***




**Photograph 2: Zone of inhibition of garlic against *Salmonella* spp.**


**Photograph 3: Zone of inhibition of garlic against *Staphylococcus aureus***




**Photograph 4: Zone of inhibition of garlic against *Pseudomonas* spp.**

**Table 2: Antibacterial activity of spices extract in different percentage against test bacteria (the filter paper disc diffusion method)**

| Bacteria | Zone of inhibition (in mm) of spices extract (100%) against test bacteria | | |
|---|---|---|---|
| | *Garlic* | *Turmeric* | *Ginger* |
| | 100 | 100 | 100 |
| E. coli | 9 | - | - |
| *Salmonella* spp. | 13 | - | - |
| *Pseudomonas* spp. | 12 | - | - |
| S. aureus | 15 | - | - |

**Antibiotic Susceptibility Testing**

All isolates except *Staphylococcus aureus* were resistant to chloramphenicol. *E. coli* and *Staphylococcus aureus* were found to be intermediate while *Salmonella* spp. and *Pseudomonas* spp. were found to be resistance to ciprofloxacin.

*Salmonella* spp. and *Staphylococcus aureus* were susceptible whereas *Pseudomonas* spp. was resistant but *E. coli* was intermediate to nalidixic acid. *Pseudomonas*

spp. was intermediate to streptomycin while remaining was susceptible to streptomycin. Both *Salmonella* spp. and *S. aureus* were found to be susceptible to tetracycline while *Pseudomonas* spp. was resistant but *E. coli* was found to be Intermediate (Table 3).

**Table 3: Antibiotic susceptibility pattern of isolated test bacteria from buff meat**

| Organism | Zone of inhibition against antibiotic (mm) | | | | |
|---|---|---|---|---|---|
| | Chloramphenicol (30) | Ciprofloxacin (15) | Nalidixic acid (30) | Streptomycin (10) | Tetracycline (30) |
| E. coli | - (R) | 20(I) | 21(I) | 23(S) | 8(I) |
| *Salmonella* spp | -(R) | 16(R) | 23(S) | 16(S) | 15(S) |
| *Pseudomonas* spp | 22(R) | 22(R) | (R) | 20(I) | 16(R) |
| S. aureus | 24(S) | 22(I) | 19(S) | 22(S) | 24(S) |

**Determination of MIC of Spices Extract Against Test Bacteria**

MICs of three spices extracts were determined for all four test bacterial isolates (*E. coli, Salmonella* spp., *Pseudomonas* spp., *and Staphylococcus aureus*).

For garlic extract, MIC values were 125 µl/ml against *Salmonella* spp. and *Staphylococccus* spp., and 250 µl/ml against *E. coli* and *Pseudomonas* spp. For turmeric extract, *E. coli* and *Staphylococcus aureus* have similar MIC value (i.e. 1000 µl/ml) as well as *Salmonella* spp. and *Pseudomonas* spp. have also similar MIC (i.e. 2000 µl/ml). In case of Ginger extract, MIC was found to be 4000 µl/ml against all four bacteria (Table 4).

**Table 4: Minimum inhibitory concentration (µl/ml) of spices extract against test bacteria.**

| Spices extract | *E. coli* | *Salmonella* spp. | *Pseudomonas* spp. | *Staphylococcus aureus* |
|---|---|---|---|---|
| Garlic | 250 | 125 | 250 | 125 |
| Turmeric | 1000 | 2000 | 2000 | 1000 |
| Ginger | 4000 | 4000 | 4000 | 4000 |

**Evaluation of Bacterial Load in *Kachhila***

The bacterial load of fresh buff meat was found to be $26x10^4$ CFU/g. Again the bacterial load was determined from prepared *Kachhila* without adding salt and found to be $53x10^4$ CFU/g, $85x10^4$ CFU/g, $76x10^4$ CFU/g, $64x10^4$ CFU/g, $51x10^4$ CFU/g and $44x10^4$ CFU/g at 0 mins, 30 mins, 60 mins, 90 mins, 120 mins and 150 mins respectively. This showed bacterial load in *Kacchila* was decreased with time due antibacterial activities of spices used (Table 5).

**Table 5: Bacterial load per gram meat before and after use of spices in preparation of *Kachhila***

| Sample (1 g) | Time (mins) | Bacterial load (CFU/g) |
|---|---|---|
| Meat | Market meat before addition of spices without salt | 26x 104 |
| *Kachhila* | 0 mins | 53x104 |
| *Kachhila* | 30 min | 81x104 |
| *Kachhila* | 60 mins | 76x104 |
| *Kachhila* | 90 mins | 64x104 |
| *Kachhila* | 120 mins | 51x104 |
| *Kachhila* | 150 mins | 44x104 |

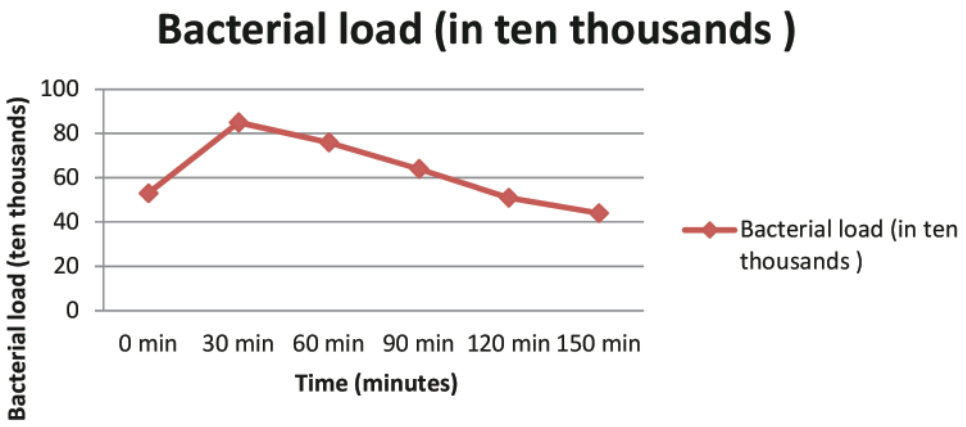



**Figure 1: Time (mins) wise activity of spices on bacterial load (CFU/g) in *Kachhila***

## DISCUSSION

Buffalo meat has gained importance in the recent years because of its domestic needs and export potential. Buffalo meat is well comparable to beef in many of the physicochemical, nutritional, functional properties and palatability attributes (Anjaneyulu et al. 1990). Furthermore, its utility in meat processing is on increase because of higher content of lean meat and less fat. The microbiological condition of fresh raw meat of local market of Dharan can be assumed to be heavily contaminated with spoilage and pathogenic organisms (Bantawa et al. 2018). The increased resistance of isolates against commonly used antibiotics may be due to the indiscriminate use of common antibiotics. The variation on the rate of resistance can be related to the difference in time and place. Another reason for the difference in resistance rates might be a rapid change in antibiotic sensitivity patterns of bacteria within a short period (Bantawa et al. 2019). The prevalence of antibiotic-resistant bacteria is increasing due to the haphazard use of antibiotics in human therapy, animal farming and other prophylactic usages (Addis et al. 2011).

Resistance enables bacteria to escape from being killed by antibiotics and reduces the ability to treat infections (Spellberg et al. 2008). Therefore, antibiotics resistance has been considered one of the greatest threats to medicine (Walker et al. 2009). Meat also plays an important role in the transfer of antibiotics resistant genes in term of antibiotic residues.

Foodborne pathogens are the leading causes of illness and death in less developed countries (Blaser 1997). Therefore, nowadays, the awareness has been growing on the public health impact of zoonotic foodborne pathogens transmitted from animal originated food in especially developing countries (Zhao et al., 2001). The growing concern about food safety has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria. Spices are one of the most commonly used natural antibacterial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour (Punir and Jain 2010). Many of the spices and herbs used today have been valued for their antibacterial effects and medicinal powers in addition to their flavor and fragrance qualities (Bin et al. 2007)

A wide range of technologies is available for the extraction of active components and essential oils from medicinal and aromatic plants. The choice depends on the economic feasibility and suitability of the process to the particular situation. Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper than modern medicine (Duhan et al. 2013).

This study was performed to evaluate the antibacterial activity of aqueous extracts of some common spices against common gram positive and gram negative bacteria found in buff meat used in preparation of *Kachhila*. This study showed aqueous extracts of garlic had a strong effect against all bacteria, while that of turmeric had a weak effect. However, the Ginger extracts could show no zone of inhibition against any test bacteria.

Different serogroups responded differently to the garlic extract at different concentrations. At 100% concentration *E. coli* serogroup O1 was least sensitive (18 mm) and serogroup O22 and O25 were more sensitive (30 mm). Both enterohemorrhagic *E. coli* (EHEC, serogroup O157) and enterotoxigenic *E. coli* (ETEC, serogroup O8) were highly sensitive to garlic extract (Indu et al. 2006).

The results showed that the bacterial load in prepared *Kachhila* decreased with time, however, in first 30 mins, the bacterial load was found to be increased and then decreased. It was basically due to antibacterial property of spices used in *Kachhila* which is itself a good source of nutrients for bacterial growth and at the same time, spices could not show antibacterial property due to deficient antibacterial extract from spices. These findings proved antibacterial property of spices which increased shelf life of *Kachhila* by decreasing bacterial load that might cause meat spoilage in sufficient numbers along with time. From these results, Bacterial growth in *Kachhila* was found to be resultant of growth promoting factors (Meat nutrients, moisture temperature etc) and growth retarding factors (spices extract). As spices have antibacterial property, they can be used for other various food items for better taste and preservation. According to Venugopal et al. (2018), addition of herbs and spices to the food preparations helps to keep a check on the concentration *Escherichia coli* in the body.

The inhibitory effect of these spices was compared with that of 5 antibiotics (chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin and tetracycline) and the results are discussed.

According to Indu et al. (2005), the antibacterial activity of the spices was less evident in paper disc method than that of the agar cup assay method; however garlic extract (100%) maintained good antibacterial activity against all the test organisms.

The mechanism of antibacterial action of spices and derivatives is not yet clear. Proposed hypothesis are : hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, followed by partition in the lipid bilayer; perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes; membrane disruption; destruction of electrons transport systems and cell wall perturbation (Punir and Jain 2010). In various previous studies, the antibacterial properties of spices are mostly attributed to lipophilic essential oils (Nikolic et al. 2014). However, hydrophilic antioxidants are also common in spices (Masuda et al. 2015), such as polyphenols, many of which possess excellent antioxidant as well as good antibacterial activity (Coccimiglio et al. 2016).

It is considered that microbial contamination and lipid oxidation are the two major factors resulting in food spoilage (MozaffariNejad et al. 2014). Hydrophilic extract of spice has good antibacterial and antioxidant activities; hence, can be used as natural food preservatives. For instance, extracts of cinnamon, oregano, and especially clove, were confirmed to be effective for retarding lipid oxidation and reducing pathogen numbers in real food matrices like cheese and raw pork (Shan et al. 2011; Shan et al. 2009). Besides, spice extracts could be applied in foods not only to increase shelf-life but also enhance health benefits of foods because probiotic bacteria like lactic acid bacteria (LAB) were less affected by the presence of these phenolic rich spice extracts, (Chan et al. 2018).

The results demonstrated that the *E. coli* was more resistant to the spices extract than *S. aureus*. Gram-negative bacteria have an outer membrane rich in lipopolysaccharides (LPS) having high hydrophobicity, as well as a unique periplasmic space. The complex composition and spatial structure of lipopolysaccharides form a barrier for penetration of mostly for hydrophobic molecules and antimicrobial agents. Similarly, the presence of enzymes in periplasmic space may break down intrusive molecules, preventing the antibacterial drugs entering intracellular environment. Hydrophobic molecules can pass through cell wall of gram-positive bacteria easier than the gram-negative bacteria because cell wall of the gram-positive bacteria contained only peptidoglycan (Ababutain 2011; Shan et al. 2007).The antibacterial activity seemed to be bacteria-dependent, and Gram-positive bacteria were more susceptible to the tested spice extracts than Gram-negative bacteria(Benmeziane etal. 2018;Nagy et al.2015)

Active compound in Garlic is Allicin (Rahman et al. 2006) which has antibacterial properties. Active compound in Ginger is phenyl propanoid derived compounds particularly gingerols and shogaols. Some volatile compounds which are responsible for antimicrobial activities in ginger were á-pinene,

borneol, camphene, and linalool (Sa-Nguanpuag et al. 2011). Similarly the active compound in Turmeric (Curcuma longa) is Curcumin (Majeed et al. 1996).

Based on this finding, Spices possess good natural antimicrobial agents against both gram-positive and gram-negative bacteria. The extracts of spices should be further analyzed to isolate the specific antibacterial component in them.Various experiments should be conducted to prove spices as food preservatives. Clinical trials should be carried out to explore the potential of the extracts in the treatment of the infectious diseases.

More studies should be carried out for synergistic inhibitory effects as more effective antimicrobial agents. Also, the knowledge on efficacy of combined extracts may be extended from culinary food applications to pharmacology and food chemistry.

## CONCLUSIONS

It concluded that the antibacterial effect of aqueous extract of Garlic extract was the stronger in comparison, followed by Turmeric and Ginger against four test bacteria isolated from buff meat namely; *E. coli, Salmonella* spp., *Pseudomonas* spp., *Staphylococcus aureus*. Therefore this study revealed that spices used in *Kachhila* have antibacterial property and enhance the shelf life of *Kachhila*. Spices can be used as alternative natural food preservatives rather than chemicals. Various spices have different types of active ingredients that suppress the growth of bacteria present in food. n health. Therefore, s

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Methicillin Resistant *Staphylococcus aureus* in Health Care Workers of a Tertiary Care Infectious Disease Hospital in Nepal

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## ABSTRACT

**Objectives:** Acquisition of *mecA* gene in infectious strains of Methicillin resistant *Staphylococcus aureus* (MRSA) are considered as one of the potential virulence factors that enables the host bacteria to carry out several nosocomial and community-acquired infections. The main aim of this study was to determine the prevalence of MRSA, their antibiogram and *mec*A gene in the bacterial isolates obtained from the asymptomatic healthcare workers (HCWs) working in Sukraraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu Nepal.

**Methods:** This prospective cross-sectional study involved the collection of nasal and hands swab of 125 randomly selected HCWs from December 2019 to February 2020. Conventional microbiological methods were used to isolate and identify *S. aureus*. Antimicrobial susceptibility testing was done by modified Kirby Bauer disc diffusion method. MRSA was confirmed by using cefoxitin disc. Detection of mecA gene in the chromosome which was extracted by Phenol: Chloroform: isoamyl alcohol DNA extraction method, amplified by using PCR and visualized by running agarose gel electrophoresis.

**Results:** The overall and MRSA carriage rate among the HCWs was found to be 28% (35/125) and 10.4% (13/125) respectively. *S. aureus* carriage rate was highest among sanitation staffs (34.2%) followed by pharmacy staffs (33.3%), laboratory personnel (18.8%), doctors (9.1%) and nurses (7.5%). Similarly, 34.2% (13/38) of the *S. aureus* isolates were resistant to methicillin, 31.6% (12/38) were inducible-clindamycin resistant and 63.2% (24/38) of them were multi-drug resistant (MDR). All the 13 MRSA isolates harbored the *mecA* gene.

**Conclusions:** Carriage rate of MRSA among HCWs was high and alarming, indicating the prompt need of intervention measures to curb the growth and spread of resistant isolates in the hospital settings. Effective surveillance (of infectious diseases) and establishment of advanced diagnostic facilities can assist in estimating the actual burden of the MRSA which in turn helps to formulate and implement the appropriate policies and infection-control programs to address the increasing antimicrobial resistance in the country.

**Keywords:** *MRSA, Health care workers, Staphylococcus aureus, Nasal carriage, Nepal.*

## INTRODUCTION

*Staphylococcus aureus*—a human commensal and opportunistic pathogen—constitutes the major causative agent of several bacterial infections. Despite the advancement and availability of several antibiotic therapies, staphylococcal infection is still remains as one of the most frequent infections in hospitalized patients causing a wide variety of clinical manifestations ranging in severity from superficial infections such as cutaneous infections to severe invasive diseases like bacteremia (Chakolwa et al. 2019).

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Antibiotics are the miracle drugs in treatment of the infections caused by pathogenic strains of bacteria. However, due to extensive and irrational use of antibiotic has led to the emergence of antimicrobial resistance (AMR), a condition in which bacteria shows the resistance against the antibiotics prescribed against it (Mazzon 2016). Like all other AMR problems, methicillin-resistant *S. aureus* (MRSA) has also emerged as a major barrier in the management of nosocomial and community-acquired infections. Following the introduction of penicillin in 1940, *S. aureus* showed resistance against penicillin due to its ability to produce penicillinase, an enzyme that hydrolyses and inactivates the penicillin group of antibiotics. First case of penicillin-resistant *S. aureus* strain was detected in 1942. Methicillin, a semi synthetic penicillin was then developed in the late 1950s to treat penicillin resistant *S. aureus* but the widespread use and misuse of methicillin and other semi-synthetic penicillin led to the emergence of methicillin-resistance, first case reported in 1961 (Barber 1961). Methicillin resistance is chiefly mediated due to the acquisition of a new penicillin binding protein PBP-2' (expressed by an exogenous gene, *mec*A) which has low affinity to the most β-lactam antibiotics (Archer 1998). There occurs a wave in the outbreaks associated with MRSA strains which is associated with higher mortality rates, prolonged hospital stays and unwanted economic burden due to the increased cost of treatment (Lakhundi and Zhang 2018). An individual colonized with MRSA has a fourfold increased risk of subsequent infection than by other susceptible strains of *S. aureus* (Safdar and Bradley 2008).

MRSA once confined to hospitals, health care environments and patients frequenting such facilities, now has become a serious problem for communities due to its emergence as a major cause of the community-associated infections (Khatri et al. 2017; Lakhundi and Zhang 2018). Since mid-1990s, sudden increase in the number of MRSA infections reported in the communities has been associated with the recognition of new MRSA strains. Such novel strains are often called as community-associated MRSA (CA-MRSA) strains. These strains which principally used to cause skin and soft tissue infections are now responsible for hospital care-associated infection as well (David and Daum 2010).

HCWs serve as the link between hospitals and the communities, which plays a significant role in cross-contamination of nosocomial and community acquired infections (El Aila et al. 2017). Asymptomatic colonization of MRSA among HCWs is a prerequisite for subsequent endogenous infection and dissemination of the strains to the hospital environment (Abimana et al. 2019). Early identification of MRSA carriers among HCWS may help to minimize the potential outbreaks in several hospitals.

Drug-resistant, often multidrug-resistant strains of the commensal and opportunistic bacteria including MRSA, methicillin resistant *S. epidermidis* (MRSE), vancomycin resistant *Staphylococcus aureus* (VRSA) and some strains of *Pseudomonas* spp, *Haemophilus* spp, *Streptococcus* spp are the major problem in the infection control strategies. Aside from socioeconomic burden and augmented risk of dissemination, these resistant strains may go undetected in the resource poor settings of Low- to lower-middle income countries (LMICs) which results the emergence of novel strains furthering the challenges in the fight with AMR (Thapa et al 2020). A number of studies are conducted in Nepal which have estimated the prevalence of MRSA ranging from 26.1% to 57.1% (Ansari et al. 2014; Kumari et al 2008; Rijal et al. 2008; Khanal and Jha 2010; Raut et al. 2017; Bhomi et al. 2016; Rijal et al. 2008; Shahi et al. 2018; Thapa et al. 2020; Kandel et al. 2020). Although there seems a large number of studies are based on MRSA but almost all studies are merely relied on the phenotypic detection. Therefore, there is still a paucity of researches to estimate the molecular detection and characterization of the resistant genes. This study explores the prevalence of MRSA strains, their antibiogram and the molecular detection of *mec*A gene in order to corroborate the need and importance of molecular detection techniques and their reliability in the precise detection of resistant strains.

## MATERIALS AND METHODS

### Study design, study site and sample population

This prospective cross-sectional study was conducted for a period of three months from December 2019 to February 2020. Sample collection and processing was conducted at Sukraraj Tropical and Infectious Disease Hospital (STIDH), Teku while the molecular detection of the *mec*A gene was carried out at Central Department of Microbiology, Tribhuvan University, Kathmandu. All purposively selected HCWs above 18 years of age from the hospital who consented to provide socio-demographic information along with nasal and hand

swab samples, were included in the study. Similarly, HCWs under 18 years of age, who were unable provide written consent for socio-demographic information were excluded from the study. A total of 250 (n=125 nasal; n=125 hand) swab samples were obtained from 125 HCWs, each of the study participant provided a nasal swab and a hand swab.

**Collection and transport of samples**

Swab samples were collected aseptically by using sterile cotton swabs pre-moistened with sterile normal saline, following standard methodology (Cheesbrough 2012). Briefly, the swab was rotated inside the anterior nares of each nostril for 2-3 times with slight finger pressure on the outside of the nose in order to assure good contact between swab and the chamber of the nostril. Without contaminating the swab, the procedure was repeated for the second nostril and the swabs were transported to the laboratory with the labels of subject's identification number and other required information (Cheesbrough 2012). No transport medium was used as the microbiology department and sample collection sites were adjacent to each other. Drying of swab was prevented by keeping the swab in tubes containing nutrient broth.

**Isolation and Identification of *S. aureus***

Swab samples were inoculated into mannitol salt agar (MSA) and blood agar (BA) and were incubated at 37$^{o}$C for 24 hrs. *S. aureus* was identified on the basis of colony characteristics, Gram's staining, and biochemical assays such as catalase, oxidase and coagulase test (Cheesbrough 2012).

**Antibiotic susceptibility testing and screening of MDR *S. aureus*:**

Antimicrobial 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: amoxicillin (AMX 10µg), cefoxitin (CX 30µg), ciprofloxacin (CIP 5µg), clindamycin (CD 2µg), cotrimoxazole (COT 25µg), erythromycin (E 15µg), gentamicin (GEN 10µg), penicillin (P 10µg), tetracycline (TE 30µg), cloxacillin (COX 10µg) and ceftriaxone (CTR 30µg). Results were interpreted on the basis of CLSI guidelines (2019). Isolates showing non-susceptibility (either resistant or intermediate) to at least one agent in three or more antimicrobial categories were identified as MDR (Magiorakos et al 2011). Confirmed *S. aureus* isolates were preserved by using 20% glycerol in TSB for further analysis.

**Screening of MRSA**

Methicillin resistance was detected by using cefoxitin disk of 30µg on MHA plates by Kirby-Bauer disc diffusion method and was interpreted according to CLSI guidelines (2019). Isolates showing zone of inhibition of ≤ 21 mm around cefoxitin disc (30 µg) were considered as MRSA (CLSI 2019).

**Detection of inducible-clindamycin resistant (ICR)**

Inducible-clindamycin resistant was detected by double disk approximation test (*D*-test) as per CLSI (2019) guidelines. In this test, a 0.5 McFarland's standard suspension of *S. aureus* was prepared and plated onto MHA. An erythromycin disk (15 µg) and clindamycin disk (2 µg) were placed 15 mm apart edge-to-edge on MHA plate. Plates were analyzed after 18 hours of incubation at 37°C. Isolates were considered inducible-clindamycin resistant when an isolate was resistant to erythromycin but sensitive to clindamycin showing flattening of the zone of inhibition of ≥21mm around clindamycin producing a "*D*" shaped blunting towards erythromycin disk (*D*-test positive).

**Extraction of DNA**

All phenotypically confirmed MRSA isolates were treated under Phenol: chloroform: isoamyl alcohol extraction method for the detection of *mec*A gene. In this method, isolates were grown in Luria Bertani (LB) broth at 37°C in an orbital shaker at 120rpm for 24 hours. 1.5 ml of liquid culture was transferred to microfuge tube of 1.5ml volume. Then, the bacterial cells were lysed with 3-5 mg/ml lysozyme in the presence of 1/10 volume of 10 % Sodium Dodecyl Sulfate (SDS) at high P$^{H}$ and the lysate was then neutralized. Subsequent deproteinization with 1:1 Phenol: Chloroform was done and then genomic DNA was precipitated with ethanol by spinning at high speed (Shrestha and Adhikari 2014).

**PCR Amplification of mecA gene**

Thus extracted mecA gene was further amplified by polymerase chain reaction (PCR). In PCR test, the crude lysates were used as a DNA template whereas (*mecA* PF1)5′- ACT GCT ATC CAC CCT CAA AC-3′ and (*mecA* PR1) 5′- CTG GTG AAG TTG TAA TCTGG-3′ were used as forward and reverse primer respectively (Vatansever et al. 2016). A final 10 µl

solution was prepared by mixing up of 5 µl master mix, 1 µl each of forward and reverse primer, 1 µl DNA, and 2 µl nuclease free water. The amplification cycle consisted of initial denaturation at 95°C for 120 seconds, denaturation at 95°C for 30 seconds, annealing at 56.2°C for 30 seconds, extention at 72°C for 20 seconds, and 29 cycles of amplification at 72°C for 5 minutes. The amplified products were then subjected to gel electrophoresis using 1.5 % agarose gel stained with ethidium bromide.The final product (163-bp DNA fragment) was then visualized under UV light. The presence of the gene was confirmed by comparing with a positive control using 100 bp DNA ladder (Molecular Biology, Thermo Fisher Scientific Company) in the gel run. The band of 163-bp was considered positive for the *mecA* gene (Vatansever et al. 2016; Oliveira and de Lencastre, 2011).

**Statistical analysis**

All the data were analyzed by using IBM SPSS statistics 23.0 version software. Frequency and percentage for descriptive and Chi Square test with cross tab for inferential statistics were used. A p-value of <0.05 was considered as statistically significant.

## RESULTS

**Distribution of bacterial growth in swab samples**

Among the 250 swabs processed, 241 (96.4%) samples showed the growth of bacteria in which 81.2% (203/250) of them were coagulase-negative Staphylococci (CONS) while 15.2% (38/250) showed growth of *S. aureus* (Figure 1*)*. Out of 38 positive samples, 42.1% (n=16) were isolated from nasal swab while 57.9% (n=22) were isolated from hands swab. About 10.4% (13/125) of the HCWs had colonization with *S. aureus* only, 15.2% (19/125) of them had hands colonization alone while 2.4% (3/125) of them had both nasal and hands colonization. However, 72% of the staff's both nasal and hand swabs were free from *S. aureus.*

**Antibiotic resistance of *S. aureus***

Regarding antimicrobial susceptibility pattern of all 38 *S. aureus* isolates, a high proportion of *S. aureus* isolates (73.7%) showed resistance towards amoxycillin. Also, 68.4%, 60.5%, 47.3%, 36.8%, 34.2%, 15.8%, 12%, 7.9% and 7.9% of the isolates were resistant to erythromycin, cloxacillin, ciprofloxacin, ceftriaxone, cefoxitin, gentamicin, clindamycin, cotrimoxazole and tetracycline respectively (Figure 2).

**Phenotypic screening of MRSA**

Out of 38 *S. aureus* isolates, 34.2% (13) were resistant to methicillin. Among which, 61.5% (8/13) isolates were obtained from nasal swab whereas 38.5% (5/13) were obtained from hand swab (Figure 3).

**Distribution of S. aureus and MRSA according to age and gender of the subjects**

Among the total studied participants, 24 were males and 101 were females. Respectively 32.7% (33/101) and 20.8% (5/24) female and male participants were found to have harbored *S. aureus*. Similarly, 10.9% (11/101) and 8.35% (2/24) of the male and female subjects respectively harbored MRSA strains.

This study included HCWs of the age between 16-80 years old. Colonization with MRSA was highest (6.3%; 2/32) among the age group >50 years, followed by the age group of 26-50 years (5.6%; 8/144) and 15-25 years (4.1%; 3/74). However, there was no significant association between the bacterial load (MRSA) and, gender and age groups (p=0.67) (Table 1).

***S. aureus* and MRSA carriage among different groups of health Profession**

Among the total staffs, the highest percentage of sanitation staffs (34.2%; 13/38) were colonized with *S. aureus* followed by pharmacy staffs (33.3%; 2/6) and lab personnel (18.8%; 6/32). However, MRSA carriage was observed highest in pharmacy staffs (16.7%; 1/6) followed by lab personnel (12.5%; 4/32), sanitation staffs (7.9%; 3/38) and nurses (5%; 4/80). This distribution of the bacterial load and MRSA were not found to be statistically associated with the various professions (wards) of the HCWs (p= 0.152) (Table 2).

**Distribution of isolates in different departments of the hospital**

In this study, the rate of *S. aureus* carriage was highest among pharmacy staffs (33.3 %; 2/6) followed by laboratory staffs (20.3 %; 13/64) and ICU staffs (16.7 %; 1/6) and staffs from the emergency department (16.7%; 3/18). However, MRSA colonization was higher in HCWs from pharmacy (16.7%; 1/6) and ICU (16.7%; 1/6) followed by HCWs from ward staffs (6.9%; 5/72) and lab staffs (6.3%; 4/64) respectively. This distribution was not statistically significant with p-value of 0.628, which suggests that MRSA carriage rate and departments of duty of HCWs are independent of each other (Table 3).

**Distribution of MRSA and MSSA on the basis of duration of employment**

Highest number of *S. aureus* was seen in those HCWs who served the hospital for less than 10 years as 52.6% (20/38) of the *S. aureus* and 35% (7/20) of the MRSA isolates were recovered from them. This was followed by the service year of 20-30 years with 23.7 % (9/38) *S. aureus* load and 22.2 % (2/9) of MRSA. Those who served for 20-30 years showed the rate of 23.7% (9/38) of *S. aureus* while the rate was 22.2% (2/9) of MRSA. It was then followed by 10-20 years and 30-40 years of employment with 21.1% (8/38) and 2.6% (1/38) of *S. aureus* carriage respectively. However, the distribution was statistically insignificant as (p=0.532) (Figure 4).

**Antibiotic susceptibility pattern of MSSA and MRSA**

All (13) of the MRSA isolates were resistant towards penicillin, amoxycillin, cefoxitin and ceftriaxone. 12 (92.3%) isolates were resistant to erythromycin. 92.3% (12/13) of the total MRSA isolates were susceptible to cloxacillin, clindamycin and gentamicin. Almost equal portion (84.6%; 11/13) of the MRSA isolates were susceptible to both Cotrimoxazole and Tetracycline. However, 60% (15/25) of MSSA isolates were resistant to amoxycillin followed by 56% (14/25) for erythromycin. All of the MSSA isolates were susceptible towards cefoxitin and cloxacillin. 96% (24/25) of MSSA were susceptible for ceftriaxone, cotrimoxazole and tetracycline. 92% (23/25) and 80% (20/25) of them were susceptible towards clindamycin and gentamicin respectively (Table 4).

**Prevalence of multi-drug resistant and inducible -clindamycin resistant *S. aureus* isolates**

Among 38 *S. aureus* isolates, 24 (63.2%) of them were MDR. Similarly, all of the MRSA isolates were MDR. Out of 38 *S. aureus* isolates, 12 (31.5%) isolates were screened as inducible-clindamycin resistance. Among 12 isolates, 7 were MRSA (Table 5).

**Prevalence of *mec*A gene among MRSA isolates**

All of the 13 MRSA isolates were confirmed to have harbored *mec*A gene (Table 6). The *mec*A gene was detected under gel electrophoresis with product size 163 bp (Figure 5).

## DISCUSSION

Healthcare workers (HCWs) are one of the major reservoirs of *S. aureus* as they often serve as the interface between hospitals and communities. Therefore, HCWs are required to screened for carriage of pathogenic and resistant strains of *S. aureus* so that potential nosocomial and community acquired infections and can be prevented and the chain of transmission can break at the earliest. As MRSA has emerged as a serious public health problem, screening of HCWs allows appropriate management of the colonized staff members. In this study, nearly one fifth of the HCWs were colonized with the commensal, *S. aureus*. Of the positive individuals, one-third (34.2%) of the isolated bacteria were the strains of MRSA—all of those isolates harbored the *mecA* gene.

Some previous studies have reported the prevalence of *S. aureus* and that of MRSA among the HCWs as 23.7% and 4.6% respectively (Albrich and Harbarth 2008; Khatri et al. 2017). In this study, the overall prevalence was slightly higher than the average rate which may be due to negligence on infection control guidelines, safety and sanitation measures among them. Moreover, nasal carriage rate of *S. aureus* and MRSA among the HCWs was found to be 12.8% (16/125) and 6.4% (8/125) respectively. Similarly, the carriage rates of *S. aureus* and MRSA in the hands of HCWs were 17.6% (22/125) and 4% (5/125) respectively. The findings of our study are in line with the previous study by Khatri et al. (2017), in which the nasal carriage rates of *S. aureus* and MRSA were 18.3% and 7.5% respectively. Also, 72.7% of *S. aureus* colonization was reported by Lama et al. (2017), 15.7% by Khanal et al. (2015), 20.37% by Sah et al. (2013), 25% by Shakya et al. (2010). The nasal carriage rate of *S. aureus* in this study was lower than the studies conducted elsewhere in Nepal. Prevalence of *S. aureus* in this study when compared internationally also shows variation. A study conducted by Shibabaw et al. (2013) from Northeast Ethiopia and Abimana et al. (2019) from Central Uganda reported the rate of 28.8% from each of the studies. Lower prevalence than our study was reported from Kenya with 18.3%; Zambia (17.1%), Kuwait (21%) and India (21.4%) (Omuse et al. 2012). Higher prevalence was from Iran (31%), Gaza (31%), Germany (33.8%), Chile (34.9%), Libya (39%), Central Uganda (41.9%), Tanzania (41.4%) and Nigeria (64%) (Shibabaw et al 2013; Abimana et al 2019; El Aila et al. 2017). Similarly, nasal MRSA carriage rate was also lower than the findings by Shakya et al. (2010) with 10% and Khatri et al. (2017) with 7.5% but was higher than those reported by Shrestha et al. (2009) with 2.3% and Khanal et al (2015) with 3.4% from Nepal. MRSA prevalence in present study is higher than some overseas findings from Iran (5.3%) and Zambia (5.7%) but lower than Ethiopia (12.7%), Libya (19%),

Egypt (13.5%), and Gaza (25.5%) (Chakolwa et al 2019). These differences in the prevalence of *S. aureus* and its strains between countries and hospitals are probably due to differences in the quality and size of samples, variation in sampling techniques, microbiological procedures, different interpretation guidelines, local infection control standards and the local prevalence of MRSA. Moreover, different levels of commitment while performing laboratory works by the investigator also contribute to these differences (El Aila et al 2017, Chakolwa et al 2019).

Socio-demographic characteristics including age, gender, profession, length of healthcare services, and services in different departments within the same hospital have been reported to influence the carriage of *S. aureus* (Kandel et al. 2020). Also in this study, significant differences in the prevalence of *S. aureus* among HCWs have been well documented with the variation in such characteristics listed in the afore-mentioned sentence.

In the antimicrobial susceptibility assay, most of the isolates were susceptible to carbapenems and gentamicin. Higher susceptibility of *S. aureus* isolates towards these antibiotics was also reported in previous studies (Thapa et al 2020; Kandel et al 2020; Sah et al 2013) and augmented resistance to gentamicin was also reported in another studies (Thulunga et al. 2015). Similarly, AST of MRSA isolates showed resistance to most of the antibiotics used except carbapenems, vancomycin and gentamicin. Our findings resonate well with some of other findings reported earlier (Thapa et al. 2020; Kandel et al. 2020; Sah et al. 2013; Belbase et al. 2017; Rijal et al. 2008). However, in another study, ciprofloxacin was effective against MRSA isolates (Shrestha 2013).

Furthermore, this study revealed nearly one-third (31%) of *S. aureus* isolates as inducible-clindamycin resistance which could be easily misidentified as clindamycin susceptible in Kirby-Bauer disk diffusion method. Therefore, D-test should be routinely performed to all the *S. aureus* isolates in clinical microbiology laboratory to guide clinicians for appropriate use of clindamycin. The prevalence of inducible-clindamycin resistance among *S. aureus* observed in this study is higher than the prevalence reported by many other researches done in Nepal as well as done internationally. From Nepal, Adhikari et al. (2017) reported 11.48%, 12.4% by Ansari et al. (2016), 12.1% by Sah et al. (2013), 3% by Mishra et al. (2013), 28.7% by Kumari et al. (2008). Varying prevalence rates of inducible-clindamycin resistance have been reported from worldwide.

*mecA* gene responsible for conferring the drug-resistant to the MRSA and MDR isolates of S. aureus. Detection of *m*ecA gene serves as an evidence of the presence of MRSA among entire *S. aureus* isolates. This statement has been further reinforced by various findings from Sudan (Maimona et al. 2014), Saudi Arabia (Meshref and Omer, 2011), Iraq (Al-Zu'bi et al 2004), Japan (Hotta et al. 1999), India (Mehndiratta et al. 2009), Australia (Cloney et al. 1999) and USA (Murakami et al. 1991). In comparison to those studies, burden of *mecA* was similar in our study as all of the MRSA isolates were tested positive for the gene. This could be due to the increased awareness, augmented infection controls in the health facility under the study. However, other intrinsic factors also need to be assessed in future studies which might have been competing and inhibiting the expression of *mecA* in MRSA isolates in large number of study findings (Kandel et al. 2020). Globally, a number of studies have documented the absence of *mecA* in MRSA isolates (Aziz et al. 2014).

A study from Nigeria reported the absence of 5 major SCCmec types, gene products of PBP2 and mecA genes in phenotypically confirmed MRSA isolates. This finding informs the existing of other intrinsic factors such as the probability of hyper-production of β-lactams, responsible for conferring the resistance (Olayinka et al. 2009). There can also be the possibility of the alterations in various amino acids in protein binding proteins cascade (PBPs 1, 2 and 3). Such alterations have been reported to cause 3 amino acid substitutions (with identical or different amino acids) in all variants of PBP (Ba X et al. 2014). Hence, the existence of several other intrinsic factors aside for mecA suggests that detection of the gene alone cannot assure the detection of resistance. This important point needs to be pondered by the regional and reference laboratories while formulating and implementing the policy. Moreover, a more strict policy should be considered to discourage the over-the-counter (OTC) use of drugs and irrational prescriptions and use of antibiotics among HCWs and patients. Furthermore, HCWs, irrespective of the absence of clinical complaints are advised to be routinely screened for the potential carriage and transfer of the pathogenic strains of the bacteria.

## CONCLUSIONS

More than one-fifth of the HCWs were colonized with *S. aureus* while one in ten isolates were MRSA and all of the MRSA tested positive for *mecA* gene in this study. The findings in this study reinforce the need for more commitment towards infection control measures that meet the standard protocols and aims at reducing the spread of infection by MRSA among susceptible individuals. Augmentation of diagnostic facilities along with antimicrobial stewardships can be recommended to combat the burgeoning spread of resistant bacteria.

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## CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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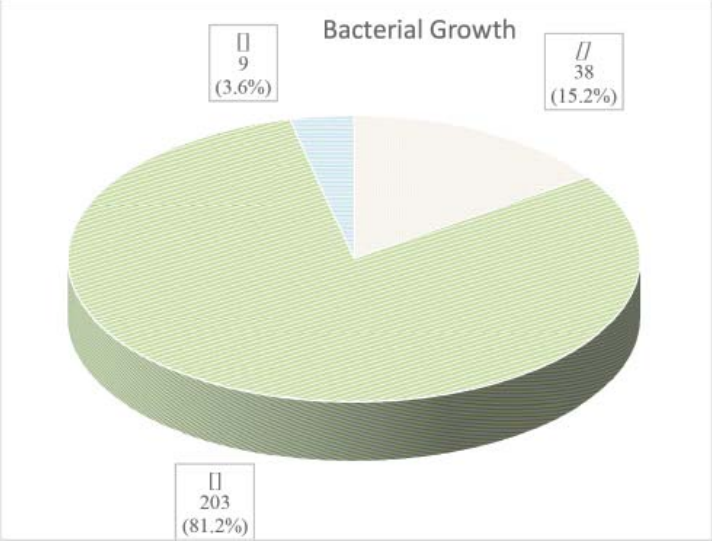


**Figure 1: Distribution of bacterial growth**

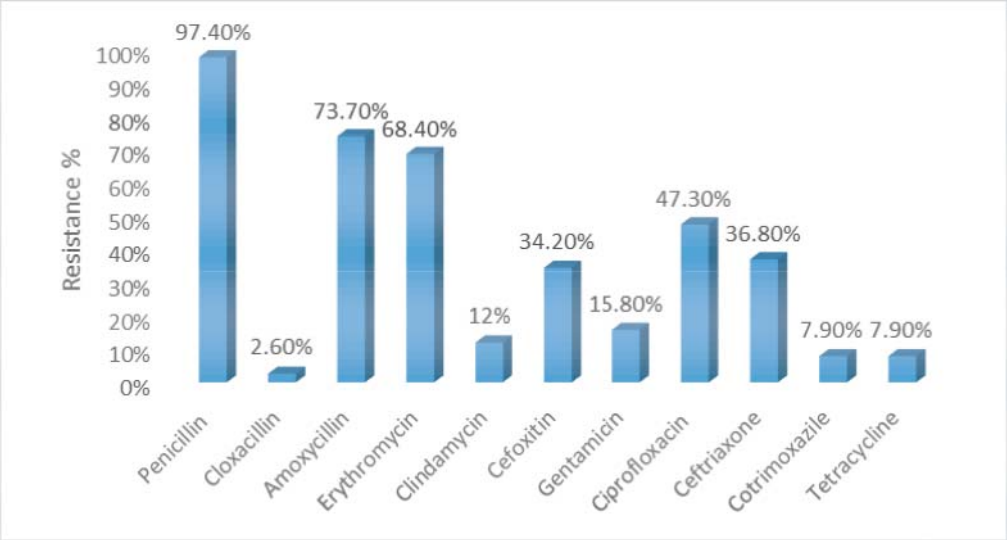


**Figure 2: Antibiotic resistance pattern of *S. aureus* isolates**

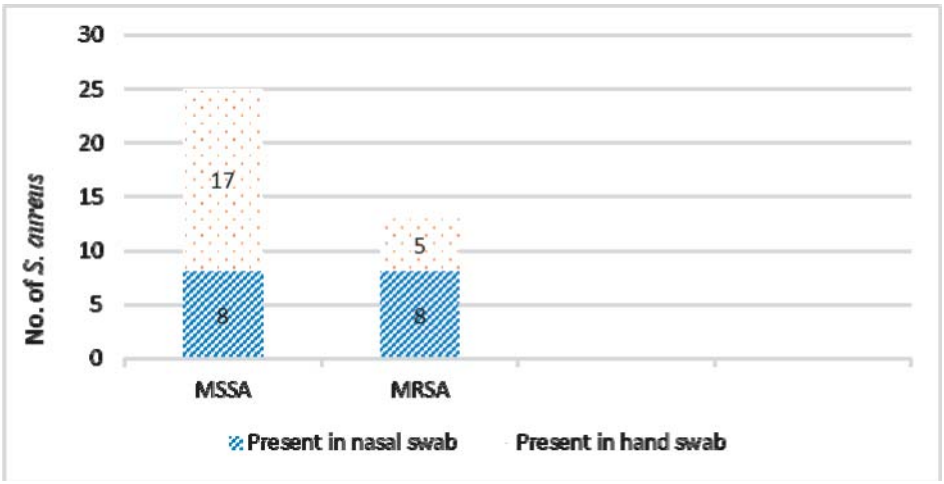


**Figure 3: MSSA and MRSA among *S. aureus* isolates**

**Table 1: Age and gender wise distribution of *S. aureus* and MRSA**

| Age of health care workers | Total no. of samples N | Gender of the health care workers | | | | Total MRSA |
|---|---|---|---|---|---|---|
| | | Male (n =24) | | Female (n =101) | | |
| | | S. aureus | MRSA | S. aureus | MRSA | |
| | | N (%) | N (%) | N (%) | N (%) | N (%) |
| 18-25 | 74 | 0 | 0 | 6 (8.1) | 3 (4.1) | 3 (4.1%) |
| 26-50 | 144 | 3 (2.03) | 1 (0.7) | 23 (16) | 7 (4.9) | 8 (5.6%) |
| Above 50 | 32 | 2 (6.3) | 1 (3.1) | 4 (12.5) | 1 (3.1) | 2 (6.3%) |
| Total | 250 | 5 | 2 | 33 | 11 | 13 (5.2%) |

**Table 2: Distribution of *S. aureus* and MRSA carriage among different groups of health professions**

| Profession of health care workers | Total no. of samples N | No. of *S. aureus* N (%) | MRSA N (%) |
|---|---|---|---|
| Doctor | 22 | 2 (9.1 %) | 0 |
| Nurse | 80 | 6 (7.5 %) | 4 (5 %) |
| Lab Personnel | 32 | 6 (18.8 %) | 4 (12.5 %) |
| Pharmacist | 6 | 2 (33.3 %) | 1 (16.7 %) |
| Health Assistant | 18 | 3 (16.7 %) | 0 |
| Sanitation Staff | 38 | 13(34.2 %) | 3 (7.9%) |
| Interns | 30 | 3 (10 %) | 0 |
| Others | 24 | 3 (12.5 %) | 1 (4.2%) |

**Table 3: Distribution of *S. aureus* and MRSA isolates in different departments**

| Department of duty | Total no. of samples N | No. of *S. aureus* N (%) | MRSA N (%) |
|---|---|---|---|
| Emergency | 18 | 3(16.7 %) | 1(5.6 %) |
| OPD | 36 | 4 (11.1 %) | 0 |
| Ward (Gastro + Male + Cabin) | 72 | 11(15.3 %) | 5(6.9 %) |
| ICU | 6 | 1 (16.7 %) | 1(16.7 %) |
| ART Department | 26 | 1 (3.8 %) | 0 |
| Laboratory | 64 | 13 (20.3 %) | 4(6.3 %) |
| Pharmacy | 6 | 2 (33.3 %) | 1(16.7 %) |
| Immunization | 10 | 1 (10 %) | 0 |
| Others | 12 | 2 (16.7 %) | 1(8.3 %) |

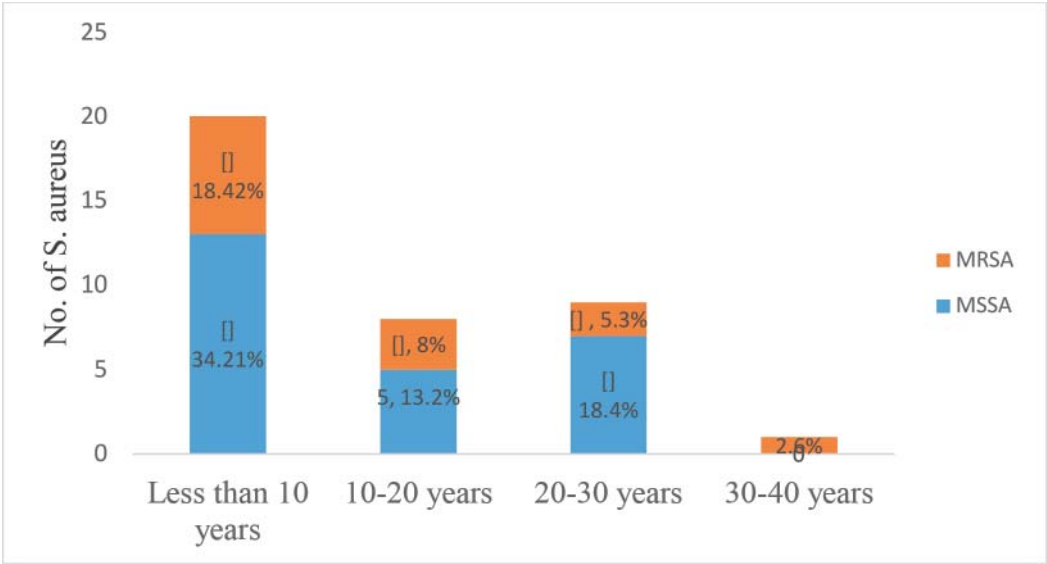


**Figure 4: MSSA and MRSA distribution according to years of employment**

**Table 4: Antibiotic susceptibility patten of MSSA and MRSA**

| Antibiotic disc | MSSA (N=25), n (%) | | MRSA (N=13), n (%) | |
|---|---|---|---|---|
| | Sensitive (S) N (%) | Resistant (R) N (%) | Sensitive (S) N (%) | Resistant (R) N (%) |
| Penicillin | 1 (4%) | 24 (96%) | 0 | 13 (100%) |
| Cloxacillin | 25 (100%) | 0 | 12 (92.3%) | 1 (7.7%) |
| Amoxycillin | 10 (40%) | 15 (60%) | 0 | 13 (100%) |
| Erythromycin | 11 (44%) | 14 (56%) | 1 (7.7%) | 12 (92.3%) |
| Clindamycin | 23 (92%) | 2 (8%) | 12 (92.3%) | 1(7.4%) |
| Cefoxitin | 25 (100%) | 0 | 0 | 13 (100%) |
| Gentamicin | 20 (80%) | 5 (20%) | 12 (92.3%) | 1 (7.7%) |
| Ciprofloxacin | 14 (56%) | 11 (44%) | 6 (46.2%) | 7 (53.8%) |
| Ceftriaxone | 24 (96%) | 1 (4%) | 0 | 13 (100%) |
| Cotrimoxazole | 24 (96%) | 1 (4%) | 11 (84.6%) | 2 (15.4%) |
| Tetracycline | 24 (96%) | 1 (4%) | 11 (84.6%) | 2 (15.4%) |

**Table 5: Prevalence of multi-drug resistant and inducible -clindamycin resistant *S. aureus***

| No. of *S. aureus* | MRSA N (%) | MDR N (%) | MDR isolates excluding MRSA, N (%) | D-test Positive N (%) | MRSA with Positive D-test, N (%) | MSSA with Positive D-test,N (%) |
|---|---|---|---|---|---|---|
| 38 | 13 (34.2%) | 24 (63.2%) | 11 (29%) | 12 (31.6%) | 7 (18.4%) | 5 (13.2%) |

**Table 6: Detection of *mec*A gene in MRSA**

| Sample | No. of MRSA isolates N | No. of *mec* A positive MRSA N (%) |
|---|---|---|
| Nasal swab | 8 | 8 (100%) |
| Hand swab | 5 | 5(100%) |

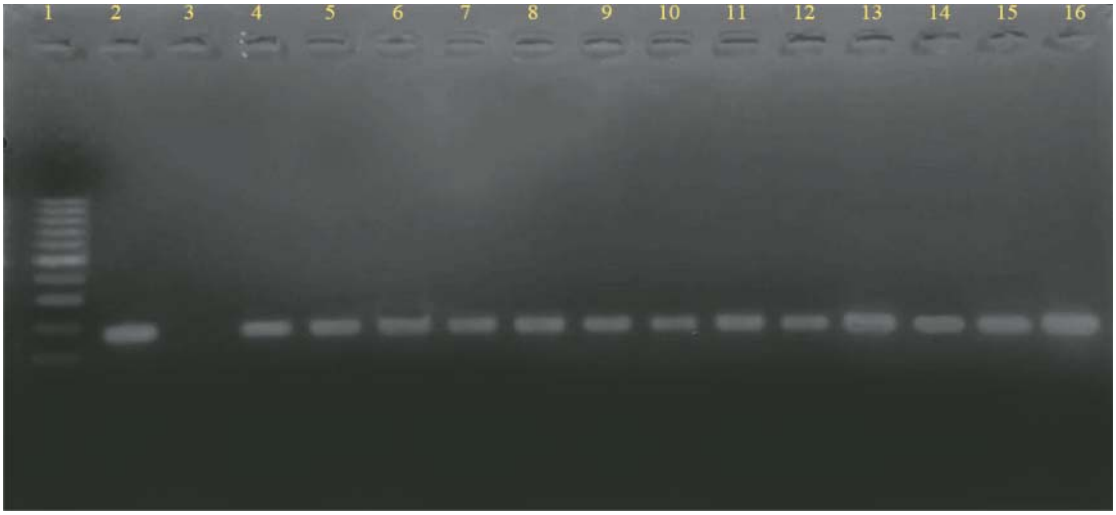


**Figure 5: Confirmation of *mec*A gene by Gel documentation of PCR products. Lane: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4-16: isolates with positively amplified *mec*A gene (163bp).**

# Antibiotic Susceptibility Pattern of *Salmonella Enterica* Serovars Typhi and Paratyphi A Isolated From Patients Suspected of Enteric Fever

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## ABSTRACT

**Objectives:** The study aimed to assess the antibiotic susceptibility profile of *Salmonella* spp isolated from patients suspected of enteric fever.

**Methods:** This cross-sectional prospective study was carried out from April to June, 2014among 484 patients clinically suspected of enteric fever visiting Bir Hospital, Kathmandu, Nepal. Blood sample collected from each patient was processed for culture in bile broth. Identification of *Salmonella* spp was done by conventional microbiological techniques including colony characteristics, Gram's staining and biochemical tests. Antibiotic susceptibility testing of identified isolates was done by Kirby-Bauer disk diffusion method following the 2014 CLSI guideline.

**Results:** Out of 484 blood samples, 36 (7.43%) cases showed the growth of *Salmonella* spp; of which 27 (75%) were *Salmonella enterica* serovar Typhi (ST) and 9 (25%) were *Salmonella enterica* Paratyphi A (SPA). Among the *Salmonella* isolates, 5.55% were multidrug resistant and 41.66% were fluoroquinolone resistant. More than 80% of isolates were sensitive to chloramphenicol, amoxicillin, and cotrimoxazole whereas 58%, 50% and 6% of isolates were sensitive to fluoroquinolone antibiotics i.e. ciprofloxacin, ofloxacin and nalidixic acid respectively. All the isolates were susceptible to ceftazidime. All SPA and 89% of ST were sensitive to azithromycin.

**Conclusion:** Higher percentage of susceptible isolates to chloramphenicol, cotrimoxazole, and amoxicillin suggests the reconsideration of these antibiotics for the treatment of enteric fever. Azithromycin can be considered as drug of choice for the treatment of enteric fever.

**Keywords:** Enteric fever, *Salmonella* isolates, MDR, antibiotic susceptibility

## INTRODUCTION

Enteric fever, a febrile disease caused by the *Salmonella enterica* serovars Typhi and Paratyphi A, B, and C, causes 21 million new infections and claims 161,000 lives each year worldwide (WHO 2019). It is a systemic disease, endemic in developing countries like Nepal (Crump and Mintz 2010). Definitive diagnosis of enteric fever is done by the isolation of organisms from the blood and bone marrow (Gasem et al. 1995). Serological tests (Widal test) can also be done, however, they are not reliable due to false-positive results. DNA probes and PCR can be used to detect organism from the blood, however, their use in the developing countries is not feasible due to high cost (Parry et al. 2002).

Initially, ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole (cotrimoxazole) had been used as a first-line drug for the treatment of enteric fever. *Salmonella enterica* serovars Typhi and Paratyphi resistant to these three first-line antibiotics are called as multidrug-resistant (MDR) strains (Crump et al. 2015).

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With the increase in MDR strains, fluoroquinolones (ofloxacin, nalidixic acid, ciprofloxacin etc.) became the drug of choice for the treatment of enteric fever. However, the dramatic increase in fluoroquinolone-resistant strains has been observed after 2000 (Mirza and Khan 2008). Therefore, third-generation cephalosporins (cefixime, cefalexin, ceftazidime etc.), and azithromycin have been used recently for the treatment of MDR, and fluoroquinolone-resistant *Salmonella* strains (Effa et al. 2011) though sporadic cases of resistance have been observed for ceftriaxone and azithromycin (Kobayashi et al. 2014, Parry et al. 2015).

In Kathmandu, the burden of the enteric fever is high and is the leading cause of febrile illness (Karkey et al. 2008). Lack of proper diagnosis of disease, on one hand, and continuous development of antimicrobial resistance, on the other hand, are the issues of major concern in the countries of low economic settings like Nepal (Parry et al. 2011). Therefore, this study aims to assess the antibiotic resistance among *Salmonella* spp isolated from febrile cases in a tertiary care hospital of Kathmandu, Nepal.

## MATERIALS AND METHODS

This cross-sectional prospective study was carried out from April to June, 2014 among 484 clinically suspected enteric fever patients visiting Bir Hospital, a tertiary hospital of Nepal. From each patient, 5-10 ml of blood sample was collected aseptically by vein puncture and inoculated directly into a bottle containing bile broth. Each sample was incubated at 37°C for 48 hours and subcultured into Blood Agar (BA) and Mac-Conkey Agar (MA). Each sample was cultured till 7 days to consider as negative. Identification of *Salmonella* spp was done based on colony characteristics, Gram's staining, catalase, oxidase and other biochemical tests (Cheesbrough 2009). The antibiotic susceptibility pattern of isolates was done in Muller Hinton Agar by Kirby-Bauer disk diffusion method following CLSI guidelines (CLSI 2014). The antimicrobial susceptibility of 10 antimicrobial agents- amoxicillin (amx) (30µg), chloramphenicol (cpl, 30µg), cotrimoxazole (TMP-SMX, 25µg), nalidixic acid (nal, 30µg), ciprofloxacin (cip, 5µg), ofloxacin (ofx, 5µg), cefixime (cfm, 5µg), azithromycin (azm, 15µg), cefalexin (cfx, 30µg), and ceftazidime (caz, 30µg) (Hi-Media Laboratory Ltd, Mumbai, India) were performed. The results were interpreted as sensitive, intermediate, or resistant based on the size of zone of inhibition and comparing it with the standard chart provided by the manufacturing company. (CLSI, 2014).

## RESULTS

Out of 484 blood samples, 36 (7.43%) were positive for *Salmonella* spp of which 27 (75%) were *Salmonella enterica* serovar Typhi (ST) and 9 (25%) were *Salmonella enterica* serovarParatyphi A (SPA).

Most of the isolates were from patients in the age group 21-30 years followed by the age group 11-20 years. Similarly, male patients (8.27%) were found to be more susceptible to *Salmonella* infection than female patients (6.18%) but was not statistically significant (Table 1).

**Table 1: Age and gender wise distribution of total and positive cases for *Salmonella* spp**

| Age group in years | Number of cases tested | | Number of positive cases | | | |
|---|---|---|---|---|---|---|
| | | | Salmonella spp | | Salmonella Typhi | Salmonella Paratyphi A |
| | Male | Female | Male | Female | | |
| <10 | 1 | 3 | 0 | 0 | 0 | 0 |
| 11-20 | 51 | 39 | 7 | 4 | 8 | 3 |
| 21-30 | 79 | 41 | 15 | 6 | 16 | 5 |
| 31-40 | 49 | 27 | 0 | 1 | 1 | 0 |
| 41-50 | 33 | 20 | 1 | 0 | 0 | 1 |
| 51-60 | 27 | 32 | 0 | 0 | 0 | 0 |
| 61-70 | 29 | 19 | 1 | 1 | 2 | 0 |
| >70 | 21 | 13 | 0 | 0 | 0 | 0 |
| Total | 290 | 194 | 24 | 12 | 27 | 9 |

More than 80% of isolates were sensitive to chloramphenicol, amoxicillin, and cotrimoxazole whereas 58%, 50% and 6% of isolates were sensitive to fluoroquinolone antibiotics i.e ciprofloxacin, ofloxacin and nalidixic acid respectively. All the isolates were sensitive to third-generation cephalosporin, ceftazidime. To azithromycin, 89% of ST were sensitive and 11% were intermediately sensitive whereas 100% of SPA were sensitive (Table 2).

**Table 2: Antibiotic susceptibility pattern of *Salmonella* isolates**

| Antibiotics | *Salmonella* Typhi (n=27) | | | *Salmonella* Paratyphi A (n=9) | | |
|---|---|---|---|---|---|---|
| | Sensitive n (%) | Intermediate n (%) | Resistant n (%) | Sensitive n (%) | Intermediate n (%) | Resistant n (%) |
| Amoxycillin | 22 (81) | - | 5 (19) | 7 (78) | - | 2 (22) |
| Cotrimoxazole | 26 (96) | - | 1 (4) | 8 (89) | - | 1 (11) |
| Chloramphenicol | 26 (96) | - | 1 (4) | 8 (89) | - | 1 (11) |
| Ciprofloxacin | 12 (44.5) | 3 (11) | 12 (44.5) | 6 (67) | - | 3 (33) |
| Ofloxacin | 11 (41) | 2 (7) | 14 (52) | 5 (56) | - | 4 (44) |
| Nalidixic acid | 2 (7) | - | 25 (93) | - | - | 9 (100) |
| Cefixime | 25 (93) | - | 2 (7) | 9 (100) | - | - |
| Cefalexin | 24 (89) | 3 (11) | - | 6 (67) | - | 3 (33) |
| Ceftazidime | 27 (100) | - | - | 9 (100) | - | - |
| Azithromycin | 24 (89) | 3 (11) | - | 9 (100) | - | - |

Among the *Salmonella* isolates, 5.55% were multidrug resistant and 41.66% were fluoroquinolone resistant (Table 2).

**Table 3: Multidrug resistant (MDR) and Fluroquinolone resistant (FQR) *Salmonella* isolates**

| Salmonella serovar | Total isolates | MDR n (%) | FQR n (%) |
|---|---|---|---|
| *Salmonella* Typhi (n=27) | 27 | 1 (3.71%) | 12 (44.44%) |
| *Salmonella* Paratyphi A (n=9) | 9 | 1 (11.11%) | 3 (33.33%) |
| Total | 36 | 2 (5.55%) | 15 (41.66%) |

## DISCUSSION

Higher prevalence of *Salmonella* Typhi and *Salmonella* Paratyphi A in our study was similar to that reported by Petersiel et al (Petersiel, Shresta et al. 2018).. In contrast, Pokhrel et al. found that the prevalence of ST (47%) infection was lower than SPA (53%) (Pokharel et al. 2006). A similar study reported that the paratyphoid fever was associated with flood and contaminated street vendor's food, whereas, typhoid fever was associated with household contamination (Woods et al. 2006). So, we expect most of the patients in our study were associated with household contamination. The proportion of enteric fever positive male patients (66.67%) was higher than female patients (33.33%) . A similar study had the proportion of enteric fever positive male patients higher than female patients (Amatya et al. 2007). Males have greater chances of acquiring enteric fever than females, probably due to the indiscriminate eating habits of the male in the roadside locations. The age group 21-30 years had the highest culture positivity (58.33%) similar to previous studies in Nepal (Adhikari et al. 2012).

Amoxicillin,cotrimoxazole, and chloramphenicol had been used previously as the first-line drug against the *Salmonella* infection. However, resistance to these first-line drugs has been reported(Ochiai et al. 2008). We found only 5.55% of the MDR *Salmonella* isolates (ST-3.71%, SPA-11.11%). Chloramphenicol was used previously (since the 1940s) as a gold standard for the treatment of enteric fever, however, due to the emergence of resistance, it is no longer a drug of choice for the treatment of enteric fever (Mandal et al. 2004). Our study shows that both the serovars of *Salmonella* were highly susceptible to chloramphenicol (ST-96%, SPA-89%) which is in agreement to another similar study conducted in Nepal (Amatya et al. 2007). The sensitivity of ST and SPA toward cotrimoxazole was found to be 96% and 89% respectively; similar findings were obtained by two different studies (Murdoch et al. 2004, Amatya et al. 2007). Both the MDR isolates were resistant to cotrimoxazole which is in agreement with the finding of Amatya et al. (100% resistant) (Amatya et al. 2007). Antibiotic susceptibility pattern of ST and SPA for amoxicillin were 81% and 78% respectively, this pattern corresponds with the finding by Amatya et al (ST- 75% and SPA- 49%) (Amatya et al. 2007). Hence, increased susceptibility of ST and SPA towards amoxicillin, cotrimoxazole, and chloramphenicol over the period in Nepal (Karki et al. 2013) mandates the reconsideration of these antibiotics for the treatment of enteric fever.

The resistance to chloramphenicol and amoxicillin during the 1990s led to the extensive use of fluoroquinolones, such as ciprofloxacin, ofloxacin, and nalidixic acid, effective against ST and SPA (Parry et al. 2002). Our study showed that 44.44% of ST and

33.33% of SPA were resistant to fluoroquinolone antibiotics. Various studies in a different part of the world showed the higher effectiveness of the above-mentioned fluoroquinolone antibiotics against ST and SPA (Gales et al. 2002, Wain et al. 2003, Maskey et al. 2008), contradicting our study. However, in agreement with our findings, Pokharel et al. found the decreased susceptibility of ST and SPA against fluoroquinolone(Pokharel et al. 2006). Similarly, Karki et al. in a review article reported the decreased susceptibility of ST and SPA against a range of fluoroquinolone antibiotics (Karki et al. 2013). The susceptibility towards ciprofloxacin (ST- 44.5%, SPA-67%) in our study corresponds to the study by Pokharel et al. (ST- 57%, SPA- 0%), while incongruent to a study by Amatya et al. (ST-93.59%, SPA-79.54%) (Pokharel et al. 2006, Amatya et al. 2007). The development of resistance towards ciprofloxacin in Nepal is due to the easy availability of ciprofloxacin in the drug store, self-prescription by the patients, and incomplete course of treatment (Pokharel et al. 2006).

Nalidixic acid resistance is considered as the phenotypic marker for the reduced susceptibility to fluoroquinolone. Our study shows that the resistance against nalidixic acid was the highest (ST-93%, SPA-100%) among all the tested antibiotics. In agreement with our study, a recent study was done by Adhikari et al. in the year 2011, found the increased resistance of nalidixic acid antibiotic (ST-82.9%, SPA-91.33%) (Adhikari et al. 2012). Similarly, Maskey et al. in 2008 (ST-49%, SPA-86%), and Neopane et al. in 2007 (ST-73.3%, SPA-94.9%) reported the growing resistance of ST and SPA to nalidixic acid antibiotic (Neopane et al. 2007, Maskey et al. 2008). Nalidixic acid had been used as anindicator of decreased ciprofloxacin susceptibility. Our study also shows a similar trend, however, it is suggested that the determination of minimum inhibitory concentration (MIC) as a reliable indicator (Crump et al. 2003).

The third-generation cephalosporins (cefixime, cefalexin, and ceftazidime) were effective against ST and SPA, as the susceptibility of both *Salmonella enterica* serovars were quite higher. Susceptibility of ST was 89% and the SPA was 100% to cefixime.Astudy reported the susceptibility of ST and SPA to cefixime was found to be 75% and 100% respectively (Amatya et al. 2007). Cefixime, an oral antibiotic, is widely used for the treatment of enteric fever as a first-line drug (Pandit et al. 2007). As we found two ST isolates resistant against cefixime, which is a worrisome finding, similar to anotherstudy(Qamar et al. 2014). Therefore, further research should be done before blindly prescribing third-generation cephalosporins for the treatment of MDR and FQR *Salmonella* isolates. Ceftazidime was most effective as both ST and SPA were found to be 100% susceptible to it. In a similar study done in ST, Hasan et al. reported that ceftriaxone and ceftazidime were the most effective antibiotics (100% susceptibility) (Hasan et al. 2011). However, mode of administration (intravenous and intramuscular) make ceftazidime a less famous antibiotic, with regards to difficulty in administering to the outpatients.

Furthermore, both ST (89% susceptible, 11% intermediate susceptible) and SPA (100% susceptible) are found to be highly susceptible to azithromycin antibiotic. In agreement with our study, Kumar et al. found the susceptibility of ST and SPA to azithromycin to be 93.6% and 100% respectively (Kumar et al. 2008). Similarly, another study shows that the antibiotic susceptibility of STwas found to be 81.25% to azithromycin(Hasan et al. 2011). Another study also highlighted that azithromycin is better in terms of fever clearance, and relapse rate for the treatment of enteric fever caused by MDR and FQR *Salmonella* isolates (Shah 2009). Hence, our study revealed that azithromycin can be an alternative solution for the MDR and FQR *Salmonella* isolates.

## CONCLUSION

Higher percentage of susceptible isolates to chloramphenicol, cotrimoxazole, and amoxicillin suggests the reconsideration of these antibiotics for the treatment of enteric fever. Azithromycin can be considered as drug of choice for the treatment of enteric fever.

## ACKNOWLEDGMENTS

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# Prevalence of Methicillin-Resistant *Staphylococcus aureus* Isolated from Clinical Samples at Narayani Samudayik Hospital, Chitwan, Nepal

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## ABSTRACT

**Objectives:** The main objective of this study was to determine the prevalence of Methicillin Resistance *Staphylococcus aureus* (MRSA) and MDR bacteria isolated from various clinical specimens from the patients attending Narayani Samudayik Hospital, Chitwan

**Methods:** A cross sectional study was carried in NPI-Narayani Samudayik Hospital, Chitwan from June to December 2017. Altogether, 3610 clinical specimens mainly pus, blood and urine were collected, streaked on Mannitol Salt Agar and Blood Agar and incubated at 37˚C for 24 hours. The confirmed colonies of *S. aureus* were sub-cultured on Nutrient Agar. The antibiotic susceptibility pattern of all isolates *S. aureus* was determined by Kirby Bauer disc diffusion method. Isolates resistant to cefoxitin (30mcg) were confirmed as MRSA.

**Result:** Among 3610 total clinical samples, 17.6 % (635/3610) showed growth and 95(14.96%) *S. aureus* were isolated. Higher number of *S. aureus* was isolated from pus sample (93.15%). Out of 95 *S. aureus* isolates, 55 (57.89%) were identified as MRSA while 40 (42.10%) were MSSA. Vancomycin, ceftriaxone and chloramphenicol were found to be most effective antibiotic against isolates. Whereas, the least effective antibiotic was cefoxitin followed by amoxiclav, oxacillin and amoxicillin.

**Conclusion:** This study concludes that the overall prevalence of MRSA and MDR among the bacterial isolates is higher compared to other studies. So, it is recommended to monitor the antibiotic susceptibility pattern of pathogens regularly and study the epidemiology of such isolates.

**Key words:** MRSA, *Staphylococcus aureus*, vancomycin, cefoxitin

## INTRODUCTION

*Staphylococcus aureus* has emerged as one of the most important human pathogens, and has over the past several decades, been a leading cause of hospital and community – acquired infections (Lowy, 1998). Methicillin, originally called celbenine, is a semisynthetic derivative of penicillin which is chemically modified to tolerate the degradative action of penicillinase. *S.aureus* that are resistant to methicillin or oxacillin, the penicillinase stable β-lactam antibiotic, are known as Methicillin resistant *S. aureus* i.e MRSA. MRSA grouped under HA- MRSA (Healthcare associated methicillin resistant *S.aureus*) and CA-MRSA (Community acquired methicillin resistant *S.aureus*) (Giacometti et al. 2000).

Methicillin resistance arises following the inactivation of beta lactamase enzymes, acquisition of nobel DNA, which results in production of a new penicillin-binding

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protein (PBP), known as PBP2′ or PBP2a, which has low binding affinity for methicillin and other currently available β-lactams (Deleo et al. 2010).

The frequency of infection caused by MRSA has been significantly increased in last 10 years (Stapheton and Taylor 2002) Studies conducted in Nepal showed that the prevalence of MRSA was astronomically increased from 29.1% to 61.6% in between 1990 and 2003 (Moran et al. 2006 & Khanal and Jha, 2010). Lack of effective antibiotic policy for the proper use of antibiotic has resulted to the emergence of resistant strains and use of incomplete course of antibiotic without proper prescription is one of the leading causes of dissemination of antibiotic resistant (Khanal et al. 2003). Most studies on *S. aureus* have been conducted on sample from nose and throat but only a limited number of studies have been reported on *S. aureus* from pus sample.

This study was carried out to know the recent status of Methicillin Resistant *S. aureus* in Chitwan district, Nepal as many studies done in Nepal suggest the gradual emergence of MRSA in hospital (Mishra 2013). According to Karki et al (2019) and Shrestha et al (2018), prevalence of MRSA was reported as 26.4% and 16.7% respectively in different Nepalese hospital settings. The information obtained from this study helps to guide the clinicians in choosing appropriate antibiotics and prevent the emergence of resistance to the drug which are still sensitive. Findings can be used to determine trends in antibiotic susceptibilities and guide in formulation of local antibiotic policy.

## METHODS

Clinical sample from June to December 2017 received in clinical microbiology lab of Narayani Samudayik Hospital, Chitwan were processed and all *S. aureus* isolates were included in the study. Ninety-five isolate of *S. aureus* were collected from culture sample received from different department of the hospital. The isolates were consecutive and non-repetitive (one per patient). One sample from one patient was inclusion criteria of study data. Second sample from other site of same patient was not considered for study.

**Inclusion and exclusion criteria**

Only those samples which were adequately collected and properly labeled were included in the study. Those samples which were not collected by medical officer or an experienced nurse or self-collected by patients were not included in the study.

**Sample collection, transportation and processing**

All the samples were collected by medical personnel using aseptic procedures. Pus samples were collected using sterile syringe. Blood samples were collected using sterile syringe (3ml from children and 5ml from adults) into a sterile blood collection bottle containing BHI. For urine, a wide-mouthed sterile leak-proof container was provided for collection. About 10-15 ml of midstream urine was collected (Cheesbrough 2006). All the samples were properly labelled and transferred to microbiology laboratory for further processing (Mahon et al 2014).

Samples were culture on blood agar, Mannitol salt agar, MacConkey agar for 24 hours. Blood culture was inoculated in Brain Heart Infusion broth and sub culture on 24 and 72 hour on BA and MSA. Identification of organism was carried out by standard laboratory operating procedure (Gram Staining, Catalase test, Coagulate test).

The antibiotic susceptibility pattern of all the strains was determined by modified Kirby Bauer Disc diffusion method against the following antibiotic: amoxiclav (30 mcg), cefoxitin (30 mcg), chloramphenicol (30 mcg), Tetracycline (30 mcg), vancomycin (30 mcg), erythromycin (15 mcg), gentamycin (30 mcg) and ceftriaxone (30mcg). Screening for methicillin resistance was performed by cefoxitin disc diffusion method and interpreted according to CLSI guidelines (CLSI 2014). Briefly, isolates with zone of inhibition (ZOI ≥ 22mm) were identified as methicillin-susceptible (MSSA) and isolates with ZOI ≤ 21mm identified as methicillin-resistant (MRSA). As a reference strain, *S. aureus* ATCC 25923 was used in this study. The obtained data were analyzed using the Microsoft Excel 2010.

## RESULTS

Out of 3610 clinical samples, 635 (17.59%) showed bacterial growth. Among them, 465 (73.2%) were *Escherichia coli* and only 95 (14.96%) isolates were identified as *S. aureus.* Other identified isolates were *Pseudomonas* spp., *Klebsiella pneumoniae*, *Enterococcus* spp. and coagulase negative Staphylococci. The majority of *S. aureus* were isolates from pus (59.13%) followed by blood (10%) (Table 1).

**Table 1: Distribution of *S. aureus* in clinical samples**

| Clinical samples | Total no. of samples | Culture positive cases N (%) | S. aureus isolated from culture positive cases N (%) | S. aureus from total samples N (%) |
|---|---|---|---|---|
| Pus | 200 | 115 (62.8%) | 68 (59.13%) | 34% |
| Blood | 1350 | 50 (3.57%) | 5 (10%) | 10 % |
| Urine | 2060 | 512 (24.85%) | 22 (4.29%) | 1.06% |
| Total | 3610 | 635 (17.59%) | 95 (14.96%) | 2.63% |

*N; Number of isolates*

A total of 635 positive cases were obtained consisting 285 females and 350 males. Out of this, 95 *S. aureus* comprising 41 females and 54 males were isolated. The isolates in females were 14.38% and that in males were 15.42% (Table 2).

**Table 2: Distribution of *S. aureus* in male and female patients**

| Gender | *S. aureus* N (%) | Other than *S. aureus* N %) | Total N (%) |
|---|---|---|---|
| Female | 41 (14.38%) | 244 (85.61%) | 285 (44.88%) |
| Male | 54 (15.42%) | 296 (84.57%) | 350 (55.12%) |
| **Total** | **95 (14.96%)** | **540 (85.04%)** | **635 (100%)** |

As described in figure 1, higher number of isolates were obtained from the age group <10, 30-39, 40-49 years with an incidence of 27.36%, 15.78 % and 13.68% respectively. Few isolates were from age group above 70 years (2.10%).

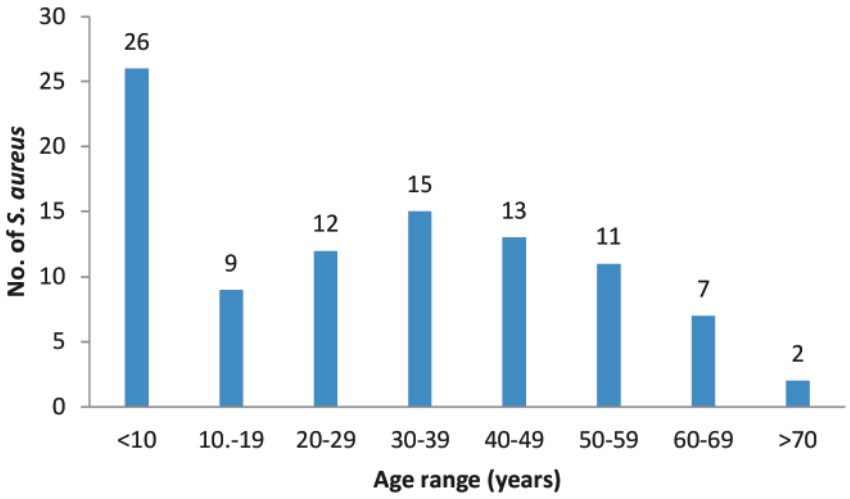


**Figure 1: Age-wise distribution of patients with *Staphylococcus aureus* isolates**

Fifty-five isolates were found to be MRSA. As shown in Table 3, out of 55 MRSA isolates 46 (83.64%) were MDR. Only 5 (12.5%) among 40 (42.10%) MSSA were MDR (Table1).

**Table 3: MDR pattern among *S. aureus* isolates**

| Drug resistance | MRSA N (%) | MSSA N (%) | Total |
|---|---|---|---|
| MDR | 46 (83.64%) | 5 (12.5%) | 51 |
| Non- MDR | 9 (16.36%) | 35 (87.5%) | 44 |
| **Total** | **55 (100%)** | **40 (100%)** | **95** |

The antimicrobial susceptibility pattern of *S. aureus* are summarized in **Table 4.** Out of 95 isolates of *S. aureus*, 55 (57.89%) were MRSA. Most of the *S. aureus* isolates were sensitive to ceftriaxone 85 (89.47%), vancomycin 83 (87.36%) and chloramphenicol 71 (74.73%). So, these were the more effective anti-staphylococcal drugs. Most of them were resistant to amoxiclav 70 (73.68%) followed by amoxycillin 60 (63.15%), cefoxitin (57.89%) and oxacillin 52 (54.73%).

**Table 4: Antibiotic susceptibility patterns of *S. aureus* (n=95)**

| Antibiotic | Susceptibility pattern (n=95) | | |
|---|---|---|---|
| | **Sensitive N (%)** | **Intermediate N (%)** | **Resistant N (%)** |
| Amoxiclav | 25 (26.31%) | - | 70 (73.68%) |
| Amoxycillin | 15 (15.78%) | 20 (21.05%) | 60 (63.15%) |
| Cefoxitin | 40 (42.10%) | - | 55 (57.89%) |
| Ceftriaxone | 85 (89.47%) | 3 (3.15%) | 7 (7.36%) |
| Chloramphenicol | 71 (74.73%) | 20 (21.05%) | 4 (4.21%) |
| Ciprofloxacin | 65 (67.36%) | 7 (7.36%) | 23 (24.21%) |
| Erythromycin | 47 (49.47%) | - | 48 (50.52%) |
| Gentamicin | 29 (30.52%) | 22 (23.15%) | 44 (46.31%) |
| Levofloxacin | 32 (33.68%) | 25 (26.31%) | 38 (40%) |
| Oxacillin | 36 (37.89%) | 6 (6.31%) | 52 (54.73%) |
| Tetracycline | 69(72.63%) | - | 14 (14.73%) |
| Vancomycin | 83(87.36%) | 10 (10.52%) | 2 (2.10%) |

## DISCUSSION

MRSA has emerged as a serious public health problem globally as it has the ability to acquire antimicrobial resistance over time, and it will continue to be a problem in the future. Today, most of the MRSA are multi-drug resistant i.e., resistant to a number of drugs, thus causing a clinical problem as antibiotic treatment becomes useless. Present study showed prevalence rate of MRSA to be 57.89%. The study done in Kathmandu valley reported 44.9% as MRSA from nosocomial *S. aureus* (Shrestha et al. 2009). Rajbhandari et al (2008) also reported 54.9% MRSA isolates in Bir Hospital. MRSA was isolated at the rate 75.5% from clinical samples in a study conducted by Rijal et al in Pokhara Valley (Rijal et al 2008). Similar study done in western parts of Nepal by Tiwari et al (2009) also reported alarming high rate of MRSA isolate (69.1%) which the authors has attributed to indiscriminate use of antibiotics and its accessibility in these.

Above studies show considerable variations between institutions, often in the same geographical areas, exist, demonstrating that MRSA prevalence, in some settings, significantly exceeds previous estimate. There could be many explanations for these differences: infection control measures, antibiotic prophylaxis and treatments used in each ward/hospital and, not less important, the clonal and often epidemic nature of these microorganisms (Betty et al. 2002).

Present study also shows maximum number of *S. aureus* and MRSA isolation from pus (48/55) ascertaining the role of the organism as cause of pyogenic infection. Kumari et al and Pandey et al have also reported that the isolation of *S. aureus* is higher from pus samples (Kumari et al. 2008; Pandey et al. 2012).

Most of the studies suggest that tests with cefoxitin are more reliable those with oxacillin because cefoxitin is a potent inducer of the *mecA* regulatory system and widely used as a surrogate marker for detection of *mecA* gene-mediated methicillin resistance (Aliberti et al. 2016). In the present study the MRSA isolates showed a highest level of resistance towards cefoxitin (100%), amoxiclave (83.63%), erythromycin (52.72%) and gentamycin (45.45%). Tetracycline have excellent tissue penetration and demonstrate good staphylococcal activity at clinically achievable levels with a reported cure rate of 83% in MRSA skin and soft tissue infections (Idrees et al. 2009). The present study shows 89.47% of *S. aureus* being sensitive to ceftriaxone, 87.36% to vancomycin and 74.73% to tetracycline. This roughly correlates with the finding of Thapa et al (2008) who have reported 73.84% of *S. aureus* as sensitive to tetracycline.

The multi-drug resistant phenotype is a particular characteristic of the methicillin-resistant *S. aureus* strains. It has added to the burden of hospital personnel to control infection associated with MDR-MRSA. Present study shows alarmingly high rate of MDR strain among MRSA isolates (83.64%). Similar studies have reported MDR-MRSA to be as 100% (Kayastha 2010), 92% (Thapa 2011), 75.86% (Pandey et al 2012). Indian literature also shows the isolation of MDR-MRSA as high as 72.1% (Tiwari et al 2008).

Present study shows alarmingly high rate of MDR strain among MRSA isolates (90.19%). The result is consistent with the previous reports in which MDR-MRSA isolates were confirmed as 100% (Kayastha 2010), 92% (Thapa 2011) and 75.86% (Pandey et al. 2012) and 93.1% (Karki et al. 2019) from clinical samples. According to Gopalakrishnan (2010) the incidence of MRSA varied from 25 % to 50% in India.

Though these MDR strains are not found with additional virulence properties, their characteristics multidrug resistance restricts the option available to treat infections caused by this organism (Voss and Doebbeling 1995).

## CONCLUSION

The prevalence of *S. aureus* was found to be 2.63% and majority of them were sensitive to ceftriaxone. More than half of *S. aureus* were found to be MRSA and among them, 83.64% were found to be MDR. MRSA infection is still one of the most life-threatening infections as such infections are difficult to treat. Further detection and molecular characterization of the gene (*mec A*), phage typing and analyses of the plasmids of MRSA is necessary.

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

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# Methicillin Resistant and Biofilm Producing *Staphylococcus* species Isolated from Different Clinical Specimens and Antibiotic Susceptibility pattern of isolates

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## ABSTRACT

**Objectives:**To determine prevalence of Methicillin Resistant *Staphylococcus aureus* in different clinical specimens and biofilm production along with antimicrobial susceptibility pattern of isolates.

**Methods:**Cross-sectional study was conducted from September 2019 to February 2020at Sukraraj Tropical and Infectious Disease Hospital. Total 3091 clinical specimens like blood, urine, sputum, pus, swab, body fluid were processed. Identification was done on the basis of colony characteristics, gram staining, culture in Mannitol Salt Agar, coagulase and oxidation fermentation test. Antibiotic susceptibility test and biofilm detection were performed by Kirby Bauer's disc diffusion methods and Tissue Culture Plate technique (TCP) respectively. Methicillin resistant *Staphylococcus* species were detected by using Cefoxitin disc.

**Results:** Out of 52 *Staphylococcus* species, 39 were *Staphylococcus aureus* and 13 were Coagulase negative *Staphylococcus* species. Highest numbers of *Staphylococcus* species were isolated from blood Sixteen (30.8%) were Methicillin resistant *Staphylococcus aureus* (MRSA) and 5(9.6%) were Methicillin resistant Coagulase negative *Staphylococcus* species. There was no significant association (p=0.25) between age group and prevalence of MRSA, MSSA, MRCoNS and MSCoNS. Methicillin resistant *Staphylococcus* species were resistant to antibiotics like amoxicillin, cloxacillin, erythromycin and higher sensitivity was found in gentamycin. Among 52 Staphylococcal isolates, 11(21.1%) were biofilm producers and 41(78.9%) were non biofilm producers. 90.9%of 90.9% of Biofilm producing *Staphylococcus* species were resistant towards penicillin and erythromycin

**Conclusion:** The study shows Methicillin resistant *Staphylococcus* species were resistant to most antibiotics and rate of resistance was slightly higher in biofilm producing isolates comparing to other isolates. resistance. Regular surveillance of methicillin resistance *Staphylococcus* species and routine screening of biofilm production is important.

**Key words**: *Staphylococcus* species, TCP, MRSA, Biofilm, Antibiotic susceptibility.

## INTRODUCTION

*Staphylococcus* species, gram positive cocci, are common causes of human infections like wound infections, septicemia and toxic shock syndrome. They are responsible for variety of diseases like infection of heart (endocarditic), infection of bone (osteomyelitis), central nervous system infections such as brain abscesses & pneumonia. Depending on the strains and the site of infection, they can cause invasive infections and/or toxin-mediated diseases. The pathophysiology varies greatly depending on the type of *S. aureus* infection. Different mechanisms for evasion of the host immune response include the production of an antiphagocytic capsule, sequestering of host antibodies or antigen masking by Protein A, biofilm formation, intracellular survival, and blocking chemotaxis of leukocytes.

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Biofilm is an assemblage of microbial cells irreversibly with a surface and enclosed in a matrix of primarily polysaccharide material (Donlan 2002). Biofilm formation is recognized method to establish and maintain infections and increase its persistence and boosts level of antimicrobial resistance.Biofilm are associated with many medical conditions like indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and uro-genital infections(Reid G 1999). Important character of biofilm is their increased tolerance to the antimicrobial agents (Wimpenny et al 2000). Resistance may be due to delayed penetration of antimicrobial agent, altered growth rate of biofilm and other physiological changes (Donlan and Costerton 2002).

With the current emergence of antimicrobials resistance MRSA has been able to evolve rapidly and create new clinical problems. MRSA has ability to survive in the presence of penicillin-like antibiotics, which normally prevent bacterial growth by inhibiting synthesis of cell wall material. MRSAmediates through an altered protein called low affinity penicillin binding protein (PBP2a). PBP2a is encoded by *mecA* gene and is present in chromosomal mobile genetic element called Staphylococcal cassette chromosome mec (SCCmec). *mecA* gene is a resistance gene which stops β-lactam antibiotics from inactivating the enzymes (transpeptidases) critical for cell wall synthesis. In fact, many strains of MRSA exhibit resistant to both β-lactams and aminoglycosides.

Production of biofilms can be a marker of virulence (Jain and Agarwal 2009) and MRSA biofilms becomes resistant to almost all available antimicrobial agents used for its treatments (Gotz 2002).MRSA and biofilm producing MRSA are becoming more resistant towards almost all available antimicrobial agents commonly methicillin, ampicillin, Ofloxacin, tetracycline, ciprofloxacin, cotrimoxazole, etc. Despite the development of antimicrobial therapy Methicillin resistance *Staphylococcus* species are recognized as a major cause of nosocomial infection resulting in significant morbidity and mortality.

## MATERIALS AND METHODS

This cross sectional hospital based study was carried out at Sukraraj Tropical Infectious Disease Hospital, Kathmandu where data collection, identification of *Staphylococcus* species, antimicrobial susceptibility test and detection of Methicillin resistant *Staphylococcus* species were done and detection of biofilm formation was done in Med-Micro Research Laboratory Babarmahal from September 2019 to February 2020. A total of 3091 clinical samples including blood, urine, sputum, pus/wound swab, throat swab, body fluid were collected from outpatient suspected of different infections during this period.

**Isolation and identification**: The received specimens were immediately cultured in Blood Agar (BA), Mac-Conkey Agar (MA), Chocolate Agar(CA) and also Cysteine lactose and electrolyte deficient agar (CLED) was used for urine sample. Suspected *S. aureus* colonies were then inoculated onto Mannitol Salt Agar and incubated.Identification of *Staphylococcus* species was done on the basis of colony characteristics, gram staining, culture in Mannitol Salt Agar (MSA), and coagulase and oxidation fermentation test.

**Antibiotic Susceptibility test and confirmation of Methicillin resistant *Staphylococcus* species:** All *Staphylococcus* species isolates were subjected to *in-vitro* antimicrobial susceptibility test by Kirby-Bauer disc diffusion method using Mueller Hinton Agar (MHA) as recommended by Clinical laboratory Standard Institute. Commercially available antibiotic tested from HiMedia Company were amoxicillin (10mcg), cefoxitin (30mcg), cefixime (5mcg), ciprofloxacin (5mcg), cotrimoxazole (25mcg), coxacillin (5mcg), clindamycin (2mcg), erythromycin (15mcg), gentamycin (10mcg), nitrofurantion (300mcg), penicillin (10mcg), and tetracycline (30mcg).

Conformation of MRSA and Methicillin Resistant Coagulase negative *Staphylococcus* species MRCoNS was done by using cefoxitin (30mcg). Diameter of zone of inhibition ≤21mm was considered as methicillin resistant whereas diameter ≥22mm was considered as methicillin sensitive (CLSI 2019).

**Preservation of isolates and screening of biofilm production in *Staphylococcus* species:** Isolates were preserved in Tryptic Soya Broth with 20% glycerol in eppendorf tube and kept at -70$^0$C until subsequent tests and same eppendorf tube was transported to laboratory with ice pack for detection of biofilm formation. Biofilm formation was detecetd by Tissue Culture Plate Technique. Isolates from eppendorf tube was then sub cultured in NA or MHA. Organisms isolated from fresh agar plates were inoculated in 10 mL of Trypticase

soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 24 hrs. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained TSB with 1% glucose. 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 to remove free-floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and then 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 (Hassan et al 2011). The interpretation of biofilm production was done according to the criteria of Stepanovic et al (2007).

| Average OD value | Biofilm formation |
|---|---|
| ≤ODc/ ODc < ~ ≤ 2x ODc | Non/ Weak |
| 2xODc<~≤4xODc | Moderate |
| >4xODc | Strong |

Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control.

**Data Analysis**: Data analysis was done using computer based software program Statistical Package For The Social Sciences SPSS version 21 and p-value was calculated by using Chi Square test

## RESULTS

Among 3091 clinical samples, 239 showed culture positive with 60 (25.11%) gram positive bacteria. Out of 60 gram positive bacteria, 52 (86.67%) were *Staphylococcus* species with 39 (65%) *Staphylococcus aureus* and 13 (21.67%) Coagulase negative *Staphylococcus* species (CoNS).

Out of 52 *Staphylococcus* species prevalence of MRSA was 16(30.8%), and MRCoNS was 5(9.6%). Highest number was obtained from blood. There was no significant association (p=0.98) and (p=0.29) between type of sample and prevalence of MRSA and MSSA and MRCoNS and MSCoNS respectively (Table 1).

**Table 1: Prevalence of MRSA, MSSA, MRCoNS and MSCoNS in different clinical specimens**

| Samples | MRSA | MSSA | P-value | MRCoNS | MSCoNS | P- value | Total |
|---|---|---|---|---|---|---|---|
| Blood | 9(27.8%) | 14(42.4%) | | 5(15.2%) | 5(15.2%) | | 33(63.5%) |
| Urine | 1(50%) | 1(50%) | | - | - | | 2(3.8%) |
| Sputum | 4(36.4%) | 5(45.5%) | 0.98 | - | 2(18.1%) | 0.29 | 11(21.1%) |
| Pus | 2(40%) | 3(60%) | | - | 1(20%) | | 5(9.6%) |
| Totals | 16(30.8%) | 23(44.2%) | | 5(9.6%) | 8(15.4%) | | 52(100%) |

Out of 16 MRSA, highest prevalence was obtained from age group of 41-50 years i.e 5(31.3%). The number of MRCoNS was same in all age groups with prevalence of 50%. There was no significant association(p=0.25) between age group and prevalence of MRSA, MSSA, MRCoNS and MSCoNS (Table 2).

**Table 2: Age wise prevalence of MRSA, MSSA, MRCoNS and MSCoNS**

| Age Group | MRSA (%) | MSSA (%) | MRCoNS (%) | MSCoNS (%) | Totals (%) | P value |
|---|---|---|---|---|---|---|
| 0-10 | - | 2(8.7) | 1(20) | - | 3(5.8) | |
| 11-20 | 2(12.5) | 1(4.3) | - | 1(12.5) | 4(7.7) | |
| 21-30 | 1(6.3) | 9(39.2) | 1(20) | - | 11(21.1) | |
| 31-40 | 2(12.5) | 5(21.8) | - | 4(50) | 11(21.1) | |
| 41-50 | 5(31.3) | 2(8.7) | 1(20) | 2(25) | 10(19.2) | |
| 51-60 | 3(18.7) | 2(8.7) | 1(20) | - | 6(11.6) | |
| 61-70 | 2(12.5) | 1(4.3) | - | 1(12.5) | 4(7.7) | |
| 71-80 | - | - | - | - | - | |
| 81-90 | 1(6.3) | 1(4.3) | 1(20) | - | 3(5.8) | 0.25 |
| Totals | 16(30.8) | 23(44.2) | 5(9.6) | 8(15.4) | 52(100) | |

All MRSA isolates showed resistance towards cefoxitin and penicillin followed by amoxycillin i.e. 93.7%. In MSSA maximum resistance was shown against erythromycin with 73.9%.MRCoNS showed highest resistance was towards cefoxitin, amoxicillin and penicillin with 100%. MSCoNS shows maximum resistance against penicillin with 75% (Table 3).

**Table 3: Antibiogram of methicillin resistant *Staphylococcus* species**

| Antibiotics | MRSA resistant to antibiotics(%) | MSSA resistant to antibiotics(%) | MRCoNS resistant to antibiotics(%) | MSCoNS resistant to antibiotics(%) |
|---|---|---|---|---|
| Amoxicillin(10mcg) | 15(93.7) | 11(47.8) | 5(100) | 4(50) |
| Cefoxitin(30mcg) | 16(100) | - | 5(100) | - |
| Cefixime(5mcg) | 10(62.5) | 8(34.8) | 3(60) | 3(37.5) |
| Ciprofloxacin(5mcg) | 7(43.7) | 3(13) | 4(80) | - |
| Clindamycin(2mcg) | 3(18.7) | 4(17.4) | 3(60) | 3(37.5) |
| Cotrimoxazole(25mcg) | 9(56.3) | 6(26.1) | 2(40) | 4(50) |
| Coxacillin(5mcg) | 12(75) | 5(21.7) | 3(60) | 3(37.5) |
| Erythromycin(15mcg) | 13(81.3) | 17(73.9) | 4(80) | 4(50) |
| Gentamycin(10mcg) | 1(6.3) | 1(4.3) | 4(80) | 1(12.5) |
| Penicillin(10mcg) | 16(100) | 13(56.5) | 5(100) | 6(75) |
| Tetracycline(30mcg) | 5(31.2) | 3(13) | 3(60) | 2(25) |

Out of 39 *Staphylococcus aureus*, 29(74.4%) were weak biofilm producers, 7(17.9%) was moderate biofilm producer and 3(7.7%) was strong biofilm producer. Among 13 CoNS, 12(92.3%) was weak and 1(7.7%) was strong biofilm producer. There was no significant association (p=0.169) between biofilm formation capacity and *Staphylococcus* species (Table 4).

**Table 4: Biofilm production by *Staphylococcus* species in Tissue Culture Plate method**

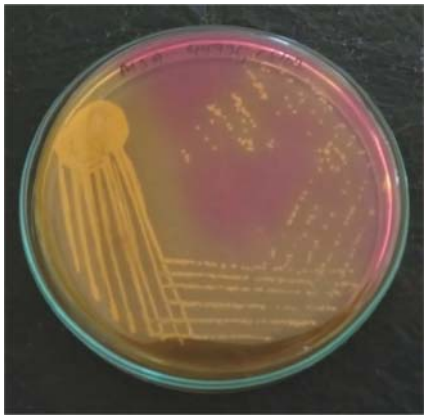
| Types of media | Biofilm formation | *S.aureus* (n=39) | CoNS (n=13) | P-value |
|---|---|---|---|---|
| TSB + 1% Glucose | Weak/Non | 29(74.4%) | 12(92.3%) | |
| | Intermediate | 7(17.9%) | - | |
| | Strong | 3(7.7%) | 1(7.7%) | 0.169 |

Biofilm producing *Staphylococcus* species shows maximum resistance against penicillin and erythromycin with 90.9% (Table 5).

**Table 5: Antibiotic Susceptibility Pattern of biofilm producing non producing *Staphylococcus* species (TSB+1% Glucose)**

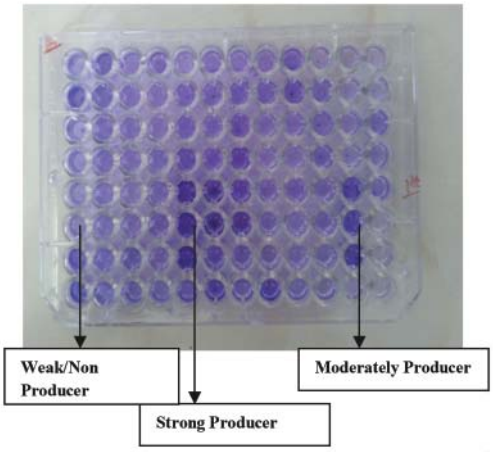
| Antibiotics | Biofilm producer (n=11) | | Biofilm Non Producer(n=41) | |
|---|---|---|---|---|
| | Resistant(%) | Sensitive(%) | Resistant(%) | Sensitive(%) |
| Amoxicillin(10mcg) | 8(72.7) | 3(27.3) | 27(65.8) | 14(34.2) |
| Cefoxitin(30mcg) | 8(72.7) | 3(27.3) | 13(31.7) | 28(68.3) |
| Cefixime(5mcg) | 4(36.4) | 7(63.6) | 18(43.9) | 23(56.1) |
| Ciprofloxacin(5mcg) | 6(54.5) | 5(45.5) | 8(19.5) | 33(80.5) |
| Clindamycin(2mcg) | 3(27.3) | 8(72.7) | 10(24.4) | 31(75.6) |
| Cotrimoxazole(25mcg) | 6(54.5) | 5(45.5) | 15(36.6) | 26(63.4) |
| Coxacillin(5mcg) | 8(72.7) | 3(27.3) | 15(36.6) | 26(63.4) |
| Erythromycin(15mcg) | 10(90.9) | 1(9.1) | 28(68.3) | 13(31.7) |
| Gentamycin(10mcg) | 3(27.3) | 8(72.7) | 4(9.7) | 37(90.3) |
| Penicillin(10mcg) | 10(90.9) | 1(9.1) | 30(73.1) | 11(26.9) |
| Tetracycline(30mcg) | 4(36.4) | 7(63.6) | 9(21.9) | 32(78.1) |

**Photograph 1: Culture of *Staphylococcus aureus* in Blood Agar (pinpoint, smooth, glistening, densely opaque colonies with butyrous consistency)**



**Photograph 2: Culture of *Staphylococcus aureus* in Mannitol Salt Agar (pinpoint yellowish colonies)**

**Photograph 3: Antiobiotic susceptibility of *S. aureus* Sensitive: Ciprofloxacin ; Resistant: Erythromycin, Amoxicillin, Cefoxitin**




**Photograph 4: Biofilm production by Tissue Culture Plate Method**

DISCUSSION

In this study 60 isolates were found to be Gram positive bacteria out of which 52 (86.67%) were *Staphylococcus* species. Among them 39 (65%) were found to be *Staphylococcus aureus* and 13 (21.67%) were Coagulase negative *Staphylococcus* species (CoNS). In the report of Kumari et al (2008), *S. aureus* occupied 83.67% out of total 98 gram positive isolates. A study of Belbase et al (2017) shows 20.9% *S. aureus* out of 364 cultures positive. In our study CoNS was second predominant among Gram positive bacteria with 21.67%. According to Abdel et al (2018), 52% were *S. aureus* and 48% were CoNS out of 150 isolates of *Staphylococcus* speciesand in study of Upreti et al (2018),*S. aureus* (56.9%) was common isolate and CoNS (7.8%) was second predominant bacteria. High frequency might be due to its ubiquitous nature and large number of virulence factors associated with it.

In this study the prevalence of MRSA was 16(30.8%), MSSA was 23(44.2%), MRCoNS was 5(9.6%) and MSCoNS was 8(15.4%). The study done in Kathmandu valley by Shrestha et al (2009) reported 45 % as MRSA from nosocomial *S. aureus*. Study done in Eastern Nepal by Kumari et al (2008) showed 26.14% MRSA. Similar study done in western parts of Nepal by Tiwari et al (2009) also had shown high rate of MRSA isolate (69.1%). Variations in prevalence of MRSA may be due to infection control measures, antibiotic prophylaxis and treatments used in each ward/hospital and clonal and epidemic nature of microorganisms (Stefani and

Varaldo 2003; Robinson and Enright 2004). Likewise prevalence of MRCoNS was 9.6% in our study which was different from the prevalence rate of Maharjan (2017) 28.7% and Begum et al (2011) 4%. In the study conducted by Singh et al (2016) the prevalence ranges from 48.2% to 60% which was higher than our studies. There was statistically no significant association (p=0.98) and (p=0.29) between type of sample and prevalence of MRSA and MSSA and MRCoNS and MSCoNS respectively. In contrast to our study, Mahmood et al (2010) reported highest prevalence of MRSA from wound swab (35.2%) and MRCoNS from urine (34%). Overall data shows lower rate of MRSA and MRCoNS than MSSA and MSCoNS in our study.

In our study the highest prevalence of MRSA was obtained from age group of 41-50 years with 31.3%. The study of Shahi et al (2018) observed highest percentage (47.6%) of MRSA was isolated from the age group of above 60 years. This might be due to the reduced immune system and use of high dose of medication. There was statistically no significant association (p=0.25) between age group and prevalence of MRSA and MRCoNS.

The antibiotic sensitivity pattern of MRSA showed maximum resistance was towards cefoxitin and penicillin with 100% followed by amoxycillin i.e. 15(93.7%). Similar type of result was reported by Tiwari et al (2009) where all MRSA strains were found resistant to penicillin and 91.9% were resistant to amoxicillin. The study conducted by Shrestha (2016) and Kumari et al (2008) also showed higher resistance to amoxicillin with 94.7% and 91.9% respectively which resembles to our study. Homogeneous resistance towards beta-lactams like amoxicillin (93.7%) and cloxacillin (75%) resistant MRSA was also observed in our study which is comparable with the study of Shahi et al (2018).This may be due to presence of intrinsically developed beta-lactamase in MRSA strain. However lower rate of resistance was reported towards gentamycin with 6.3% in comparison with the study of Belbase et al (2017) which reported 31.6% resistance to gentamycin. This may be due to intravenous route of administration and thus a less- commonly used antibiotic that makes abuse difficult (Obiazi et al 2007). In case of MSSA, maximum resistance was observed against erythromycin with 73.9% which was higher than previous study done by Sanjana et al (2010) who reported 58.6% resistance towards erythromycin. Also MSSA has showed 56.5% resistivity towards penicillin. This study showed that all MRSA isolates were significantly more resistant to antibiotics and same result was also obtained in MRCoNS.

The antibiotic resistivity pattern of MRCoNS showed maximum resistance was towards cefoxitin, amoxycillin and penicillin with 100% which is comparable with result of Sharma et al (2010) with 100% resistivity towards penicillin group of antibiotics. Similarly 80% resistance was observed against erythromycin and ciprofloxacin which was higher than Maharjan (2017) who reported resistance rate of erythromycin as 52.2% and ciprofloxacin as 73.9%. The lower rate of resistance towards erythromycin may be due to extensive use for both serious and minor Staphylococcal infections. The present study also showed that MRCoNS are comparatively more resistant to multiple antimicrobial agents than MSCoNS.

In this study, 52 isolates of *Staphylococcus* species were tested for biofilm production by Tissue Culture Plate Technique (TCP).Out of 39 *Staphylococcus aureus*, 29(74.4%) was found to be weak/non biofilm producer, 7(17.9%) was found to be moderate biofilm producer and 3(7.7%) was found to be strong biofilm producer in TSB+1% Glucose media. There was statistically no significant association (p=0.169) between biofilm formation capacity and *Staphylococcus* species. Our result can be compared with Tuladhar (2018) where 78.4%, 12.74% and 8.8% were weak/non, moderate and strong biofilm producer respectively. Our result was consistent with another study from Algeria by Lotfi et al (2014) which showed 8% strongly adherent, 20% moderately adherent, 40% weakly adherent and 32% non adherent strains. The study by Neopane et al (2018) reported 34.88% weak biofilm production, 27.90% moderate production and 6.97%strong biofilm production by the TCP method. Likewise among 13 CoNS, 12(92.3%) was found to be weak/non biofilm producer and 1(7.7%) was strong biofilm producer and there was no moderate biofilm producer in TSB+1% Glucose media. Tuladhar (2018) also reported 81.25%, 16.6%, 2.1% as weak/non, moderate and strong biofilm producer respectively which is slightly similar to our study.

With regards to biofilm producing isolates in TSB+1% Glucose media (11), maximum resistance was shown by penicillin and erythromycin with 90.9%. The isolates

were highly sensitive to clindamycin and gentamycin with 72.7%. The study of Neopane et al 2018 also showed maximum resistance towards penicillin with 86.7% and erythromycin with 50% in biofilm producing *S. aureus*. In our study, rate of resistance is slightly higher in biofilm producing isolates comparing to other isolates. These results indicate biofilm may be one of the major factors for increasing resistance. Therefore, low-concentration combination therapies can be used to eradicate biofilm-related staphylococcal infections, including those by MRSA (Wu et al 2013).

## CONCLUSION

*Staphylococcus aureus* was predominant followed by CoNS among Gram positive organisms and were frequently isolated from blood. The incidence of MRSA was high in age group 41-50. Most of the clinical isolates of methicillin resistant *Staphylococcus* species were resistance towards β-lactams like penicillin, amoxicillin, cloxacillin etc, Macrolids, Fluoroquinolones. Resistance is slightly higher in biofilm producing isolates comparing to other isolates.

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## CONFLICT OF INTEREST

The author declares no conflict of interest.

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# Effectiveness of Commonly Used Antibiotics in Combination with Honey Against Bacterial Infection

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## ABSTRACT

**Objectives**: The study was carried out to compare the inhibitory effects between commonly used antibiotics and bee honey samples, so as to correlate the inhibitory effects between bee honey alone and in combination with antibiotics.

**Methods**: This study was carried out between December 2012 to September 2013. A total of one hundred and twenty-two clinical microbiological specimens and five different floral sourced honey samples were collected between December 2012 to September 2013. Twenty-three multi-drug resistant organisms were selected. Then, AST for commonly used antibiotics, honey alone and combination of honey-antibiotics discs was done. The difference in ZOI of antibiotic contrasting with the antibiotics containing honey were statistically analysed to define the synergism.

**Results**: The inhibition due to honey is variable among bacteria types ($F=39.17$, $p<0.05$). From means plot, *Staphylococcus* and *Acinetobacter* were recognized as highly susceptible bacteria for honey ($\bar{x}$ = 21.1 ± 6.2 mm and $\bar{x}$ = 18.3 ± 3.3 mm respectively) but *Acinetobacter* species could not show synergism to honey-antibiotic combination. The tested organisms from *Enterobacteriaceae* family showed effective susceptibility to Chloramphenicol-honey mixture. Imipenem-honey combination and Gentamicin-honey combination showed significant effects against *Pseudomonas aeruginosa*.

**Conclusion**: Thus, honey can be used in various bacteria-directed infections and found to be effective in various infections. Incorporation of honey in antibiotics like Chloramphenicol, Imipenem, and Gentamicin work better in healing various infection.

**Key words**: Honey, Antibiotics, Synergism, Antibiotic-honey combination

## INTRODUCTION

Honey has four modes for the antibacterial effects (Molan 1992). They are osmophilic effect (Molan 1992), acidic pH (Nassar et al. 2012), hydrogen peroxide production due to glucose oxidase of bee gut (Irish et al. 2011, Bizerra et al. 2012), and antioxidants such as catalases, polyphenols, Maillard reaction products and ascorbic acid (Bizerra et al. 2012) and other components in nectar produced by the plants (Molan 1992).

It is bacteriostatic and bactericidal for gram positive and gram-negative bacteria (Pimentel et al. 2013). It also possesses antifungal character (Lane et al. 2019) and can be used against antibiotic resistant bacteria (Sharp 2009) like MRSA (Müller et al. 2013) and VRE (Boukraâ and Sulaiman 2009).

The protein now identified as universal stress protein A (UspA) is involved in the stress stamina response and its down-regulation could help to explain the inhibition of MRSA of Manuka honey. The level of expression was found to be changed at least two-folds following treatment with the honey (Jenkins et al. 2011).

However, a review article and meta-analysis suggests that the previous works are not sufficient to prove the

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antimicrobial effects of honey (Wijesinghe et al. 2009). In Nepal, the similar studies were performed where honey samples used were not measured properly during the tests. Thus, this study was carried out to determine the present condition of bacteria for the susceptibility towards natural compound honey and to define the statistically significant synergism with various antibiotics.

## MATERIALS AND METHODS

This study was performed between December 2012 to September 2013. One hundred and twenty-two microbiological specimens included pus specimen, wound swabs, blood, urine, body fluids were obtained for routine culture in microbiology laboratory.

Sample collection:

1. Study population: All age groups and both sexes visiting KNFH were included from whom the samples were obtained for routine culture and AST. Patients or their relatives refusing to give informed consents were excluded from the study. The samples from the patients who were already in medication were also excluded from the data.

2. Collection and transportation of clinical specimen:

   (a) Pus samples: A sterile cotton swab moistened with physiological saline was used to collect two pus swabs from each patient, one for Gram staining and other for culture. The both swabs were transported aseptically to laboratory for further processing.

   (b) Blood samples: 1 volume of blood sample collected from the patient was mixed with 5 volume of Brain Heart Infusion (BHI) and transported to laboratory for further processing.

   (c) Urine samples: About 10 to 20 ml of mid-stream urine sample was collected in a sterile container and transported to laboratory for further processing.

3. Processing of samples: Pus samples were observed for their consistency and blood contents. The physical appearance of urine samples was recorded for future reference.

   The pus samples, blood samples, and the urine samples were streaked on Nutrient Agar (NA), Blood Agar (BA) then MacConkey Agar (MA). The plates were incubated at 37 ± 0.2 °C for 24 to 48 hours. The colour, appearance, pH and turbidity of urine were evaluated during macroscopic examination of the urine sample (Vandepitte et al., 2003; Collee et al., 2001). The urine samples were cultured onto the BA and MA plates by the semi-quadrant streak technique using a standard calibrated loop having internal diameter of 4 mm. The protocol was followed as recommended by WHO (Vandepitte et al., 2003). The plates were then aerobically incubated at 37 ± 0.2 °C for 24 to 48 hours before reporting negative. Semi-quantitative counting method was performed to calculate the number of cfu $mL^{-1}$ of urine and the bacterial count was reported as:

Significant bacteriuria:

- Urine containing more than $10^5$ (100000) bacteria per ml that is $10^5$/ml urine-is an indication of UTI.
- Women with symptomatic UTI ≥ $10^5$ cfu $mL^{-1}$ :
- Men ≥ $10^3$ cfu $mL^{-1}$ (if 80% of the growth is due to single organism in both cases) (Scottish Intercollegiate Guidelines Network, 2012).

If the culture indicates presence of two uropathogens both showing significant growth, definitive identification and antimicrobial susceptibility testing of both were performed whereas in case of ≥ 3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery to laboratory (Vandepitte et al., 2003).

The culture plates were examined for the visual growth of the organisms. The colour and the morphology of the colonies were observed carefully. Then biochemical tests were performed for the isolated colonies for their identification.

Antibiotic susceptibility test (AST) by Kirby Bauer disc diffusion method was performed to select out the drug resistant organisms and multiple drug resistant organisms were selected for the study. The organism which showed resistance to at least three or more antibiotics of different classes were considered as MDR (Sahm et al 2000).For the identification of the characters, the ZOI chart provided by CLSI guidelines (2011) was followed. Five honey samples of various origins and of two varieties of bees (*Apis mellifera* and *Apis dorsata*) were used for the tests.

Honey discs were prepared by using dry sterile filter

papers of same thickness (1 mm) and same size (6 mm diameter) as the antibiotic discs on which about 50 microlitres of honey samples were dispensed individually. Same volume was poured on antibiotic discs to meet the concentration same as honey discs used. To ensure the transfer of same volume of honey, calibration of the micropipette was done at regular interval of time. The following steps were followed for the calibration. 1 ml of deionized water was carefully pipetted into the plate in a sensitive balance. Its weight was measured. After continuous repetition for 3 times, the average weight was calculated. Then the following formula was used to calculate the accuracy of the pipette.

$$accuracy\ (\%) = \frac{Pipette\ volume - average\ value}{Pipette\ volume} \times 100\ (\%)$$

All the bacterial isolates were tested for antibiotic susceptibility test by Kirby Bauer disc diffusion method with Mueller–Hinton Agar using the guidelines and interpretive criteria of the CLSI guidelines (CLSI 2011). The antibiotic discs used were Amoxycillin-Clavulanic acid (20/10µg), Ceftazidime (30µg), Ceftriaxone (30µg), Chloramphenicol (30g), Ciprofloxacin (5 µg), Cotrimoxazole (1.25/23.75µg), Gentamicin (30µg), Imipenem (10µg), and Oxacillin (1µg). The diameter of Zone of Inhibition (ZOI) was recorded for each disc. During this procedure, the measured diameter for honey discs was deducted from the diameter of the sterile discs.

The differences in Zone of inhibition (ZOI) of antibiotic contrasting with the antibiotic containing honey were statistically analysed to define the synergism. Data entry was performed using Microsoft® Excel® and data analysis was performed using Statistical Package for Social Sciences (SPSS® 16.0 for Windows®) software. The one-tailed t-statistics with α *value* 1% was implied for the statistical confirmation.

The following formula was used to calculate the test statistics t:

$$t = \frac{\bar{x}_{diff}}{\sqrt{n}}$$

$\bar{x}_{diff}$ = where

*s* = sample mean of the differences

s = sample standard deviation of the differences

n = sample size (i.e. number of pairs)

**Null hypothesis setup**: There is no difference in the use of honey and antibiotic or antibiotic alone ($\mu_1 - \mu_2 \geq 0$).

**Alternate hypothesis setup**: There is enhanced effect of honey and antibiotic than antibiotic alone ($\mu_1 - \mu_2 < 0$).

**Quality control**

Laboratory equipment like incubators, hot air oven, autoclave, refrigerator etc. were regularly monitored for their performance and immediately corrected if any deviation occurred. The temperature of the incubators and refrigerator were monitored every day. Reagents and biochemical media were checked for manufacture and expiry date and proper storage. After preparation, each media and reagent were labelled with preparation date, expiry date and stored in proper conditions. Sterility testing and performance testing were carried out using standard control strains.

**1. Quality control during isolation and identification**

Culture media that passed quality control for performance and sterility were used. During the identification, pure and isolated colony of the organism was used. After inoculation into different biochemical media, the inoculum was verified for pure culture and no contamination occurred during inoculation.

**2. Quality control during antimicrobial susceptibility testing**

Muller Hinton agar and antibiotic disc were checked for each lot number, manufacture date and expiry date and stored properly. Before use, each antibiotics disc and Muller Hinton Agar was monitored for their performance quality with E. coli ATCC 25922 and S. aureus ATCC 25923. For antimicrobial susceptibility testing, standard inoculum (matched with 0.5 McFarland solutions) was used.

## RESULTS

Twenty three culture positive samples were collected from Korea Nepal Friendship Hospital (KNFH). Among them, six (26.09%) were *S. aureus*, one was coagulase negative staphylococcus (CONS), eight (34.78%) were *E. coli*, four (17.39%) were *Klebsiella* species and again four (17.39%) were *Salmonella* species. After screening by AST, 10 samples (43.48%) were finalized as multi-drug resistant. In addition, 4 metallo-β-lactamase producers (2 *Acinetobacter* species and 2 *Pseudomonas aeruginosa*) (that were helped by Mr. Saroj Chandra Lohani, a researcher of Birendra Sainik Hospital, Chhauni) were also used for the study.

Among the 10 isolates collected from KNFH, 4 were MDR *S. aureus* (17.39%), 2 each were MDR *E. coli* (8.70%), MDR *Klebsiella* species (8.70%) and MDR *Salmonella* species (8.70%).

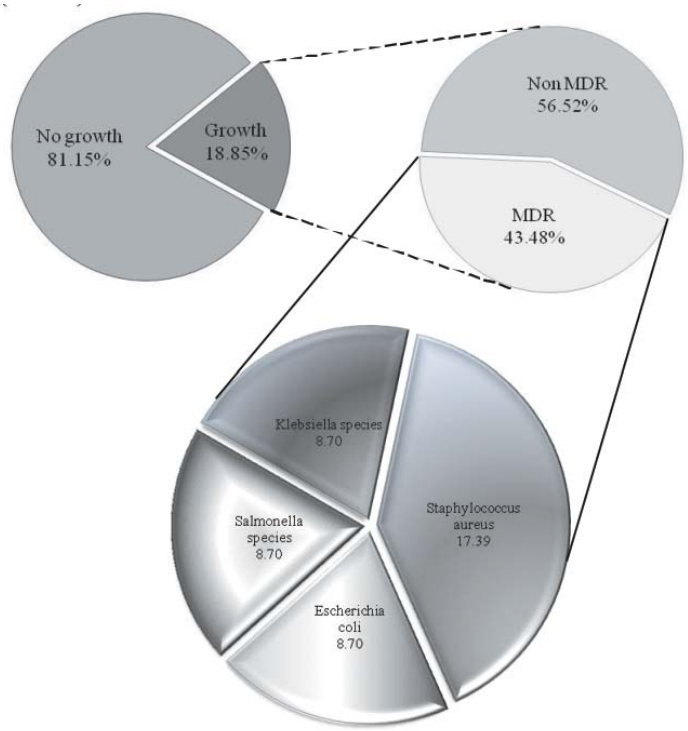


**Figure 1 Distribution of organisms in the samples with MDR isolates**

Five honey samples were also collected from The Beekeeping Shop, Lalitpur. Four of the samples were from single floral source, which nectar is collected by different colonies of *Apis mellifera.* One sample (Wild) was from mixed flower source, collected by *Apis dorsata.*

**Table 1: Physical property of honey samples**

| Honey type | Floral source | Bee variety | Colour* | Specific gravity at RT† |
|---|---|---|---|---|
| Buckwheat | *Fagopyrum esculentum* (Buckwheat) | *Apis mellifera* | 038R, 018G, 017B Or Hex#261211 | 1.4 |
| Chiuri | *Diploknema butyracea* (Nepali butter tree) | *Apis mellifera* | 079R, 071G, 032B Or Hex#4F4720 | 1.7 |
| Mustard | *Brassica campestris* (Mustard) | *Apis mellifera* | 245R, 235G, 097B Or Hex#F5EB61 | 1.5 |
| Rudilo | *Pogostemon benghalensis* (Rudilo) | *Apis mellifera* | 131R, 100G, 036B Or Hex#836424 | 1.45 |
| Wild | Wild or mixed floral source | *Apis dorsata* | 102R, 026G, 000B Or Hex#661A00 | 1.32 |

*Red Green Blue format in upper line, and hexadecimal code in lower line, data taken in average
†Room temperature

**Antibiotic susceptibility pattern of different isolates**

**1. *Staphylococcus aureus***

Oxacillin resistant (MRSA) *Staphylococcus aureus* were selected for this study. They were also Gentamicin resistant. These organisms showed variable inhibition zone when honey was incorporated with these antibiotics. In average, the susceptibility of the organism was increased by 3 mm for Chloramphenicol and Cotrimoxazole, 2 mm for Ciprofloxacin and Gentamicin. The increase by 8 mm in average was seen when honey sample was added in Oxacillin for the susceptibility test (Table 2).

**Table 2: Zone of inhibition of Antibiotic and Antibiotic incorporated with honey for *Staphylococcus aureus***

| | Mean | S.E.# | SD§ | Minimum | Maximum |
|---|---|---|---|---|---|
| Chloramphenicol | 26.5 | 0.8 | 3.59 | 21 | 30 |
| Chloramphenicol with honey | 29.65 | 1.41 | 6.33 | 17 | 40 |
| Ciprofloxacin | 23.75 | 0.85 | 3.8 | 19 | 29 |
| Ciprofloxacin with honey | 25.9 | 0.95 | 4.27 | 19 | 38 |
| Cotrimoxazole | 21.25 | 0.44 | 1.97 | 18 | 23 |
| Cotrimoxazole with honey | 24.35 | 0.7 | 3.12 | 19 | 30 |
| Gentamicin | 20 | 0.93 | 4.17 | 15 | 26 |
| Gentamicin with honey | 24.15 | 1.21 | 5.43 | 14 | 34 |
| Oxacillin | 12.5 | 0.95 | 4.26 | 7 | 17 |
| Oxacillin with honey | 20.5 | 1.84 | 8.22 | 0 | 38 |

#Standard Error of Mean §Standard Deviation

**2. *Escherichia coli***

At least one among Amoxycillin-Clavulanic acid, Ceftazidime, Ceftriaxone, Ciprofloxacin, and Cotrimoxazole resistant *E. coli* was taken to detect the synergism. Figure 2 shows that there is slight change in susceptibility pattern when honey was incorporated with the antibiotics. However, there was no statistical difference in T-score (Table 3) except gentamicin ($p>0.01$) and the correlation was strongly positive for the same antibiotic ($p<<0.01$). Other antibiotics could not show statistical significance in T-score and correlation ($p>0.01$) (Table 3).

**Table 3: T-statistics and correlation of antibiotics with antibiotics and honey for *E. coli***

| Variables | T score | p-value | R (correlation) | p-value |
|---|---|---|---|---|
| amoxyclav and amoxyclav with honey | 1.217 | 0.255 | -0.358 | 0.31 |
| ceftazidime and ceftazidime with honey | 3.337 | 0.009 | 0.25 | 0.486 |
| ceftriaxone and ceftriaxone with honey | 1.637 | 0.136 | 0.448 | 0.194 |
| chloramphenicol and chloramphenicol with honey | -0.238 | 0.817 | 0.560 | 0.092 |
| cotrimoxazole and cotrimoxazole with honey | 2.297 | 0.047 | 0.287 | 0.421 |
| gentamicin and gentamicin with honey | 3.000 | 0.015 | 1.000 | <0.001 |

**3. *Klebsiella* species**

For the study, *Klebsiella* species used were resistant to Amoxycillin-Clavulanic acid, Ceftazidime, Ceftriaxone, Ciprofloxacin, Cotrimoxazole and/or Gentamicin, but all were susceptible to Chloramphenicol. On the test, there was sudden increase in susceptibility with the combination of honey and antibiotics (Figure 2). Ceftazidime, ceftriaxone, and chloramphenicol had shown significant synergism ($p<<0.01$) and among them, chloramphenicol showed strong positive relation ($R = 82.7\%$) with honey but a slight negative relation ($R = -6\%$) between ceftriaxone and honey (Table 4).

**Table 4 T-statistics and correlation of antibiotics with antibiotics and honey for *Klebsiella* species**

| Variables | T score | p-value | R (correlation) | p-value |
|---|---|---|---|---|
| amoxyclav and amoxyclav with honey | 2.984 | 0.015 | 0.00 | 1.000 |
| ceftazidime and ceftazidime with honey | 5.155 | 0.001 | 0.342 | 0.333 |
| ceftriaxone and ceftriaxone with honey | 4.287 | 0.002 | -0.06 | 0.869 |
| chloramphenicol and chloramphenicol with honey | 10.301 | <0.001 | 0.827 | 0.003 |
| cotrimoxazole and cotrimoxazole with honey | 0.404 | 0.696 | -0.720 | 0.019 |

**4. *Salmonella* species**

Ceftazidime, Cotrimoxazole and/or Gentamicin resistant *Salmonella* species were used for this study. Except Ciprofloxacin ($R= -89.4\%$, $p<<0.01$), all other antibiotics showed enhanced inhibition zone when honey was incorporated, but could not show the significant statistical difference ($p>0.01$) (Table 5).

**Table 5: T-statistics and correlation of antibiotics with antibiotics and honey for *Salmonella* species**

| Variables | t score | p-value | R (correlation) | p-value |
|---|---|---|---|---|
| amoxyclav and amoxyclav with honey | 3.972 | 0.003 | 0.715 | 0.02 |
| ceftazidime and ceftazidime with honey | 1.374 | 0.203 | -0.186 | 0.606 |
| ceftriaxone and ceftriaxone with honey | 1.134 | 0.286 | 0.502 | 0.139 |
| chloramphenicol and chloramphenicol with honey | 2.160 | 0.059 | -0.492 | 0.148 |
| ciprofloxacin and ciprofloxacin with honey | -0.519 | 0.616 | -0.894 | 0.000 |
| cotrimoxazole and cotrimoxazole with honey | 2.067 | 0.069 | -0.279 | 0.436 |
| gentamicin and gentamicin with honey | 3.000 | 0.015 | 1.000 | <0.001 |

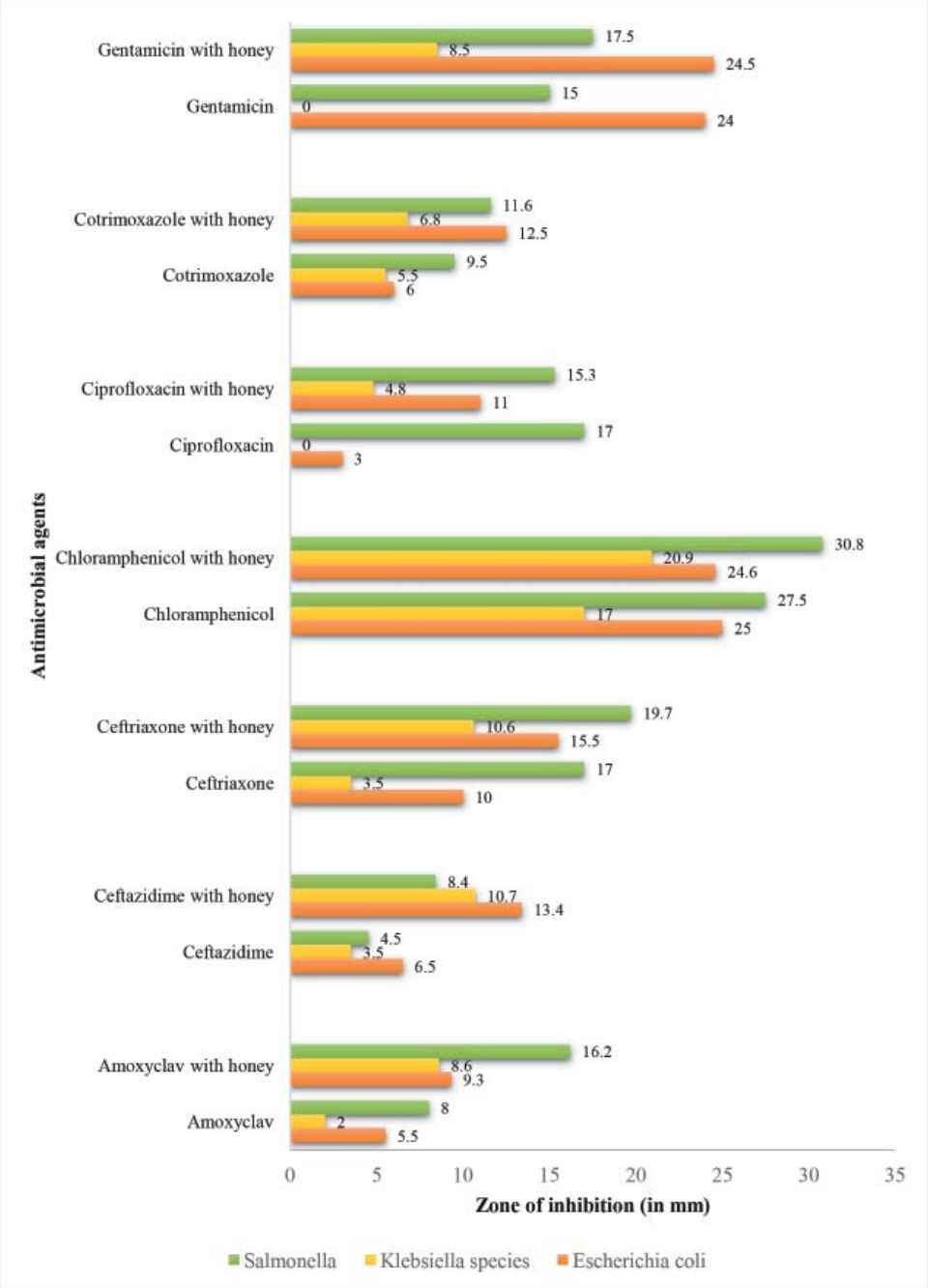


**Figure 2 Comparative chart of Zone of inhibition for *Enterobacteriaceae* between antibiotics and antibiotics with honey**

**5. *Pseudomonas aeruginosa***

When metallo $\beta$–lactamase producing (MBL) *Ps. aeruginosa* were used, there was a drastic and significant increase in zone of inhibition ($p<<0.01$) (Figure 3). But the relation was not much stronger and lies between 8% to 41.9% (Table 6).

**Table 6 T-statistics and correlation of antibiotics with antibiotics and honey for *Pseudomonas aeruginosa***

| Variables | t score | p-value | R (correlation) | p-value |
|---|---|---|---|---|
| ciprofloxacin and ciprofloxacin with honey | 8.444 | <0.001 | 0.419 | 0.228 |
| gentamicin and gentamicin with honey | 16.058 | <0.001 | 0.082 | 0.821 |
| imipenem and imipenem with honey | 8.508 | <0.001 | 0.103 | 0.776 |

**6. *Acinetobacter* species**

MBL *Acinetobacter* species showed decreased susceptibility with Ciprofloxacin and Imipenem when honey was added (Figure 3). It was found that there was 28.9% relation of honey with Ceftazidime and –4.9% with Gentamicin (Table 7).

**Table 7: T-statistics and correlation of antibiotics with antibiotics and honey for *Acinetobacter* species**

| Variables | t score | p-value | R (correlation) | p-value |
|---|---|---|---|---|
| ceftazidime and ceftazidime with honey | 3.955 | 0.003 | 0.289 | 0.417 |
| ceftriaxone and ceftriaxone with honey | 0.631 | 0.544 | 0.071 | 0.846 |
| ciprofloxacin and ciprofloxacin with honey | -0.303 | 0.768 | 0.296 | 0.406 |
| cotrimoxazole and cotrimoxazole with honey | 2.666 | 0.026 | 0.095 | 0.795 |
| gentamicin and gentamicin with honey | 0.923 | 0.380 | -0.049 | 0.894 |
| imipenem and imipenem with honey | -0.419 | 0.685 | 0.921 | <0.001 |

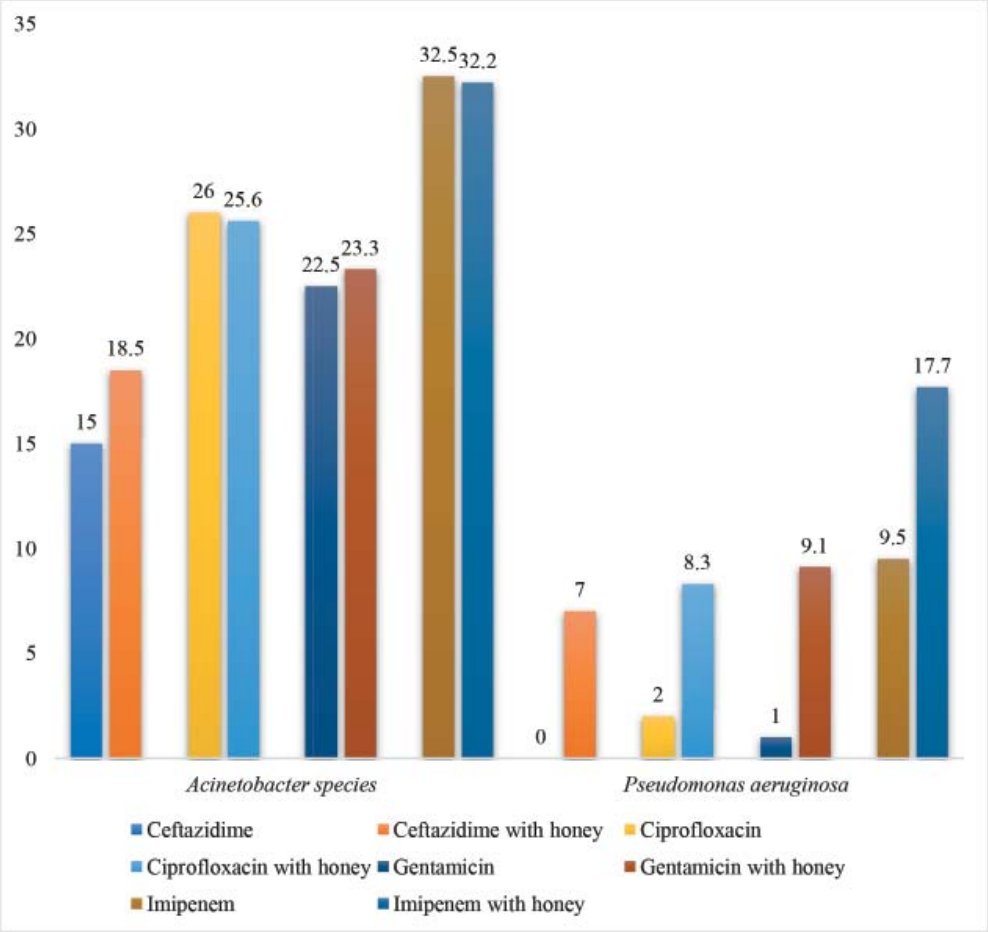


**Figure 3 Zone of Inhibition of Antibiotic and Antibiotic incorporated with honey for *Acinetobacter* species and *Pseudomonas aeruginosa***

**Susceptibility test to honey compared via ANOVA**

In this study among the 6 bacterial samples used, honey was found to be most effective against *S. aureus* ($\bar{x}$ = 21.2 ± 4.44 mm) and the effect was decreased gradually to *Acinetobacter* species ($\bar{x}$ = 18.2 ± 2.49 mm), *Pseudomonas* species ($\bar{x}$ = 7.2 ± 1.10 mm), *Klebsiella* species ($\bar{x}$ = 6 ± 2.45 mm), *E. coli* ($\bar{x}$ = 5.4 ± 3.58 mm) and *Salmonella* species showed lowest inhibition zone ($\bar{x}$ = 5.2 ± 1.30 mm) for the honey.

When the relation between the groups was compared, null hypothesis that average ZOI differences in different groups are equal is rejected. It means the inhibition is variable among the bacteria (F=39.17, p<0.05). From means plot, it was found that *S. aureus* and *Acinetobacter* species were highly susceptible whereas *Ps. aeruginosa* and organisms of *Enterobacteriaceae* family are less susceptible to honey (Figure 4).

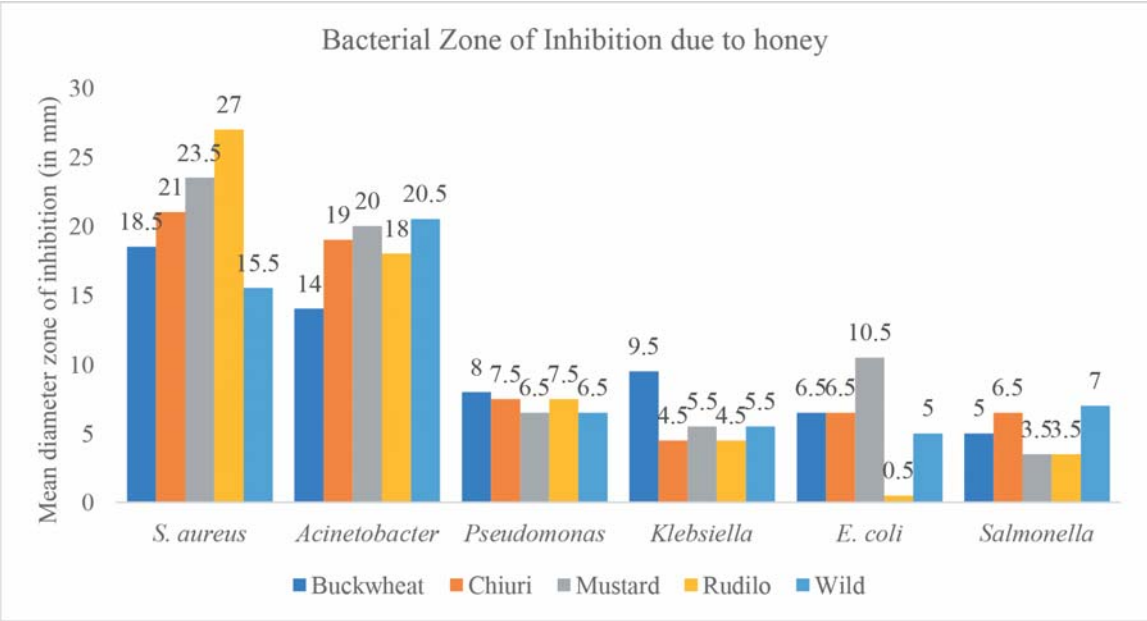


**Figure 4 Clustered bar chart of Zone of Inhibition of different bacteria by different honey samples**

## DISCUSSION

The antibacterial and antifungal effect of honey has been repeatedly noticed from ancient time. The traditional method of treatment using honey is a remarked gateway for the study of microbiology. The advancement of tools and techniques and modernization of the lifestyle has reduced the use of honey. Antibiotics are now gaining their path as the first selection for the treatment of bacterial infection. This caused uncontrollable increase in drug resistant organisms. Pokhrel (2004) found that 47.57% pathogens in sputum and 60.40% pathogens in urine were MDR. Bomjan (2005) found that 60% urinary and sputum isolates were MDR pathogens. This study shows 43.48% multi-drug resistant organisms were isolated from various samples. These data show that the distribution of MDR pathogens is different on different geographical areas within Nepal. Indiscriminate & inadequate use of antibiotics causes losing their potency against various types of organisms (Bajaj et al. 2018). Due to the different properties of honey, it is used traditionally for treatment of different infections of wound, burns and blood infections (Sharp 2009). Thus the synergism of antibiotics and honey is an interest of study.

In this study, 5 honey types derived from different floral sources were screened for their antibacterial activity. Initial screening with the Kirby Bauer disc diffusion method demonstrated that all tested honey types exhibited more or less susceptibility to all the clinical bacterial isolates used. *S. aureus* showed greater sensitivity and the members of Enterobacteriaceae were less sensitive to honey. This finding is in accordance with the findings of previous studies (Abd-El et al. 2007, Moussa et al. 2012, Ahmed et al. 2013). These results are very much important for clinical implementation such as wound and burn management (Sufya et al. 2014).

Antibacterial activity of honey can be described due

to different factors. Due to low water concentration of honey and its hygroscopic nature, its osmophilic activity extracts water from bacteria and makes the organism inactive. The acidic pH inactivates the organism enzymes for metabolism and thus the organisms are inhibited. In addition, glucose oxidase obtained from bee gut that is regurgitated by bee during honey making process, de$_{H_2O_2}$es glucose to produce hydrogen peroxide. The oxidises the organism enzyme and is inactivated and leads to its death. Different phytochemicals like methyl glyoxal and methyl syringate from the floral source is taken by bee during collecting nectar. These metabolites coordinates with the acidic pH to act for the bacterial inhibition. Recently, other components were also identified in honey that help it as antibacterial effect. Catalase, Maillard reaction products, polyphenols, ascorbic acid, bee defensin-1 protein are some of them (Bizerra et al. 2012).

In different studies, significant synergism was found between honey and antibiotics when tested in vitro. Honcrivine (honey plus acriflavine 0.1%) is efficient for debridement of wound without any inflammatory or allergic responses (Efem 2009) which is due to activation of protease due to $H_2O_2$ from honey dressing (Lane et al. 2019). In a study for 16 clinical pathogens including *S. aureus*, *Salmonella* species, *Streptococcus pyogenes*, *B. cereus* and *B. subtilis*, were up to 60% more resistant than equal reference strains, and concluded for variability in the antibacterial effect of honey (Voidarou et al. 2011). A research in Pakistan has concluded that methyl glyoxal can be a good inhibitory agent against MDR and non-MDR *Salmonella* and other Gram-negative organisms (Afzal et al. 2018).

This study found increased zone of inhibition when honey is added to antibiotics. Though the reason behind the synergism is not clear, a study described the synergism of methylglyoxal (found in natural honey) with Piperacillin, a β-lactam antibiotic. But a study in New Zealand concluded methylglyoxal is not the sole factor for Manuka honey to act synergistically with rifampicin against MRSA (Jenkins and Cooper 2012). From these studies it can be concluded that various factors are responsible for the synergistic or antagonistic action of antibiotics with honey.

In this study, honey showed synergetic effect with Gentamicin against *E coli* and *Salmonella* species, with Chloramphenicol against *Klebsiella* species (R=82.7%) and with Ceftazidime against *Acinetobacter* species. The results are incompatible with the results of a study performed by Karayil et al. in 1998, where antibiotics Gentamicin, Amikacin and Ceftazidime were synergetic with honey against *Pseudomonas* species and not with *Klebsiella* species. This leads to the mystery if the organisms in different geography can lead to different synergism or changing their genetic behaviour with time lapse.

## CONCLUSION

Honey is found to be very effective against *Staphylococcus* infection whereas it is least effective against *E. coli*. The synergetic effect of honey with different antibiotics are found to be effective against *S. aureus, Pseudomonas aeruginosa* and the members of *Enterobacteriaceae* family involved in this study but found to be less or no effective against *Acinetobacter* species.

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# Incidence of Intestinal Parasites in Government and Private School Going Children

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## ABSTRACT

**Objective:** Aim to assess the incidence of intestinal parasites in government and private school going children.

**Methods:** The work was conducted from October, 2018 to March, 2019 at Microbiology Laboratory of DAV College, Dhobighat, Lalitpur. A total of 100 stool samples of children aged between 5-12 years were collected from both government and private schools situated in Lalitpur metropolitan city, during school hours. The stool samples were examined for intestinal parasites by Saline wet mount; Iodine wet mount and Formal – ether sedimentation technique. The questionnaires accompanying the queries related to the study were filled.

**Results:** Of the total 100 stool samples examined, intestinal parasites were observed in 7% (7/100) of the total stool samples. Among the positive stool samples, 71% (5/7) of the stool samples were from government school's children whereas 29% (2/7) were from private school's children. Fifty seven pecentage 57% (4/7) girls and 43% (3/7) boys were found to be infected with intestinal parasite in the tested stool samples. Out of total parasite detected, 57% (4/7) were eggs of Ancylostoma *duodenale*, 29% (2/7) were eggs of Ascaris *lumbricoides* and 14% (1/7) were cysts of Giardia *lamblia.* The study indicates that *Ancylostoma* is the most commonly infecting parasite followed by *Ascaris* and *Giardia*.

**Conclusion**: Personal hygiene and sanitary condition were responsible for the incidence of intestinal parasites in the school going children. Environmental sanitation improvement and health education promotion will be helpful to reduce the parasitic infection rate.

**Keywords**: Intestinal parasites, Samples, Sedimentation, Sanitation, Infection

## INTRODUCTION

Parasites are those organisms which receive nourishment and shelter from another organism where they live, and host is the organism which harbours the parasites (Chatterjee, 1998). Those parasites which live on the intestine of host are known as intestinal parasites. Intestinal parasite ranges from virus, bacteria and protozoa to helminthes (Chandrasekhar et al., 2005). It is estimated that some 3.5 billion people are affected and 450 million are ill as a result of intestinal parasitic infections (Tandukar et al., 2015). Of those 450 million, at least 50% is school going children. These infections are most prevalent in tropical and subtropical regions of the developing world where adequate water and sanitation facilities are lacking (Haque, 2007). Children are the most affected due to heavy infections they harbor and because of their vulnerability to nutritional deficiencies. The distribution and prevalence of the various intestinal parasites species depend on sociogeographical, economical and inhabitant customs (Tandukar et al., 2015). In Nepal, 50% of diarrheal diseases among children are due to parasites, and hence parasitic diarrhea being major killer of Nepalese children. The prevalence ranges from 32.6% to 72.4% among school going children, suggested by majority of studies done in Kathmandu and rural hills (Tandukar

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et al., 2015). Most common intestinal parasites reported from the stools of school going children in Nepal are *Ascaris lumbricoides, Hymenolepis nana,* hookworm, *Trichuris trichiura, Giardia lambia, Entamoeba histolytica.* Of the protozoal infections, amoebiasis and giardiasis are frequently reported. About 70% of health problems are due to infectious diseases. Diarrheal disease is one the major cause for morbidity and mortality in Nepal (Yadav and Prakash, 2016).

Available prevalence data on parasitic infections in the developing countries of the South-east Asian regions including Nepal are not sufficient. The present study is thus conducted to compare the intestinal parasitic status among the government and private schools going children and figure out the relation between incidence based on the hygiene practices and economic status.

## MATERIALS AND METHODS

A cross-sectional study was carried out among the school going children of one government school and one private school, of both genders, belonging to age group 5-12 years. A total of 100 stool samples, 50 male and 50 female stool students were taken and processed for the detection of parasites from October, 2018 to March, 2019 at microbiology laboratory of DAV College, Dhobighat, Lalitpur. Unlabeled samples or sample contaminated with urine were excluded.

Fecal samples were collected in dry, clean, leak-proof, screw-capped plastic containers and the children were asked to fill up a questionnaire with the help of their teachers and parents. From the questionnaire, major information like age of child, sex of child, family size, parent's occupation, source of drinking water at home, hand washing habit, medical complications, history of taking anti parasitic drug in last six months etc. were collected. Microscopic examination was done by normal saline and iodine wet mount method and concentration was done by formalin-ether concentration technique. Thus obtained data from laboratory examinations and questionnaire were analyzed and presented.

## RESULTS

The incidence rate of the intestinal parasites among tested stool samples of school going children was found to be 7% (7/100). Out of 7% positive stool samples, 71.42% (5/7) parasites were found in students studying in government school and 28.57% (2/7) parasites were found in students studying in private school. Among detected parasites, helminthes 85.7% (6/7) were dominated over protozoans 14.2% (1/7). Where, 57.17% (4/7) positive stool samples were detected with *Ancylostoma duodenale* 28.58% (2/7) with Ascaris *lumbricoides* and 14.28% (1/7) with *Giardia lamblia*. Among 50 samples from males students, 6% (3/50) were found to be positive with intestinal parasites and out of 50 stool samples from female students, 8% (4/50) were found to be positive with intestinal parasites. Among the 100 students under this study, 48 students were found to use soap and water to wash their hands after defecation, among them 2.08% (1/48) was detected with intestinal parasites in their stool samples. Similarly, 5 students were found using ash and water to wash their hand after defecation and none of their stool samples were tested positive for intestinal parasites. Also 15 students were found using soil and water to wash their hands after defecation out of which 6.66% (1/15) stool samples these students were found to be positive for intestinal parasites. Similarly, 32 students were found using only water to wash their hand after defecation and out of which 15.6% (5/32) stool samples were found to be positive with intestinal parasites.

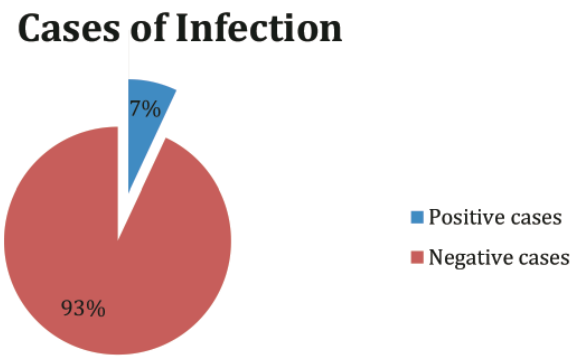


**Figure 1: General Incidence of intestinal parasites**

Table 1: Distribution of intestinal parasites based on type of school

| Schools | Positive cases in no. | Positive Cases in % |
|---|---|---|
| Government | 5 | 71.42 |
| Private | 2 | 28.57 |
| Total | 7 | 100 |

**Table 2: Incidence of type of intestinal parasite with respect to type of school**

| Species of parasites identified | Total No. of Parasite found No (%) | School | |
|---|---|---|---|
| | | Government No (%) | Private No (% |
| *Ancylostoma duodenale* | 4 (57.17%) | 3 (75%) | 1 (25%) |
| *Ascaris lumbrocoides* | 2 (28.58%) | 1 (50%) | 1 (50%) |
| *Giardia lamblia* | 1 (14.28%) | 1(100%) | 0 |
| Total | 7(7%) | 5 (71.42%) | 2 (28.57%) |

**Table 3: Incidence of Intestinal Parasites based on gender**

| Sex | Total samples examined | Total +ve cases **No (%)** | Government school **No (%)** | Private School **No (%)** |
|---|---|---|---|---|
| Male | 50 | 3(6%) | 2(66.66%) | 1(33.33%) |
| Female | 50 | 4(8%) | 3(75%) | 1(25%) |
| Total | 100 | 7(7%) | 5(71.42%) | 2(28.57%) |

**Table 4: Incidence of Intestinal Parasites on the basis of hand washing material used**

| Agent | Observation no. | + ve Cases **No (%)** | Government School **No (%)** | Private School **No (%)** |
|---|---|---|---|---|
| Soap and water | 48 | 1 (2.08%) | 1 (100%) | 0 |
| Ash and water | 5 | 0 (0%) | 0 (0%) | 0 |
| Soil and water | 15 | 1 (6.66%) | 1 (100%) | 0 |
| Only with water | 32 | 5 (15.6%) | 3 (60%) | 2 (40%) |
| Total | 100 | 7 | 5 (71.42%) | 2 (28.57%) |

## DISCUSSION

Despite the government policy for anti-helminthic program and health education program launched at community level, the intestinal parasitic infestation still remains the major cause of diarrheal diseases in Nepal (Sherchand et al., 2009). In the present study, the overall incidence of intestinal parasitic infection among school going children was found to be 7% (7/100). Our result was compared to the others research findings related to intestinal parasites in various parts of the world. A study done by Dahal et al, (2018) showed incidence of 12.4% (24/194) in Kathmandu valley, a study by Shakya et al, (2012) showed incidence of 13.9%. The higher prevalence rates were reported as 31.7% and 31.13 % (Kidane et al., 2014; Tiwari et al., 2013). These differences might be due to environmental, geographical, climatic conditions of the study place and the technique used for detection of parasites. The lower prevalence in this study may be due to the hygiene habits of children and also anti-helminthic drugs given to the children.

As per our study among the two categories of school going children, intestinal parasites were found more commonly in the children of government school 71.42% (5/7) showing higher incidence of intestinal parasite compared with private school going children 28.57% (2/7). In the studies conducted by Dahal et al, (2018) and Tandukar et al, (2013), the intestinal parasites were found higher in the children of government school (66.7%) and (73.3%) and lower in children of private school (33.3%) and (7.7%) respectively. Our finding is in agreement with both studies. This is possibly due to low socio-economic status of the government school children because of which they are unable to get avail the sanitary goods and products, their poor hygienic habits as they are not much aware about sanitary practices. Similarly, lack of sanitation prevailing in the school area can also be one of the reasons.

The overall incidence of intestinal parasites in this study was 7 (7%), out of which 4 (57.17%) were infected by *Ancylostoma duodenale* along with *Ascaris lumricoides* 2(28.58%) and with *Giardia lamblia* 1 (14.28%). Out of all positive stool samples for *Ancylostoma duodenale*, 3 (75%) were form government school children's stool and 1 (25%) was from private school children's stool. Similarly, positive stool samples of Ascaris *lumbricoides* were 1(50%) from government school children and

1 (50%) from private school children's stool. Only 1 positive stool sample showed *G. lamblia* which was from government school going children's stool. Whereas in the study done by Dahal et al, (2018) incidence rate of the parasite was same (*A. lumbricoides* 8.3%, Hookworm 8.3%). In the study conducted by Tandukar et al, (2015) there was no incidence of *Ancylostoma duodenale* but had presence of *Ascaris lumbricoides* 4(5.3%) along with *T. Trichuria* 2(2.6%). The incidence of hookworm was found in higher rate in comparison to other helminthes because children are mostly exposed to external environment without slippers due to which the worm infestation can take place through their feet. Similarly, hand to mouth transmission of the eggs is more common among children. These parasites were also common among children because of consumption of faecal contaminated water, lack of public awareness.

Among the 100 samples, female children had higher incidence rate of intestinal parasitic infection (8%) than male children (4%). This finding is similar to the finding of study conducted in Chitwan by Rai et al, (2017) in which females were more infected i.e. 24.8% female and 21.8% male. Other study conducted by Shakya et al 2012; Bhattachan et al 2015 also showed that females were more infected than males. The study was not in agreement with the study done by Kidane et al (2014) (male 58.2%; female 62.8%) and Chandi and Lakhani (2018) (male 28.75%; female 35.6%). These differences indicated that the association of gender with parasitic infection differs from one community to another and might be due to socio-behavioral activities (Khanal et al. 2016). The relatively higher incidence in female children might be mainly due to the general trend in this orthodox society where females do more household and soil related works than males. This difference also could mainly be due to living conditions.

Furthermore, an important difference was observed in hand washing practice after defecation with soap and water which had 2.08%(1/48) incidence of intestinal parasite, followed by soil and water 6.66% (1/15), ash and water 0% (0/5), and only with water 15.6%(5/32). The high incidence of intestinal parasitic infection among children using only water to wash their hands after defecation might be due to the habit of handling foods and drinks with contaminated hands. A study conducted by Tandukar et al, (2013), the intestinal parasitic infection was found higher in those children who did not follow hand washing practice after defecation (47.5%). Tandukar et al, (2013) stated that children playing in outdoor environment get in contact with parasites and not washing hands after defecation and before meal leads to the entry of parasites in the body.

## CONCLUSION

The present study reveals that incidence of intestinal parasites is comparatively higher among children of government school than the children of private school. Poverty, lack of awareness, poor environmental sanitation, raw and uncooked food consuming habit and unsafe drinking water are some of the predisposing factors highlighted by this study as causes of parasitic infections. Hence, the results of this study re-emphasize the fact that intestinal parasitic infestation among school going children in the study area is mainly water-borne and are the result of poor environmental sanitation. The burden of parasitic infestations among the school children, coupled with the poor sanitary conditions in the schools, should be regarded as an issue of public health priority. This strongly supports the need for school health programs that will involve periodic deworming, health education and improvement of school sanitation.

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# Detection of Metallo-β-Lactamases and Carbapenemase Production *Pseudomonas aeruginosa* Isolates from Burn Wound Infection

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## ABSTRACT

**Objective**: The study aims to detect carbapenemase producing *P. aeruginosa* isolated from burn wounds and confirm MBL production by Imipenem-Combined disk method.

**Method**: A total of 310 non-repeated clinical specimens including tissues, pus aspirates, and wound swabs were processed using standard microbiological procedure. Each identified isolate of *P. aeruginosa* was subjected to *in vitro* antibiotic susceptibility test by using modified Kirby-Bauer disc diffusion method. Two imipenem (10µg) disks were placed on the surface of the agar plate in which one of them was added with 5µl of 0.5M EDTA solution. The result was interpreted after 18 hours of incubation at 37°C by comparing the inhibition zone of imipenem and imipenem-EDTA disks. The increase in inhibition zone by ≥7mm with imipenem-EDTA disks than imipenem alone was considered as MBL Positive. Similarly, for detecting carbapenemase Modified Carbapenem Inactivation Method (mCIM) was used.

**Results**: *P. aeruginosa* was found to be the predominant organism (13.99%). Among 20 *P. aeruginosa* isolates resistant to imipenem and meropenem, 20% were found to be carbapenemase producer by mCIM assay and 15% were found to be MBL producers by Imipenem-Combined disk method. High percentage of MBL producing isolates of *P. aeruginosa* were found resistant towards tested antibiotics.

**Conclusion**: This study reports that the clinical isolates of *Pseudomonas aeruginosa* have the ability to produce MBL. The increasing and rapid spread of *P. aeruginosa,* as well as the rate of drug resistance among the isolates, was found to be a worrisome situation.

Keywords: Burn wound infections, Antibiotic susceptibility test, Carbapenemase, Metallo-Beta-Lacatmase (MBL), CLSI

## INTRODUCTION

Burns are more persistent cause of infection when compared with surgical wounds. This is because of prolonged hospital stay and larger area involved. In addition, burns provide an appropriate location for bacterial multiplication (Aghnihotri et al. 2004). The predominating organisms that cause burn wound infections in any burn treatment facility change over time. Initially, after the injury Gram positive bacteria inhabit the burn wound (Barret and Herndon 2003) and later Gram-negative bacteria also rapidly colonize the burn wound surface (Wysocki 2002).

*Pseudomonas aeruginosa* is accountable for serious hospital acquired infections, chiefly in burn patients (Sheikh et al. 2014). Although, a wide range of antibiotics are used for its treatment, the bacterium is

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intrinsically resistant to many antibiotics due to which the therapeutic options for treating serious infections are severely restricted. The resistance to antibacterials is developed either via mutational processes that alter the expression or through the acquisition of resistance genes on mobile genetic elements (i.e., plasmids) and/or function of chromosomally encoded mechanisms. Hence, a global health issue has arisen because of increasing number of multidrug-resistant (MDR) *P. aeruginosa* (Adachi. 2009; Madigan et al. 2012).

Carbapenems are the drug of choice used for treating infections caused by *Pseudomonas aeruginosa*, producing cephalosporinase, AmpC or extended-spectrum β-lactamases (Zavascki et al. 2010). However, the development of carbapenem-resistant *P. aeruginosa* has threatened on the use of carbapenem in the management of its infections. The most frequent reason behind resistant to carbapenem in *P. aeruginosa* is attributed to impermeability through alteration or loss of the porin OprD, increased expression of an efflux pump, or the production of class B **metallo-β-lactamases** (MBLs) (Kateete et al. 2016).

Production of metallo-beta-lactamase (MBL) enzyme has been one of the major causes of carbapenam resistance in *P. aeruginosa*. Metallo- β-lactamase mediates resistance to β-lactams by cleaving the amide bond of the β- lactam ring. MBLs can be divided into two groups, one that are chromosomally mediated and the other encoded by transferable genes (Walsh et al. 2005). The six different types of mobile MBLs, namely, IMP, VIM, SPM, GIM, SIM and AIM are known so far and the mechanism of hydrolysis varies from one kind to another (Gupta, 2008). *P. aeruginosa* is predominantly known to produce IMP and VIM type MBL (Khosravi and Mihani. 2008). The IMP and VIM genes responsible for MBL production are horizontally transferable via plasmids and can rapidly spread to other bacteria (Zubair et al. 2011).

In 2017, Carbapenem-resistant *P. aeruginosa* (CRPA) is ranked as the second most critical-priority bacterium among 20 antimicrobial-resistant bacterial species in a survey conducted by World Health Organization on multi-country antibiotic resistance (Tacconelli et al. 2018). Moreover, infection with MBL producing organism such as *P. aeruginosa* is associated with higher rates of mortality, morbidity and health care cost, especially due to inadequate empirical therapy (Picao et al. 2008; Kaleem et al. 2010). Although, there have been reports and studies involving increasing drug resistance in burn patients worldwide, the information regarding the etiology and management of burn wound infections is limited in developing countries like Nepal. Hence, the prime focus of this study is to determine proportion of MBL and carbapenemase in *P. aeruginosa* isolated from burn wound which if taken into consideration either during empirical therapy or pathogen directed therapy can substantially reduce health care associated cost and more importantly the emergence and spread of resistance itself.

## MATERIALS AND METHODS

This cross sectional hospital based prospective study was conducted in between June 2018 and December 2018 in microbiological laboratory of Nepal Cleft and Burn Center, Kirtipur Kathmandu. A total of 310 non-repeated clinical specimens including tissues, pus aspirates, and wound swabs were processed using standard microbiological procedure during the study period. The samples of patients not listed as burn victims were excluded from the study.

All the samples were collected by experienced medical personnel using standard microbiological procedures. Wound swabs were collected by using sterile cotton-wool swab taking special care to avoid the contamination by the commensal organism. Pus aspirate samples were either collected in a sterile syringe or by using a sterile cotton-wool swab. The cotton-wool swabs were then placed back into sterile tubes and capped. Tissue culture samples were collected with special care avoid contamination by commensal organisms and placed in a sterile container. Pus aspirate from the wounds was collected with the help of sterile syringe and contamination by the commensal organism was prevented with special care (Cheesebrough M 2018). After proper labeling, the samples were transported to the microbiology laboratory promptly.

The wound swabs and pus aspirate were directly inoculated into Blood Agar (BA) and Macconkey Agar (MA) with the help of a sterile loop. The inoculated agar plates were incubated aerobically overnight at 37°C. The tissue culture from burn wounds was first aseptically removed from the container and inoculated into Brain heart Infusion (BHI) broth and incubated aerobically overnight at 37°C. This was followed by subculture on BA and MA (HiMedia). Standard

microbiological procedures were followed for the identification of the isolates as described in Bergey's manual of systemic bacteriology

Each identified isolate of *P. aeruginosa* was subjected to *in vitro* antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method as recommended by CLSI guidelines on Muller Hinton Agar (CLSI. 2018). Commercially available antibiotic discs of HiMedia were used that includes amikacin (30 µg), gentamycin (10 µg), ciprofloxacin (5 µg), cefepime (5 µg), piperacillin/tazobactam (100/10 µg), cefoperazone/sublactam (75/30 µg), meropenem (10 µg), imipenem (10 µg), doxycycline (30 µg), ceftazidime (30 µg), polymyxin B (10 µg), colistin (CL). MDR isolates were detected based on their resistance to two or more antibiotics (Cheesbrough. 2006; CLSI. 2018)

Detection of metallo-beta-lactamase production by Combined Disk (CD) assay

Following the standard procedures, the test organism was inoculated on MHA plate as recommended by the CLSI guidelines. Two imipenem (10µg) disks were placed on the surface of the agar plate in which one of them was added with 5µl of 0.5M EDTA solution. The result was interpreted after 18 hours of aerobic incubation at 37°C by comparing the inhibition zone of imipenem and imipenem-EDTA disks. The increase in inhibition zone by ≥7mm with imipenem-EDTA disks than imipenem alone was considered as MBL Positive (Yong et al. 2002).

**Modified Carbapenem Inactivation Method (mCIM)**

A 1-µl loop full of test organism from an overnight agar plate was transferred to a tube containing 2 ml of trypticase soy broth (TSB) and the suspension was vortexed followed by addition of 10-µg meropenem disk to the suspension. The suspension was incubated at 35 °C for four hours at ambient air. Prior to completion of four-hour incubation, a 0.5 McFarland suspension of *E. coli* ATCC 25922 was prepared and inoculated onto MHA plate following Modified Kirby-Bauer Disk Diffusion Method. The meropenem disc was removed from TSB suspension after complete four-hour incubation with the help of a 10-µl loop, taking care to remove excess liquid from the disk. The freshly removed meropenem disc was immediately placed on the MHA plate that has been inoculated with *E. coli* ATCC 25922. The plate was incubated at 35 °C in ambient air overnight. The zone of inhibition around the meropenem disc was measured and interpreted. If the zone of inhibition was measured to be 6-15mm, the test organism was Carbapenemase positive, if the zone of inhibition was measured to be greater or equal to 19mm, the test organism was carbapenemase negative. And if the zone of inhibition was measured to be 16-18mm, the test organism was considered intermediate (CLSI. 2018).

## RESULT

Out of 310 non-repeated clinical samples collected, 72.58% showed significant bacterial growth. Overall 336 isolates were isolated from the culture positive samples of which 134 (59.56%) showed mono-microbial bacterial growth while 91 (40.44%) showed poly-microbial bacterial growth of the cases. The isolation of Gram positive and Gram negative bacteria were 26.49% and 73.51%, respectively.

Among the Gram positive bacteria, *S. aureus* (11.01%) was most commonly isolated followed by CoNS (8.93%), whereas for Gram negative bacteria 13.99% of isolates were *P. aeruginosa* followed by *K. pneumoniae* (12.8%) and *Acinetobacter calcoaceticus-baumannii* complex (10.12%).

**Table 1: Gram positive isolates among samples**

| Gram Positive isolates | Wound Swab | Tissue culture | Pus aspirate | Total |
|---|---|---|---|---|
| | No. (%) | No. (%) | No. (%) | No. (%) |
| *S. aureus* | 33 (9.82) | 4 (1.19) | 0 (0) | 37 (11.01) |
| *E. faecalis* | 11 (3.27) | 11 (3.27) | 0 (0) | 22 (6.55) |
| CoNS | 27 (8.04) | 2 (0.60) | 1 (0.30) | 30 (8.93) |
| **Total** | **71 (21.13)** | **17 (5.06)** | **1 (0.30)** | **89 (26.49)** |

Table 2: Gram negative isolates among samples

| Gram Negative isolates | Wound Swab | Tissue culture | Pus aspirate | Total |
|---|---|---|---|---|
| | No. (%) | **No. (%)** | **No. (%)** | **No. (%)** |
| *A. lwoffi* | 5 (1.49) | 7 (2.08) | 0 (0) | 12 (3.57) |
| ACB complex | 26 (7.74) | 7 (2.08) | 1 (0.30) | 34 (10.12) |
| *E. coli* | 22 (6.55) | 11 (3.27) | 0 (0) | 33 (9.82) |
| *C. koseri* | 22 (6.55) | 9 (2.68) | 0 (0) | 31 (9.23) |
| *E. aerogenes* | 14 (4.17) | 5 (1.49) | 1 (0.30) | 20 (5.95) |
| *E. cloacae* | 5 (1.49) | 2 (0.60) | 0 (0) | 7 (2.08) |
| *K. oxytoca* | 2 (0.60) | 1 (0.30) | 0 (0) | 3 (0.89) |
| *K. pneumoniae* | 30 (8.93) | 13 (3.87) | 0 (0) | 43 (12.8) |
| *P. mirabilis* | 5 (1.49) | 3 (0.89) | 0 (0) | 8 (2.38) |
| *P. vulgaris* | 3 (0.89) | 1 (0.30) | 0 (0) | 4 (1.19) |
| *S. marcescens* | 4 (1.19) | 1 (0.30) | 0 (0) | 5 (1.49) |
| *P. aeruginosa* | 37 (11.01) | 10 (2.98) | 0 (0) | 47 (13.99) |
| **Total** | **175 (52.08)** | **70 (20.83)** | **2 (0.60)** | **247 (73.51)** |

**Table 3: Mono-microbial and poly-microbial bacterial growth**

| Samples | Mono-microbial growth | Poly-microbial growth |
|---|---|---|
| | **No. (%)** | **No. (%)** |
| Wound Swab | 102 (45.33) | 66 (29.33) |
| Tissue Culture | 29 (12.89) | 25 (11.11) |
| Pus aspirate | 3 (1.33) | 0 (0) |
| **Total** | **134 (59.56)** | **91 (40.44)** |

*P. aeruginosa* showed high resistant rate towards doxycycline (91.49%) followed by ciprofloxacin (82.98%) and gentamycin (82.98%). All isolates of the bacteria were susceptible to colistin. Among the 47 *P. aeruginosa* isolates, 82.97% were found to be MDR.

**Table 4: Antibiotic susceptibility profile of *P. aeruginosa* (n= 47)**

| Antibiotics | Sensitive | Resistant |
|---|---|---|
| | **No. (%)** | **No. (%)** |
| Amikacin | 9 (19.15) | 38 (80.85) |
| Gentamycin | 8 (17.02) | 39 (82.98) |
| Ciprofloxacin | 8 (17.02) | 39 (82.98) |
| Cefepime | 12 (25.53) | 35 (74.47) |
| Piperacillin/ Tazobactam | 30 (63.83) | 17 (36.17) |
| Cefoperazone/ Sublactam | 12 (25.53) | 35 (74.47) |
| Meropenem | 21 (44.68) | 26 (55.32) |
| Imipenem | 27 (59.57) | 20 (40.43) |
| Doxycycline | 4 (8.51) | 43 (91.49) |
| Ceftazidime | 11 (23.4) | 36 (76.60) |
| Colistin | 47 (100) | 0 (0) |

Among the 20 imipenem and meropenem resistant *P. aeruginosa* isolates, 4 (20%) isolates were detected to be carbapenemase producers by modified Carbapem Inhibition Method (mCIM) while 3 (15%) isolates were detected to be MBL producers by using Combined Disk (CD) test.

Both MBL and carbapenemase producing *P. aeruginosa* showed complete resistant towards amikacin, gentamycin, ciprofloxacin, cefepime, doxycycline and ceftazidime, However, MBL producing isolates were 66.67% and 100% resistant for cefoperazone/sublactam and piperacillin/tazobactam respectively whereas that for carbapenemase were 50% and 75%. All the MBL positive isolates were MDR.

**Table 5: Antibiogram of carbapenemase, MBL producing and Non-MBL *P. aeruginosa* (n=24)**

| Antibiotics | Carbapenemase producing | MBL producing | Non- MBL |
|---|---|---|---|
| | Resistant (%) | Resistant (%) | Resistant (%) |
| Amikacin | 4 (100) | 3 (100) | 17 (94.12) |
| Gentamycin | 4 (100) | 3 (100) | 17 (100) |
| Ciprofloxacin | 4 (100) | 3 (100) | 17 (100) |
| Cefepime | 4 (100) | 3 (100) | 17 (100) |
| Piperacillin/ Tazobactam | 3 (75) | 3 (100) | 10 (58.82) |
| Cefoperazone/ Sublactam | 2 (50) | 2 (66.67) | 17 (100) |
| Doxycycline | 4 (100) | 3 (100) | 17 (100) |
| Ceftazidime | 4 (100) | 3 (100) | 17 (100) |
| Colistin | 0 (0) | 0 (0) | 0 (0) |

## DISCUSSION

Burnt areas are susceptible site for microbial colonization and proliferation within few hours of injury as the trauma and the wound local microenvironment induces immunosuppressant state (Srinivasan et al. 2009; Gonzalez et al. 2016). These organisms may further cause disseminated infection following colonization and it has been estimated that 75% of all deaths in burnt patients were associated with infections. As the etiology of burn wound changes with time, the expanded use of antibiotics leads to the development as well as selection of multidrug resistant (MDR) bacteria which results in treatment failure and intensifies the complications (Srinivasan et al. 2009; Gupta et al. 2019). The Gram negative pathogen *P. aeruginosa* presents the maximum incidence and even becomes predominate among the burn wound pathogens (Gonzalez et al. 2016). Therefore, this study was carried out to investigate the etiology of burn wound with special focus on *P. aeruginosa* and its antibiotic susceptibility pattern. In addition, carbapenem resistant *P. aeruginosa* strains were selected for testing MBL and Carbapenemase production.

In this study, out of 310 samples processed, 225 (72.58%) samples had significant bacterial growth. This culture positivity rate is lower to the other study done which have shown 87.5% and 86.5% growth rate (Rajbahak et al. 2014; Dahag et al, 2018). The lower rate of bacterial isolation in the present study may be due to the differences in the specimen size involved in those studies. Overall, 59.56% and 40.44% samples showed mono-microbial and poly-microbial bacterial growth respectively. This finding is in harmony with the studies by Dahag et al (2018) and Rajbahek et al (2014) were mono-microbial (46% and 54.4%) outweighed poly-microbial growth rate (40.5% and 45.6%). However, in a study by Ali *et al* (2017), 59.6% of samples were polymicrobial and remaining monomicrobial. The polymicrobial incidence might be because a suitable environment is created by the presence of one microorganism that enables other pathogenic microorganism to colonize the respective niche resulting in the synergistic interaction among pathogens to cause disease (Ali et al, 2017). In addition, several factors of the wound such as formation of excessive devitalised tissue, increased tension in the wound, haematoma and seromas and foreign bodies influence patients to secondary bacterial infections (Bangera et al, 2016).

Gram positive isolates accounted for 26.49% of samples while 73.51% of the isolates were identified as Gram negative, which is similar to Yousefi-Mashouf and Hashemi (2006). The Gram positive organisms which caused burn wound infections in this study were *S. aureus*, CoNS, *Enterococcus* which is similar to other study i.e. Naqvi et al. 2014. The Gram negative isolates in this study were identified to be *A. lwoffi, Acinetobacter calcoaceticus-baumannii* complex, *E. coli, C. koseri, E. aerogenes, E. cloacae, K. oxytoca, K. pneumoniae, P. mirabilis, P. vulgaris, S. marcescens,* and *P. aeruginosa.* Similar etiology is reported by Altoparlak et al (2004). The incidence of Gram negative isolates was found to be much more than Gram positive isolates in burn wound infections. As the microbial profile of burn wound infection change over time, Gram positive bacteria inhabits the burn wound for first 48 hours of injury and later, Gram-negative bacteria also rapidly colonize the burn wound surface. (Barret and Herndon. 2003; Wysocki. 2002).

Among the isolates, *P. aeruginosa* was found to be predominate which accounted for 13.99% of total isolates. This result is in concordance with previous

reports (Agnihotri et al. 2004; Gupta et al. 2019), where the same bacteria is most frequently isolated but is in contrast to other studies which report *S. aureus* as predominant organism (Lesseva and Hadjiiski, 1996; Komolafe et al. 2003). The reason that *P. aeruginosa* is most commonly identified in the burn wards may be due to the fact that organism thrives in a moist environment (Atoyebiet al. 1992). Furthermore, it is a ubiquitous microorganism and could affect individual with immunocompromised situation and responsible for nosocomial infections (Lanotte et al. 2004).

In our study, the resistant of *P. aeruginosa* towards antibiotics was alarmingly high. Resistant to piperacillin and tazobactum in our study was 36.17% which is in harmony to 38.6% in a study by Sheikh et al (2014) but in contrast to 27.8% and 19.45% in the studies by Srinivasan et al (2009) and Saaiq et al (2015) respectively. In the studies by Agnihotri et al. (2004) and Sheikh et al (2014), *P. aeruginosa* showed 53.85% and 55.20% resistant towards amikacin while that for our study was 36.17%. Similarly, 76.60% isolates were resistant for ceftazidime which was higher in comparison to other study which showed 63.72% and 66.80% resistant (Agnihotri et al. 2004; Sheikh et al. 2014). In this study resistance to carbapenem antibiotics- imipenem and meropenem were found to be at 59.57% and 44.68% respectively, while Khosravi et al (2007) reported slightly lower resistance (41%) for both imipenem and meropenem. However, Coetzee et al (2013) observed resistance of imipenem and meropenem at 90.2% and 93.4% respectively. This undoubtedly exhibits that the drugs that were previously supposed to be effective in literature against *P. aeruginosa* are becoming more resistant (Chaudhary et al. 2019)

In the present study, the most susceptible antibiotics against *P. aeruginosa* was colistin (100%). Similar results were seen in earlier studies where colistin is sensitive upto 100% (Shanthi and Sekar 2009; Viedma et al. 2012). Selective pressure from the use of antimicrobial agents is the major determinant for the emergence of resistance (Mesaros et al. 2007). This outcome suggests that colistin should keep as the reserved drug to treat MDR isolates.

Among the imipenem and meropenem resistant *P. aeruginosa,* 15% showed MBL production by CD test and 20% showed carbapenem production by mCIM test. In contrast, Saderi et al (2010) reported that 94% of imipenem resistant isolate were positive by imipenem-CD test while Saderi et al (2008) showed 39.06% of all isolates were MBLs positive by ceftazidime- CD test. In this study, MBL producing *P. aeruginosa* (by CD test) was completely resistant to most of the antibiotics used except cefoperazone/ sulbactam which showed 66.67% resistance while all the isolates were susceptible to colistin. This result is co-relates with the result published by Anvarinejad et al (2014). All the MBL positive isolates were MDR including resistance to antibiotics prescribed as the first line of treatment-cefepime, ciprofloxacin, amikacin, meropenem, imipenem, piperacillin/tazobactam, and gentamicin which co-relates with Mirsalehian et al (2017).

## CONCLUSION

*P. aeruginosa* still remains the predominant bacteria isolated from burn wound infection. The high proportion of MBL and carbapenemase producers warrants the detection of MBL and carbapenemase in routine laboratory coupled with rational use of antibiotics inorder to limit the spread of such enzymes producing organism.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests

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# Phenotypic detection of Extended Spectrum Beta lactamase production from *E. coli* and *K. pneumoniae* in urinary samples among children

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## ABSTRACT

**Objectives**: The main objective of this study was to detect antimicrobial drug resistance (AMR) and Extended Spectrum Betalactamase (ESBL) production phenotypically in *E. coli* and *K. pneumoniae* isolated from urines with significant bacteriuria.

**Methods**: This cross-sectional study was carried out in Microbiology laboratory of Kathmandu College of Science and Technology, Kamalpokhari. The urine samples from suspected urinary tract infected cases were collected from both genders of children below 15 years of age from Out and In-patient department of International Children Friendship Hospital, Maharajgunj and those with significant bacteriuria were cultured for isolating the bacterial etiology targeted as *E. coli* and *K. pneumoniae*. AMR for these two bacteria were tested and detected using Kirby Bauer Disc Diffusion technique. ESBL production was confirmed by Double Disc Synergy test (DDST) and Phenotypic Confirmatory Disc Diffusion Test (PCDDT) after screening for all the isolates showing resistance to third generation cephalosporin namely Cefotaxime and Ceftriaxone according to CLSI instructions.

**Results**: Out of 388 urine samples processed, 29.89% (116/388) showed significant bacterial growth. Five (5) different Aerobic Gram Negative bacterial species were detected and identified. *E. coli* topped the list (70.68%) followed by *K. pneumoniae* (15.52%), *K. oxytoca* (8.62%), *Proteus vulgaris* (3.45%) and *Pseudomonas aeruginosa* (1.73%). Among positively screened (44.82%) beta lactamase producers (36.2%) of total isolates were confirmed to produce ESBL. Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%). AMR was detected using Kirby-Bauer Disc diffusion technique. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. 40 out of the 42 (95.23%) ESBL producing strains showed susceptibility to the combination drug, piperacillin/tazobactam. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%).

**Conclusion**: This study concluded that there is high prevalence of multidrug resistant uropathogenic clinical strains of *E. coli* and *K. pneumoniae* with higher rates of ESBL production. A resistance to the carbepenems is also emerging. Appropriate antimicrobial regimen selection for empirical therapy is thus important for such cases. On managing the empirical antibiotics practice, one can reduce the risk of ESBL producers. There is an essence need of regular routine practice of ESBL detection.

**Keywords:** AMR, ESBL, *E.coli, K.pneumoniae*, PCDDT, DDST

## INTRODUCTION

Antimicrobial resistance among bacterial strains causing Urinary tract infections (UTIs), one of the most common bacterial infections in humans both in the community and the hospital settings, is an emerging problem, worldwide. *E. coli* and *K. pneumonia*e are the two major pathogens commonly isolated in urine, with *E. coli* being the most prevalent type accounting

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for 75-90% of UTIs. Usually these infections are treated with a variety of antibiotics, including β-lactams, β-lactam/β-lactamase inhibitors, flouroquinolones, and carbapenems (Kariuki et al. 2007; Ullah et al. 2009; Hoban et al. 2011 and Briongos-Figuero et al. 2012). However, in recent times, these uropathogens have also developed resistance to commonly prescribed antimicrobial agents; this severely limits the treatment options of an effective therapy.

Primarily, these uropathogens exerts their antimicrobial resistance against beta-lactams by producing extended spectrum beta-lactamases (ESBLs) enzymes that confers bacterial resistance to all beta-lactams except carbapenems, cephamycins and clavulanic acid (Coque et al. 2008). It is global matter of concern since infections associated with ESBL producing clinical isolates are found with higher mortality, length of stay, and health care cost and longer antibiotic therapy in comparison to that with non-ESBL producing pathogens (Schwaber and Carmeli 2007). Correct diagnosis and prompt treatment is crucial in order to prevent morbidity and mortality associated with the disease which is further backed up by frequent changing pattern of antimicrobial resistance with development of various resistant mechanisms like drug efflux, reduced uptake and production of hydrolytic enzymes like extended spectrum β-lactamases (Ghedira et al. 2004). The Infectious Disease Society of America has listed *E.coli* and *Klebsiella* species as two out of six pathogens for which new drugs are urgently needed in order to combat their growing resistance (Talbot et al. 2006) The prevalence of ESBL-positive isolates depends on a range of factors including species, geographic locality, hospital/ward, group of patients and type of infection, and large variations have been reported indifferent studies (Livermore et al. 2007).

The different studies from Nepal have indicated a variable rate of ESBL producing bacterial strains in Nepal, where Enterobacteriaceae were found 28% to 67% (Hammer et al. 2007). The acquisition and expression of ESBLs enzymes among Enterobactericeae have posed a serious public health problem in developing countries like Nepal that still lacks the facilities for urine culture and antimicrobial susceptibility testing; this clearly leads to missing ESBL isolates in our country. This might be one of the reasons that are creating deaths among urinary tract infected cases in Nepal. Thus, the regular surveillance of the drug resistance among the clinical isolates will be helpful to know the actual burden of the situation, which will help making the necessary policy to reduce the incidence of drug resistance among the bacteria primarily causing UTIs which are being difficult to treat nowadays. This study was carried out to detect antibiotic resistance and phenotypic detection of ESBL among multidrug resistance *E. coli* and *K. pneumoniae* to determine the prevalence and antibiotic resistance profile in clinically relevant urine isolates from children.

## MATERIALS AND METHODS

This cross sectional study was carried out at microbiology laboratory of Kathmandu College of Science and Technology, Kamalpokhari, Kathmandu. During the study, 388 urine samples from suspected urinary tract infected cases were collected from both genders of children below 15 years of age from Out and In-patient department of International Children Friendship Hospital, Maharajgunj. For sample collection, In case of neonates and infants, genital area was first cleaned with sterile water and wiped from front to back until area is clean. For female, urine bag was affixed over genital area, starting from the perineum and working upwards. For male, urine bag was placed over the penis ensuring a tight seal all around the bag. Urine bag was checked frequently and removed as soon as the urine is passed. The parents of toilet trained children were suggested to collect midstream clean catch urine and were transported to our laboratory using ice box as soon as possible. However, improperly labeled, unlabeled and leaked sample were excluded from the study.

For processing of each sample, microbiological protocols were followed according to standard microbiological guidelines (Cheesbrough 2006 and Forbes et al. 2007). The collected urine samples were inoculated on MacConkey agar (MA) and Blood agar (BA) using a sterile calibrated loop. The inoculated plates were then incubated aerobically at 37˚C for 24 hours. Colony count was made and plates showing more than or equal to $10^5$ colony forming units (cfu)/ml of urine was considered for positive result (Forbes et al. 2007). The plates showing significant bacteriuria were then cultured for presumptive identification of *E. coli* and *K. pneumoniae* that was carried out on basis of colonial characteristics, gram staining and biochemical tests.

The Kirby-Bauer disc diffusion method, according to the CLSI guidelines, was used to test the isolates for their antimicrobial susceptibilities using β-lactam antibiotics viz. cefpodoxime (30µg), cefotaxime (30µg), ceftrioxone (30µg), ceftazidime (30µg), cefepime (30µg), and non β-lactam antibiotics viz. gentamicin (10µg), ciprofloxacin (5µg), piperacillin/tazobactam (100/10µg), norfloxacin (10µg), amikacin (30µg), nitrofurantoin (100µg), cotrimoxazole (25µg), imipenem (10µg) and meropenem (10µg). All the antibiotic discs were procured from Hi-media, Mumbai (CLSI 2016).

In this study, if the isolates were resistant to at least one agent of three different classes of commonly used antimicrobial agents, they were regarded as multidrug resistant (MDR) (Magiorakos et al. 2012). If the zone of inhibition (ZOI) was ≤25mm for Ceftriaxone, ≤22mm for Ceftazidime, and/or ≤27mm for Cefotaxime, the isolate was considered a potential ESBL producer as recommended by CLSI and further tested by confirmatory methods.

For Double disc synergy test, Mueller Hinton agar was inoculated with the standard (0.5 McFarland) inoculum of the test isolate. Ceftazidime (30 µg) disc was placed on agar 15 mm away from the center of amoxicillin-clavulanic acid (20 µg/10 µg) disc. Extension of zone of inhibition towards amoxicillin-clavulanic acid was interpreted as ESBL producer.

For phenotypic confirmatory test, disks of third generation cephalosporins alone and disks of third generation cephalosporins plus clavulanic acid are required for the phenotypic confirmatory test that uses combination disk method according to CLSI guidelines. Ceftazidime (30 µg) disk alone and ceftazidime + clavulanic acid (30 µg + 10 µg) disk; and cefotaxime (30 µg) disk alone and cefotaxime + clavulanic acid (30 µg + 10 µg) disk were used in this study. The disks were placed at a distance of at least 25mm on a carpet culture of the isolate on MHA plate. Differences in zone diameters of cephalosporins alone and in combination with clavulanic acid were recorded after incubation for 16-18 hours at 37°C. The increase in zone diameter equal to or greater than (≥) 5mm around cephalosporin plus clavulanic acid disk compared to cephalosporin alone indicates ESBL production by the organism.

The data of the case record forms were entered in the worksheet of Microsoft Excel. Frequency and percentages were analyzed as descriptive findings.

## RESULTS

Out of a total of 388 mid-stream urine specimens (202 from male and 186 from female child) screened for significant bacteriuria, a total of 116 (29.89%) were found to have significant growth from which bacterial isolates were obtained. Among total, 82/116 (70.68%) and 18/116 (15.52%) samples showed the growth of *E. coli* and *K. pneumoniae*, respectively. Rests of the bacterial isolates were *Proteus vulgaris* (4/116), *Pseudomonas aeruginosa* (2/116) and *K. oxytoca* (10/116). Initial screening of these isolates for ESBL production showed 42/82 of *E. coli* and 8/18 of *K. pneumoniae* strains to be ceftriaxone resistant. Confirmation test (PCDDT) revealed 34/82 (41.46%) of *E. coli* and 8/18 (44.44%) of *K. pneumoniae* isolates to be ESBL positive as shown in the following table.

**Table 1: Distribution of bacterial isolates in urine sample**

| Bacterial isolates | Number(%) |
|---|---|
| ***E. coli*** | 82(70.68) |
| ***K. pneumoniae*** | 18(15.52) |
| ***K. oxytoca*** | 10(8.62) |
| ***Proteus vulgaris*** | 4(3.45) |
| ***Pseudomonas aeruginosa*** | 2(1.73) |
| **Total** | **116(100)** |

**Table 2: Phenotypic confirmation of ESBL producer from potentially screened isolates**

| Isolates | Total Screened for ESBL | Confirmed ESBL producers (%) |
|---|---|---|
| ***E. coli*** | 42 | 34(41.46) |
| ***K. pneumoniae*** | 8 | 8(44.44) |
| ***K. oxytoca*** | 2 | 0 |
| **Total** | **52(44.82%)** | **42(36.20)** |

Among total ESBL isolates, highest rate of ESBL producers were from age group below 5 years (66.66% ) followed by age group 6-10 (23.80%) and 11-15 (9.52%). ESBL production was more (52.38%) from bacterial isolates of male child than female (47.62%).

**Table 3: Distribution of ESBL producers according to age and gender of children**

| Age-Group (years) | Gender | | Bacterial isolates | Gender | | ESBL producers (%) |
|---|---|---|---|---|---|---|
| | Male | Female | | Male | Female | |
| ≤5 | 46 | 32 | 78 | 18 | 10 | 28(66.66) |
| 6-10 | 16 | 8 | 24 | 4 | 6 | 10(23.80) |
| 11-15 | 2 | 5 | 7 | 0 | 4 | 4(9.52) |
| Total | 68 | 45 | 116 | 22 (52.38) | 20 (47.62) | 42 (100) |

Among total bacterial isolates from Outpatient department, ESBL was produced from 46.87% isolates whereas from inpatient isolates only 23.07% produce ESBL.

**Table 4: Distribution of ESBL producers from patients attending different departments**

| Patient department | Bacterial Isolates | ESBL producers(%) |
|---|---|---|
| **Out-Patient** | 64 | 30(46.87) |
| **In-Patient** | 52 | 12(23.07) |
| **Total** | **116** | **42** |

Among total MDR isolates, 67.74% produce ESBL and among MDR *E. coli* 73.91 % produce ESBL and among MDR *K. pneumoniae*, 80% produce ESBL whereas *K. oxytoca* did not produce ESBL.

**Table 5: Distribution of ESBL producers among MDR isolates**

| Bacterial isolates | No. of MDR isolates (%) | ESBL producer (%) |
|---|---|---|
| ***E. coli*** | 46(74.19) | 34(73.91) |
| ***K. pneumoniae*** | 10(16.13) | 8(80) |
| ***K. oxytoca*** | 6(9.68) | 0(0) |
| **Total** | **62(100)** | **42(67.74)** |

Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%)

**Table 6: In vitro susceptibility of ESBL producers to β-lactam antibiotics**

| Antibiotics | E. coli n=34 | E. coli n=34 | Total N=42 Susceptible No. (%) |
|---|---|---|---|
| Ceftazidime | 8 | 8 | 10(23.80) |
| Ceftriaxone | 1 | 1 | 1(2.38) |
| Cefotaxime | 6 | 6 | 7(16.67) |
| Cefepime | 5 | 5 | 6(14.28) |
| Cefpodoxime | 4 | 4 | 5(11.90) |

Upon testing of Susceptibility pattern for ESBL and non ESBL producers to non β-lactam antibiotics, a co-resistance to the non-β lactam antibiotics was observed more with the ESBL producers. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. 40 out of 42 (95.23%) ESBL producing strains showed susceptibility to piperacillin/tazobactam, a combination drug. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%) among ESBL producers while ESBL non-producers have shown absolute sensitivity towards meropenem, imipenem and piperacillin/tazobactam.

**Table 7: Antibiotic susceptibility of ESBL producers and non-producers towards non β-lactam antibiotics**

| Antibiotics | ESBLproducers (n=42) | | Non-producers (n=74) | |
|---|---|---|---|---|
| | Susceptible No. (%) | Resistant No. (%) | Susceptible No. (%) | Resistant No. (%) |
| Norfloxacin | 2 (4.77) | 40 (95.23) | 45 (60.8) | 29 (39.2) |
| Ciprofloxacin | 5 (11.9) | 37 (88.1) | 48 (64.8) | 26 (35.2) |
| Amikacin | 34 (80.9) | 8 (19.1) | 69 (93.24) | 5 (6.76) |
| Gentamicin | 11 (26.19) | 31 (73.81) | 65 (87.83) | 9 (12.17) |
| Co-trimoxazole | 8 (19.04) | 34 (80.96) | 33 (44.59) | 41 (55.41) |
| Nitrofurantoin | 38 (90.47) | 4 (9.53) | 70 (94.59) | 4 (5.41) |
| Piperacillin/Tazobactam | 40 (95.2) | 2 (4.8) | 74 (100) | 0 (0) |
| Meropenem | 38 (90.47) | 4 (9.53) | 74 (100) | 0 (0) |
| Imipenem | 39 (92.85) | 3 (7.15) | 74 (100) | 0 (0) |

## DISCUSSION

The occurrences of ESBLs among clinical isolates vary greatly worldwide and geographically and are rapidly changing overtime. The prevalence of ESBL producers was 36.20 % (*E.coli*= 41.46% and *K. pneumoniae* = 44.44%) in this study. It correlates with a study done in India (Babypadmini and Appalaraju 2004) which reported nearly 40% of urinary isolates of *E. coli* and *K. pneumoniae* were ESBL positive. Findings from other studies in Nepal have shown ESBL production ranging from 18% to 62.7 %. (Shrestha et al. 2011; Poudyal et al. 2011; Thakur et al. 2013). Variation might have occurred due to low number of samples studied from different geographical locations. Similarly, variation in prevalence of ESBL producing organisms was found in other countries. Significant increase in ESBL organisms were published from India (Sasirekha et al. 2010 and Sharma et al. 2012) Pakistan (Ullah et al. 2009) Nigeria (Yusha'u et al. 2010).

Previous studies from Nepal have reported the prevalence of the ESBL producing bacteria ranging from 13.5% to 33.2%. Chander and Shrestha (2013) reported the ESBL prevalence rate to be 13.5% whereas, recently, Neupane et al. (2016) have reported 33.2% of ESBL producers in their study. Similarly, Ansari et al. (2015), Khanal et al. (2013), have reported 24% and 25% ESBL producers, respectively. These findings show less prevalence with those of our study. However, extremely lower rates of ESBL production have been documented from Japan, Korea, and United States (Paterson and Bonomo 2005; Yan et al. 2014). The differences in the ESBL rates may be attributable to the geographic difference, antimicrobial stewardship programme, and infection control practices.

In this study, ESBL production was more (52.38%) from isolates of male child than female (47.62%). However, Females showed a higher rate of isolation of ESBL producing *E. coli* (60%) and *K. pneumoniae* (62.5%), which discords the findings as reported earlier (Oladeinde et al. 2011 and Ahmed et al. 2012 ). The result is inconsistent because ESBL production and pathogenic nature may not differ according to gender. However, in our country, preference is given to male child and parents take care of them with so much love and take them to hospital in a higher frequency. This may be the reason behind higher inclusion of male child in this study.

The highest bacterial isolates were found in children less than 5 years age, including the prevalence of ESBL organisms which was above 66.66%. The reason for this may be due to the immunological status of the children below 5 years of age who are more vulnerable to infections, malnourished child, and child living in poor sanitation. Similar result was observed in a study done by Kayastha et al. (2020) in Nepal. The higher prevalence of bacterial growth in outpatients, in this study indicates the emergence of ESBL producing pathogens from community that may spread creating difficulties in treating the patients with drug resistance. However, this result is contrary to a study in Nepal (Kayastha et al. 2020) which showed greater prevalence in inpatients which may have been added by nosocomial infections associated with prolonged hospital stay, intensive care unit admission, extensive use of invasive medical devices, and overconsumption of antibiotic among inpatients.

MDR was found in 53.44% (62/116) of the urinary isolates, among them, major MDR producer was *E. coli.* Among total MDR isolates, 67.74% produce ESBL and among MDR *E. coli* 73.91% produce ESBL and among MDR *K. pneumoniae*, 80% produce ESBL. Production of different β-lactamase, hydrolyze β-lactam ring of

antibiotic, like TEM-1, TEM-2, SHV-1 and many other plasmid-mediated β-lactamases confers high level of resistance to drug in *E. coli*. Furthermore, different efflux pumps and target site mutation at *gyrA* and *parC* are responsible for fluoroquinolones resistance (Sharma et al. 2018).

Among ESBL producing isolates, highest susceptibility was seen to Ceftazidime (23.80%) followed by Cefotaxime (16.67%). The ESBL producing isolates were least susceptible to Ceftriaxone (2.38%). The high rates for non-ESBL mediated ceftriaxone resistant isolates may be due to their different mechanisms for resistance such as the production of ampC β lactamase, metallo-betalactamase, etc. (Dalela et al. 2012). This further limits the therapeutic options available to treat these infections. In our study, false susceptibilities to ceftazidime and cefotaxime were observed in 23.8% and 16.67% of the ESBL producers. This could be due to the reason that the optimal substrate profile varies from one ESBL enzyme to another (Wong 2001). Hence, the susceptibility panels with only one extended spectrum cephalosporin cannot predict the resistance to the other extended spectrum cephalosporins (Rice and Jao 1991).

Due to the difficulty in detection of ESBL by the current clinical methods, many of these strains have been falsely reported to be susceptible to the widely used broad-spectrum β-lactams, ESBLs constitute a serious threat to the β-lactam therapy (Mackenzi et al. 2002). The ESBL producers are intrinsically resistant to all the cephalosporins even if they exhibit an in vitro susceptibility. The ESBL production coexists with the resistance to several other antibiotics.

In the study, upon testing the susceptibility pattern for ESBL and non-ESBL producers to non β-lactam antibiotics, a co-resistance to the non β-lactam antibiotics was observed more with the ESBL producers. Comparatively less resistance to amikacin and nitrofurantoin (19.1% and 9.53% respectively) was seen among ESBL producers. In the study, 95.23% ESBL producing strains showed susceptibility to the combination drug, piperacillin /tazobactam. The resistance to meropenem was observed to be less (9.53%) as compared to that to imepenem (7.15%).

A co-resistance to the quinolones and the aminoglycosides is common. We found such an associated resistance of ESBL producers to co-trimoxazole (80.96%), gentamicin (73.81%) and the flouroquinolones (88.10-95.23%). Another study reported 91.17%, 100% and 94.91% resistances respectively to gentamicin, cotrimoxazole and ciprofloxacin in the ESBL producers (Gupta et al. 2007). The high resistance to the non β-lactam antibiotics of the ESBL producing strains poses a threat of treatment failure by these drugs and it also minimizes the therapeutic choice to the carbapenems. Hence, the emerging resistance to the carbepenems is a phenomenon of great concern for combating the infections of the multidrug resistant bacteria (Parveen et al. 2010). Although β- lactam/ β- lactamase inhibitor combinations have been suggested as the treatment option for ESBL producers, these drugs must be given in high doses in lower frequency, so that serum and tissue levels of these combinations are higher with a correspondingly higher clinical success rate. (Adam 2002).

## CONCLUSION

This study concluded that there is high prevalence of multidrug resistant uropathogenic clinical strains of *E. coli* and *K. pneumoniae* with higher rates of ESBL production. These strains show lower rate of sensitivity to β-lactam antibiotics even they are capable of producing ESBL and higher rate of sensitivity to combination therapy. A resistance to the carbepenems is also emerging. Appropriate antimicrobial regimen selection for empirical therapy is thus important for such cases. On managing the empirical antibiotics practice, one can reduce the risk of ESBL producers. There is an essence need of regular routine practice of ESBL detection.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Evaluation of Ground Water Quality of Kathmandu Valley and Antibiotic Susceptibility test against *Klebsiella pneumoniae*

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## ABSTRACT

**Objectives:** The aim of this study was to assess quality status of ground water in Kathmandu valley and describe the antibiotic susceptibility of the isolated *Klebsiella pneumoniae.*

**Methods:** A total of 100 samples were collected from different places of Kathmandu valley with 50 each from two different groundwater sources namely boring and well. This study was conducted from June to September, 2019 at Environment and Climate Study Laboratory, Nepal Academy of Science and Technology (NAST). The physicochemical analysis of the samples was done according to standard methodology. Membrane filtration technique was performed for the enumeration of total coliform and different biochemical tests were performed for isolation and identification of *Klebsiella pneumoniae* followed by Kirby-Bauer disc diffusion method for antibiotic susceptibility test.

**Results:** This study reveals the poor microbiological aspects of ground water sources as 98% of total water samples crossed the standard value for total coliform count. The pH, turbidity, ammonia, nitrate and iron content were found to be higher than Nepal Drinking Water Quality Standard (NDWQS 2005) in 15%, 26%, 34%, 7% and 26% of total water samples respectively. The chloride and arsenic content in all the water samples were within the NDWQS, 2005. The 12 out of 18 isolates of *Klebsiella pneumoniae* from ground water source were highly resistant against first generation Cefazolin however, 15 out of 18 isolates were sensitive to Chloramphenicol. Additionally, four isolates showed zone of inhibition in intermediate range provided by Clinical and Laboratory Standard Institute (CLSI) guideline towards Imipenem and Meropenem.

**Conclusion:** This study concludes that ground water sources were heavily contaminated by coliform bacteria and most of the physicochemical aspects were under standard limit. Although *Klebsiella pneumoniae* isolated from ground water were not multidrug resistant, one isolate was recorded to be resistant to Meropenem. These results alarm for circulation of antibiotic resistance in environmental bacterial isolates. Therefore, the appropriate water purification methods should be applied before consumption and should be examined periodically.

**Keywords:** Total coliform count, Antibiotic resistance, ground water, *Klebsiella pneumoniae*

## INTRODUCTION

Groundwater resources are generally considered a reliable source of water for multi-purpose uses. Kathmandu valley, a largest urban center in the Nepal has experienced rapid growth in population in recent years and nearly half of the valley's water supply is derived from groundwater (Khatiwada et al. 2002). Due to huge gap in demand and supply of water in the valley, majority of households own either bore hole or well to extract ground water. This implies increased stress on quality and quantity of groundwater in the valley.

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Because of heavy dependence on ground water sources for drinking water and domestic uses, chemical and microbial contamination of ground water is a serious problem in Kathmandu valley. The contamination is mainly due to anthropogenic causes of pollution. It is assumed that ground water in Kathmandu is polluted due to sewage line, septic failures, open pit latrines, leaching from landfill sites, and direct disposal of domestic and industrial wastes to the surface water (Shrestha et al. 2012). It is notable that nearly 70% households are not connected to sewer system in Kathmandu discharging in open drains and river systems (Shrestha et al. 2017). Globally, over 2 billion people still rely on unsafe water and 4.2 billion rely on sanitation facilities, where their excreta is leaked untreated into the environment (WHO 2019). It is speculated that degradation of surface and shallow groundwater quality has encouraged people to extract of deep groundwater in search of alternative, safe, and reliable source (Shrestha et al. 2016). It is important to note that many studies of shown substandard quality of Kathmandu's groundwater in terms of high concentration of chemical pollutants (Emerman et al. 2010, Koju et al. 2014, Gwachha et al. 2020, Chapagain et al. 2009) and presence of microbial indicators and water-borne pathogens (Diwakar et al. 2008, Manandhar et al. 2010, Shrestha et al. 2018). However, the chemical and microbial quality of private non-piped groundwater has not been viewed separately. In this context it is noteworthy to understand the quality situation of such water so that the necessary mitigation approaches could be recommended to the users.

The pollution of water resources and unsafe drinking water increases the risk of mortalities due to water borne diseases like diarrhea, dysentery, hepatitis as well as many protozoan and helminths infection (WHO 1997). The presence of coliform in drinking water is considered as a possible threat or indicative of microbiological water quality deterioration (Rompre, et al. 2002). We reported various types of gram negative enteric bacteria including *Escherichia coli, Enterobacter* spp, *Citrobacter* spp, *Klebsiella* spp, in the ground water of Kathmandu valley (Bajracharya et al. 2007, Jayana et al. 2009, Prasai et al. 2007). In this period of antibiotic resistance, *Klebsiella pneumoniae* belonging to Enterobacteriaceae family, one of the most concerning pathogens involved in antibiotic resistance and together with other important multi-drug resistant pathogens, it has been classified as an ESBL organism (Navon-Venezia, et al, 2017). According to WHO, the occurrence of Extended Spectrum β Lactamase-producing *K. pneumoniae* has reached now endemic rates of up to 50% in many parts of the world, and up to 30% resistance rates in the community demonstrating its widespread nature (WHO, 2014). It is now well understood that water environment is reservoir of antibiotic resistant bacteria and their resistant genes (Joshi 2017).

In this study, we examined private non-piped groundwater sources to assess the selected chemical and bacteriological quality indicators; and analyzed the statistical correlation among quality indicators. In view point of increasing environmental antibiotic resistance; we also tested resistivity of *K. pneumoniae* against certain antibiotics including carbapenems. This study provides a picture of quality issues of non-piped groundwater sources in the valley.

## MATERIALS AND METHODS

All chemicals used were of analytical grade. All aqueous solutions were prepared using 18.2 MΩ water (Millipore, Milli-Q). All glassware was soaked in nitric acid solution (10%) for at least 24.0 hours followed by three times rinsed with distilled water and dried at 60 °C for 4.0 hours before use. The water samples were collected from Kathmandu, Bhaktapur and Lalitpur. All the experiments were conducted in Environment and Climate Study Laboratory of Nepal Academy of Science and Technology (NAST). The water samples were analyzed for physicochemical and microbiological quality according to Standard method for the examination of water and wastewater (APHA 2005). The water samples were tested immediately on arrival to the laboratory. In case when the immediate analysis was not possible, the samples were preserved at 4 °C (WHO 2006). The temperature and the pH of the water samples were analyzed by Thermo Scientific Orion Star A111 pH meter. The electrical conductivity was measured by the Mettler Toledo conductivity meter. Turbidity was measured by using HANNA nephelometer. Similarly, for the chemical analysis, the concentration of chloride and total hardness were analyzed by Argentometric titration and EDTA titration, respectively. The iron content was analyzed phenanthroline method by using Agilent Technology Cary UV-Vis spectrophotometer at 510 nm. The standard test kits were used for the determination

of ammonia (Standard Visocolor alpha kit), arsenic (Standard Quantofix kit) and nitrate (Standard Visocolor alpha kit) detection through indication of different color range.

The total coliform count was performed by using standard membrane filter technique in which 100 mL of water sample was filtered through sterile membrane filters having 0.45 µm pore size and 47 mm diameter. The membrane filter retained with microbial biomass was aseptically transferred to M-Endo agar and incubated at 37 °C. The bacterial colonies were enumerated after 24 hours. The pinkish colonies with golden green metallic sheen from M-Endo agar were sub-cultured on MacConkey agar and Nutrient agar. *Klebsiella pneumoniae* was presumptively identified based on results gram staining and different biochemical tests including Indole test, Methyl Red test, Voges-Proskauer test, Citrate utilization test, Urease test, Triple Sugar Iron test, Catalase test and Oxidase test.

The identified *K. pneumoniae* isolates were taken for the antibiotic susceptibility test by disc diffusion method, also known as Kirby Bauer disc diffusion. The inoculum was prepared by suspending the organisms into 2 mL of sterile saline (0.9% w/v NaCl) and the turbidity of this inoculum was adjusted to 0.5 McFarland standards. The inoculum was cultured at 37 °C on Mueller Hinton agar (MHA) media with sterile cotton swab. *K. pneumoniae* isolates were tested against five antibiotics - Cefazoline (30 µg), Cefepime (30 µg), Chloramphenicol (30 µg), Imipenem (10 µg), and Meropenem (10 µg). The zones of inhibition (mm) were measured at 18-24 hours of incubation. The antibiotic susceptibility was interpreted based on CLSI guidelines (CLSI, 2018).

In order to estimate statistical relationship among ground water quality parameters, Pearson's correlation coeeficient was calculated using OriginPRO 2018 software.

## RESULTS

A total of one hundred ground water samples were collected for assessment from June to September, 2019. The collected samples were differentiated as well water 50 and boring water 50. The result values for individual parameters were compared with NDWQS, 2005 as shown in Figure 1.

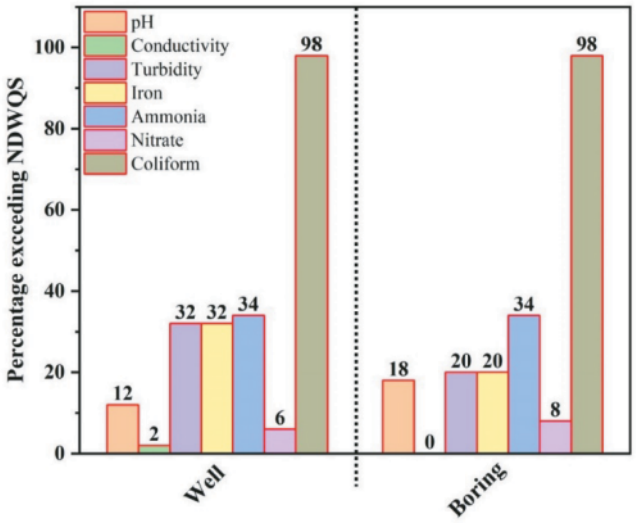


**Figure 1: Percentage of water samples exceeding NDWQS, 2005 for given parameters**

The temperature for both the sources ranged from 27 ˚C to 28.2 ˚C. The pH value varied from 5.7 to 8.8 as shown in Figure 2. The physical parameters such as pH, conductivity and turbidity of 12%, 2% and 32% respectively of well water samples exceeded the value of NDWQS, 2005. The chemical parameters as iron, ammonia and nitrate of 32%, 34% and 6% respectively of well water samples exceeded standard limit. Similarly, pH of 18% and turbidity of 20% boring water samples were crossed the standard limit. The chemical parameters like iron, ammonia and nitrate of 20%, 34% and 8% respectively of boring water samples exceeded NDWQS, 2005. However, hardness, chloride and arsenic were found within limit for both the ground water sources.

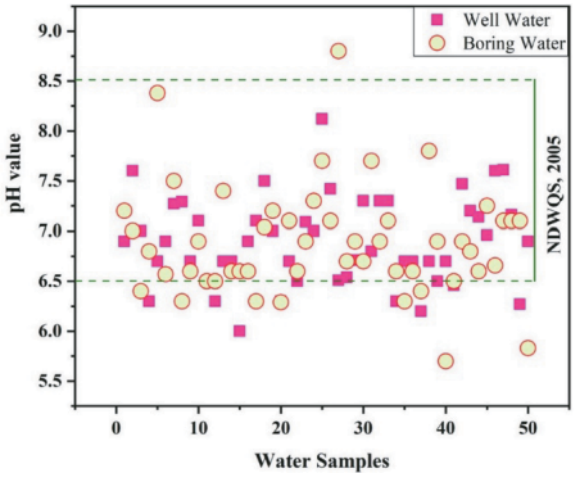


**Figure 2: The pH value of ground water samples**

Pearson's correlation coeeficient was determined to explore relationship among ground water quality parameters as given in Table 1. In this study, conductivity was positively correlated with hardness, chloride, ammonia and nitrate significantly ($p<0.05$). Likewise, turbidity was positively correlated with hardness and iron ($r=0.72$) significantly. Furthermore, hardness was positively correlated with chloride ($r=0.40$), ammonia ($r=0.44$) and ($r=0.33$) nitrate significantly. Interstingly, chloride showed significat negaive correlation with arsenic though coefficient value was low ($r= -0.20$). However, none of physicochemical parameters were significatly correlated with biological (coliform count) parameter.

**Table 1: Pearson's correlation coefficient ($r$) among quality parameters of ground water samples ($n=100$)**

| Parameters | Conductivity | Turbidity | Hardness | Chloride | Iron | Arsenic | Ammonia | Nitrate | Coliform |
|---|---|---|---|---|---|---|---|---|---|
| **pH** | -0.06 | -0.02 | -0.05 | 0.07 | -0.09 | 0.13 | -0.04 | 0.00 | -0.11 |
| **Conductivity** | | 0.09 | **0.61*** | **0.23*** | 0.10 | 0.08 | **0.41*** | **0.26*** | 0.10 |
| **Turbidity** | | | **0.21*** | 0.08 | **0.72*** | 0.12 | 0.17 | -0.07 | 0.10 |
| **Hardness** | | | | **0.40*** | 0.10 | 0.06 | **0.44*** | **0.33*** | 0.12 |
| **Chloride** | | | | | 0.06 | **-0.20*** | 0.11 | **0.27*** | -0.04 |
| **Iron** | | | | | | 0.11 | 0.13 | 0.02 | 0.07 |
| **Arsenic** | | | | | | | **0.21*** | 0.10 | 0.09 |
| **Ammonia** | | | | | | | | 0.02 | -0.01 |
| **Nitrate** | | | | | | | | | 0.14 |

The values indicate Pearson's correlation coefficient ( $r$).Bold face *indcates statistcally significant ($p<0.05$) correlation.

As for the microbiological test, the total coliform count in 98% of both the sources exceeded the NDWQS limits. We selectively targeted *K. pneumoniae* isolates for further study. *K. pneumoniae* was recovered from 18 ground water samples. Antibiogram of *K. pneumoniae* revealed higher degree of susceptibility towards the tested antibiotics. The frequency of the isolates susceptible to most of tested antibiotics ranged in between 72.2 to 83.3% with susceptibility to Cefepime (83.3%), Imipenem (77.8%), Meropenem (72.2%), and Chloramphenicol (77.8%) as demonstrated in Figure 3(a). However, in contrary Cefazoline was highly resisted by the *K. pneumoniae* isolates (83.3%). As shown in Figure 3(b), *K. pneumoniae* isolated from well water were more resistant to Carbapenem group including Imipenem (22.2%) and Meropenem (16.7%). Contrary to this isolates from boring water were resistant towards antibioltics - Cefepime (11.1%), Cefazoline (44.4%). Interestingly, none of the boring water isolates of *K. pneumoniae* were resistant towards Imipenem.

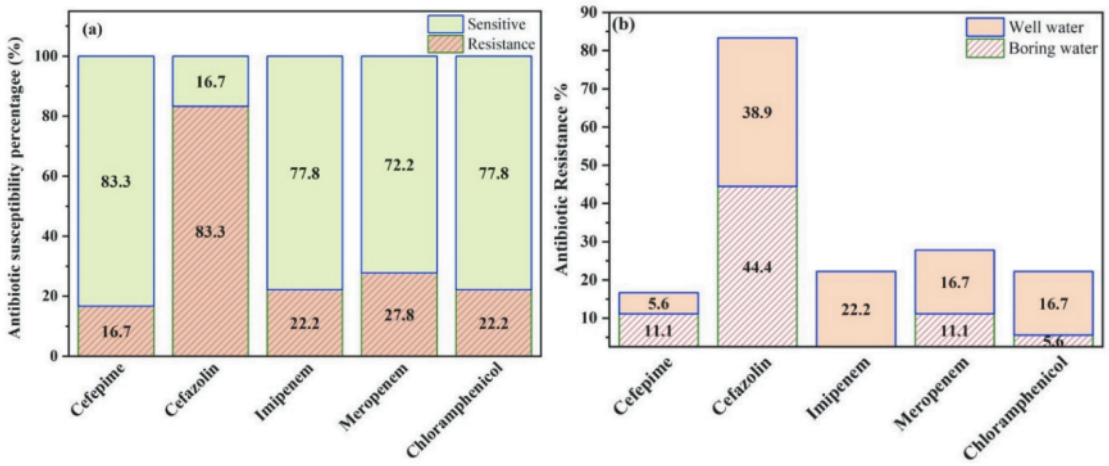


**Figure 3: (a) Susceptibility pattern against *K. pneumoniae isolates towards selected antibiotics (b) Antibiotic resistance pattern in well and boring water***

## DISCUSSION

This study was focused to assess the quality of private non-piped ground water sources in Kathmandu valley. The quality was compared with drinking water quality standards of Nepal (NDWQS 2005) to elucidate the results for physiochemical and microbiological parameters in ground water samples. The water samples were analyzed for physical (pH, turbidity, conductivity, and temperature), chemical (hardness, chlorine, iron, arsenic, nitrate, ammonia) and microbiological quality parameters (Total Coliform Count and identification of bacteria).

We found moderate temperature ranged (27 - 28.2 °C) for all the ground water samples, though water temperature may vary with seasonal variation. High water temperature enhances the growth of microorganisms and may increase taste, color and corrosion problem (WHO 2004). This correlates with the fact that such water is likely to support the growth of bacteria, algae and other life forms. However, several other factors are also crucial for microbial life in water. The pH is an important water quality parameter. Even though pH is not directly related to health risk, it is very crucial in chlorination process. When the pH exceeds 8, disinfection is less effective, while low pH is acidic and cause corrosion of metal pipes (WHO 2017). Conductivity of well water (2%) is comparatively higher than boring water, which indicates that there may be higher presence of dissolved solids in well water. The specific conductance measures the presence of dissolved solids such as chloride, nitrate, sulfate, phosphate, sodium, magnesium, calcium, and iron which indicates water pollution (Murphy 2007). Nearly 20% of well water samples and 32% of boring water samples crossed the permissible limit for turbidity and iron content; this may be caused by the presence of suspended and colloidal matter. This proportional increase in concentration of turbidity and iron content indicates the correlation between them. In a similar study, water with high turbidity had offensive color, taste, odor and also correlates with iron content of water sample and inhibits chlorination (Dietrich and Burlingame 2015). Turbidity is also considered as indirect indicator for the presence of microbes (WHO 2006). Hardness and chloride of most of all water samples were found within the limit proposed by NDWQS, 2005, however, only one (2%) water sample from well contained hardness above the standard. In a previous study done for the treated water in Kathmandu valley, similar results for hardness and chloride were observed (Maharjan, et al. 2019). Arsenic concentration in ground water samples was within limit of NDWQS, 2005, except one well-water sample and four boring water samples which contained higher concentration of arsenic (0.025 mg/L). It is not surprising as previous studies have reported even higher concentration of arsenic (max. 2.8 mg/L) in certain deep ground water sources from Kathmandu valley (Emerman et al. 2010, Gwachha et al. 2020).

Most of the tested water samples contained nitrate within the standard value, however, its concentration in 6% of well and 8% of boring water samples exceeded the standard value. Nitrate itself is not toxic but the effects are hazardous as it is converted to nitrite by

microbial action which may cause Blue baby Syndrome in infants (WHO 1997). The result showed that 34% of well water and 34% of boring water samples exceeded the standard limit for ammonia concentration. This implies higher concentration of ammonia in the ground water of the valley. Therefore, the household treatment options for ammonia removal are highly recommended. For instance, simple aeration of groundwater can also significantly reduce the level of ammonia and iron (Pacini et al. 2005, Zhang et al. 2019).

Pearson's correlation analysis revealed that hardness of the groundwater had significant ($p<0.05$) positive correlation with conductivity, turbidity, chloride, ammonia and nitrate. Similarly, conductivity itself was positively correlated with chloride, ammonia and nitrate along with hardness. This is important for water quality testing laboratories that higher conductivity in groundwater may predict higher values for aforementioned chemical parameters. It may help to reduce the number of parameters for testing. However, none of the tested chemical parameters were significantly correlated with coliform count. This indicates widespread contamination of coliforms in the environment and their unpredictability with chemical factors.

As a second focus of this study, the bacteriological water quality was assessed by enumerating total coliforms bacteria, and detecting *K. pneumoniae* in ground water samples. The results revealed that tested ground water samples were loaded with considerable numbers of total coliforms, most of them having too numerous to count (>300 CFU/mL). Considering detection of coliforms in ground water since long time (Koju et al. 2014, Bajracharya et al. 2007) and increased contamination level in this study, it can be anticipated that fecal bacteria might be well adapted in deep aquifers. The bacterial pollution of ground water might be mostly due to sewage infiltration and seepage from the polluted river flowing, unhygienic practices such as unsanitary septic tank constructed near the water sources (WHO 1997). Previous studies evaluating different water sources revealed that the samples were highly contaminated by total coliform bacteria (Maskey et al. 2020, Maharjan et al. 2018, Bishankha et al. 2012, Acharya et al. 2019, Shakya et al. 2012, Ghaju Shrestha et al. 2017). Therefore, fecal coliforms or other bacteriophage indicators should be adopted for water quality testing. This is one of limitation of this study. Coliform count may not represent fecal contamination accurately.

We could recover *K. pneumoniae* from 18 ground water samples. *K. pneumoniae* is important pathogen in clinical settings in particular causing lower respiratory infections. Antibiotic resistant *K. pneumoniae* have garnered increasing concern. In this study, we found *Klebsiella pneumoniae* was highly sensitive to Chloramphenicol as 15 isolates were sensitive and it was highly resistance to Cefazolin, which is a first-generation Cephalosporin as 12 isolates showed resistivity. *K. pneumoniae* didn't show the resistivity towards Imipenem. However, four isolates showed intermediate range of zone of inhibition to Imipenem. Furthermore, four isolates were in intermediate range and one isolate was resistance to Meropenem. Imipenem and Meropenem belonging to Carbapenem family are the most effective drug. The study held to assess the ESBL producing *Enterobacteriaceae* isolates in packaged water bags sold as drinking water in Kinshasa, the capital of Democratic Republic of Congo, reports that 150 *Enterobacteriaceae* isolates were recovered out of which 56% isolates were *K. pneumoniae*, 30.6% were *Enterobacter spp*, 4.7% *Citrobacter spp* and 3.3% *E. coli*. Eight isolates (5.3%) were confirmed as ESBL producers (Boeck, et al. 2012). Hence, proper treatment option and regular monitoring of the drinking water should be implemented as the poor water quality has direct effects on public health.

## CONCLUSION

The present work evaluated the quality of ground water for domestic purposes. This study concludes that most of the physicochemical aspects were under standard limit, however none of the water samples had an unquestionable quality. There was heavy contamination by coliform bacteria in ground water, indicating higher level of fecal pollution. This implies the maximum possibility of residing water borne enteric pathogens in the ground water of Kathmandu. These results may predict the possible epidemic outbreak of water borne diseases in Kathmandu valley. Although, *Klebsiella pneumoniae* isolated from ground water were not multi drug resistant, however, majority of isolates were resistant to Cefazolin. During the study, one isolate was recorded to be resistant to carbapenem (meropenem). This results alarm for circulation antibiotic resistance in environmental bacterial isolates. Further studies are recommended

to investigate environmental (water) circulation of antibiotic resistance in Kathmandu. For the human consumption, it is very important to apply proper treatment options before using the ground water for drinking purpose. The awareness among people and proper sanitation practice can help to reduce the risk of epidemic outbreak.

## ACKNOWLEDGEMENTS

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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# Characterization of Intestinal Parasitosis in pregant women at Ram Janaki Hospital, Janakpurdham

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## ABSTRACT

**Objectives:** The objective of this study was designed to focus the prevalence, detection and identification of intestinal parasites and its associated factors among pregnant women.

**Methods:** Total 264 stool samples were collected in a labeled dry, clean disinfectant free wide mouthed plastic container during antenatal visits at Ram Janaki Hospital, Janakpurdham and were examined by macroscopically and microscopically. The detection and identification of protozoal cysts, oocysts, trophozoites and helminthic eggs or larva was done by wet preparation and formal-ether sedimentation concentration technique. The data was analysed using SPSS 20 version and Microsoft Excel 2007. A Chi-square test was performed to predict the parasite detection using predictor variables. The p-values <0.05 was considered as significant.

**Results:** The prevalence of intestinal parasitosis among pregnant women was 42%. There was positive association of symptoms of intestinal parasitosis among pregnant women ($p < 0.05$). The most predominant intestinal parasites among study participants were *E. histolytica* (20%) slightly dropped by *G. lamblia* (16%) followed by Hook worm (13%) and *A. lumbricoides* (11%). The correlation between all the variables with intestinal parasites presence and absence was statistically significant ($p<0.05$) but statistically insignificant for age and consumption of green leafy vegetables ($p>0.05$).

**Conclusion:** The overall prevalence of intestinal parasitosis was relatively moderate. Lack of awareness, low hygienic and sanitation habits regarding parasitic infections were the major determinant factors for higher prevalence. Improving sanitation, awareness creation and public health programes should be organized at regular interval in community.

**Keywords:** Helminths, Hygiene, Infestation, Intestinal parasites, Pregnancy, Sanitation

## INTRODUCTION

Pregnant women are one of the most vulnerable groups and often experience more severe infections due to their immune suppression during their pregnancy (Yakasai and Umar, 2013). Intestinal parasitic infections caused by protozoa and soil helminths which are transmitted faeco-orally through contaminated sources (Yadav and Prakash 2016; Rai et al. 2002). Most common intestinal parasites reported from Nepal are *Ascaris lumbricoides*, *Hymenolepis nana, Hookworm, Trichuris trichiura, Giardia lamblia* and *Entamoeba histolytica*. Of the protozoal infections, amoebiasis and giardiasis are most frequently reported. *Ascaris lumbricoides, Trichuris trichiura* and Hookworms, collectively referred to as soil-transmitted helminths (STHs) which are the most common intestinal parasites (Yadav and Prakash 2016; Mehraj et al. 2006).

Globally, approximately, 4.5 billion people are at risk, more than 1 billion people become infected, and 450 million are ill from STHs (WHO, 2014). High prevalence of STHs is mainly related to poverty, poor living conditions, personal and environmental

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hygiene, sanitation, and water supply facilities (Ohaeri and Orji, 2013) low literacy rate, the habit of eating raw vegetables, walking barefoot, malnutrition and hot and humid tropical climate are some of the factors associated with the STH infections (Derso et al. 2016 ). Intestinal parasitic infections disturb pregnancy, directly or indirectly lead to a spectrum of adverse maternal and fetal/placental effects (Dotters-Katz et al. 2011). Infected pregnant women develop malnutrition; maternal anemia; total energy, protein, folate, and zinc loss (Stephenson et al. 2020); low pregnancy weight gain (Khor, 2003) and increased vulnerability to other infections (Steketee, 2003). STH infections also show adverse outcomes on the offspring such as low birth weight, intrauterine fetal growth restriction, and perinatal mortality (Steketee, 2003).

STHs is a significant community health problem, especially in developing countries of both Asia and Africa (De Silva et al. 2013). Soil transmitted helminthes infections are endemic in the communities where poor environmental sanitation and poor personal hygiene play an important role in transmission of STH infections. *A. lumbricoides* and Hookworms cause morbidity in humans in different ways by affecting nutritional equilibrium, inducing intestinal bleeding, inducing malabsorption of micronutrients, reducing growth, reducing food intake, causing complications such as obstruction rectal prolapsed, abscess and affecting congenital development (Mehraj et al. 2006).

Intestinal parasites (especially helminths) can be tissue dwelling or intestinal but all induce a dramatic expansion of the Th2 lymphocyte subset (Finkelman and Urban, 2001). It remains unclear whether these Th2-derived responses, including IgE, eosinophilia, and mastocytosis are important in the protective immune response to the parasite, or are responsible for immune-mediated pathology, or both but at least is a paraclinical marker of infection (Finkelman and Urban, 2001).

To the best of our knowledge, institution-based information revealed that infection with protozoa and geohelminths is the primary disease among pregnant women (Hailu et al., 2020) but the prevalence and factors associated with parasitic infections are still unknown in Nepal. Therefore, this study was carried out to find out the prevalence of intestinal parasites among pregnant women attending for antenatal check ups at a tertiary care hospital and its association with various socio-demographic factors which will provide an opportunity to recommend the prevention, control and treatment of the intestinal parasites.

## MATERIAL AND METHODS

### Study design and area

This cross-sectional study was conducted among the pregnant women attending for antenatal care checkup at Department of Obstetrics and Gynecology at Ram Janaki Hospital, Janakpurdham. All the laboratory procedures were carried out at Microbiology Department of Clinical Pathology and Laboratory Medicine at Ram Janaki Hospital, Janakpurdham located in Dhanusha district at Province No. 2 of Nepal from June 2018 to September 2019. The random sampling technique was applied on 264 pregnant women.

### Data collection

During the process of specimen collection from study participants, a structured questionnaire accompanying the queries about their sociodemographic variables (age, residence, occupation and religion), clinical history, hygienic practice and nutritional behavior were collected by face-to-face interviews. The data was collected by trained midwifery health professionals.

### Sample collection

The stool samples were collected from the pregnant women. The containers were labeled with name, code number, date and time of collection. A labeled dry, clean disinfectant free wide mouthed plastic container was distributed to all study participants during antenatal visits to bring about 10 gms stool sample. They were advised not to contaminate the stool with water and urine. The collected stool samples were immediately preserved with 10% formalin solution.

### Inclusion and Exclusion Criteria

Asymptomatic pregnant woman were included after informed consent. Women refusing to give consent and those who received prior treatment with anti-parasitic drugs before two weeks, severely ill or with previous diagnosis of infectious diseases as HIV/AIDS, HBV infection, syphilis, or toxoplasmosis were not enrolled in the study. Also, those participants who came with stool samples contaminated with water and urine were excluded from study.

### Laboratory investigation

The collected stool samples were examined by macroscopic and microscopic examination.

**Macroscopic examination**

The stool samples were observed for color, consistency, presence of blood and mucus, presence of adult worms and segments and other abnormalities.

**Microscopic examination**

The detection and identification of protozoal cysts, oocysts, trophozoites and helminthic eggs or larva by wet (normal saline and iodine) preparation and formal-ether sedimentation concentration technique (FECT) employed for all the stool specimens.

**Formal ether concentration techniques (FECT)**

About 0.5 gm stool sample was transferred into 10 ml of normal saline in a glass container and mix thoroughly. Two layers of gauze were placed in a funnel and strained the contents into a 15 ml centrifuge tube. Then, 2.5 ml of 10% formaldehyde and 1 ml of ether were added to a test tube. The test tubes were mixed well and centrifuged at 1,000 rpm for 3 minutes. The supernatant was removed and sediment was further proceeding for wet mount preparation.

**Normal saline and Iodine mount techniques**

The sediment was mixed well, prepared on two slides one with 2ml of normal saline and the other with 2ml of iodine solution, and covered with cover slide and detected under a microscope at 10X and 40X.

**Ethical consideration**

Informed verbal consent was obtained from the participants prior to the study before preceding the questionnaire and specimen collection. Work approval letter was taken from Ram Janaki Technical Institute and Ram Janaki Hospital, Janakpurdham, Nepal.

**Statistical analysis**

A descriptive analysis was done for the positivity among different age groups. The obtained data was analysed using SPSS 20 version and Microsoft Excel 2007. A Chi-square test was performed to predict the parasite detection using predictor variables for hand wash before eating, hand wash after defecation, green leafy vegetable consumption, use of latrine and footwear. The association of parasitic infection with sanitary practices and socio-demographic factors were also assessed by using the Chi-square test. The p-values < 0.05 was considered significant.

## RESULTS

**Macroscopic examination of stool sample**

Table 1 shows 35.98% stool samples had normal color, 64.01% had abnormal color, 27.65% had normal consistency and 72.34% had abnormal consistency in pregnant women. Blood, mucus and worm were detected in 31.06%, 24.62% and 14.77% stool samples respectively whereas they were not detected in 68.93%, 75.37% and 85.22% stool samples of pregnant women respectively.

**Table 1: Macroscopic examination of stool sample**

| Properties | Macroscopic examination | | | | Total |
|---|---|---|---|---|---|
| | Normal (%) | Abnormal (%) | Presence (%) | Absence (%) | |
| Color | 95 (35.98) | 169 (64.01) | - | - | |
| Consistency | 73 (27.65) | 191 (72.34) | - | - | |
| Blood | - | - | 82 (31.06) | 182 (68.93) | 264 |
| Mucus | - | - | 65 (24.62) | 199 (75.37) | |
| Worm | - | - | 39 (14.77) | 225 (85.22) | |

**Prevalence of intestinal parasitosis among pregnant women**

The prevalence of intestinal parasitosis among pregnant women was 42% as shown in figure 1.

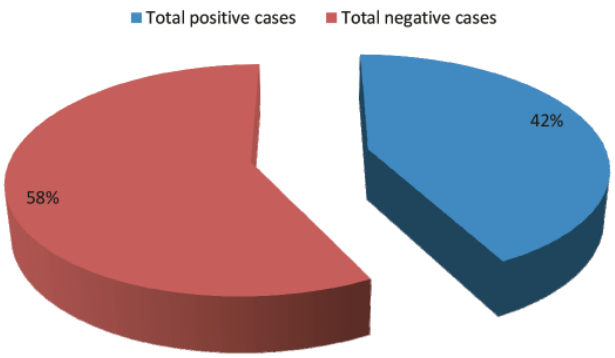


**Figure 1. Prevalence of intestinal parasitosis among pregnant women**

**Sociodemographic characteristics of pregnant women and its association with intestinal parasites detected and not detected**

Table 2 reflects the relationship of age among pregnant women with intestinal parasites detected and not detected which was statistically insignificant (p >0.05). Similarly, the relationship of religion, residence, occupation and education among pregnant women with intestinal parasites detected and not detected was statistically significant (p <0.05).

**Table 2: Sociodemiographic characteristics of pregnant women and its association with intestinal parasites detected and not detected**

| Age (years) | Intestinal parasites detected (n=112) (%) | Intestinal parasites not detected (n=152) (%) | Total (N= 264) | Statistics |
|---|---|---|---|---|
| 15-19 | 23 (20.53) | 18 (11.84) | 41 (15.53) | |
| 20-24 | 32 (28.57) | 36 (23.68) | 68 (25.75) | |
| 25-29 | 19 (16.95) | 33 (21.71) | 52 (19.69) | **$x2$ = 5.88 p = 0.20** |
| 30-34 | 15 (13.39) | 28 (18.42) | 43 (16.28) | |
| >35 | 23 (20.53) | 37 (24.34) | 60 (22.72) | |
| **Religion** | | | | |
| Hindu | 54 (48.21) | 143 (94.07) | 197 (74.62) | **$x2$ = 71.62 p= 0.00001** |
| Muslim | 58 (51.78) | 9 (5.92) | 67 (25.37) | |
| **Residence** | | | | |
| Rural | 97 (86.60) | 86 (56.57) | 183 (69.31) | **$x2$ = 27.33 p= 0.00001** |
| Urban | 15 (13.39) | 66 (43.42) | 81 (30.68) | |
| **Occupation** | | | | |
| Employed | 13 (11.60) | 59 (38.81) | 72 (27.27) | **$x2$=24.06 p= 0.00001** |
| Unemployed | 99 (88.39) | 93 (61.18) | 192 (72.72) | |
| **Education** | | | | |
| Literate | 52 (46.42) | 45 (29.60) | 97 (36.60) | **$x2$ = 7.85 p =0.005** |
| Illiterate | 60 (53.57) | 107 (70.39) | 167 (63.25) | |

**Symptomwise distribution of positive cases and its association**

The association of positive cases with the symptoms of intestinal parasites among pregnant women was statistically significant (p < 0.05) as shown in table 3.

**Table 3: Symptomwise distribution of positive cases and its association**

| Parameter | Total no. (%) | Positive cases (%) | Statistics |
|---|---|---|---|
| **Symptomatic** | 189 (71.59) | 91 (81.25) | |
| **Asymptomatic** | 75 (28.40) | 21 (18.75) | **$x$ 2 = 3.85 p = 0.04** |
| **Total** | **264** | **112** | |

**Trimester wise distribution of study population and its association with parasites detected**

Table 4 shows the association of trimesters among pregnant women with positive cases was statistically difference (p > 0.05).

**Table 4: Trimester wise distribution of study population and its association with parasites detected**

| Trimesters | Total no. (%) | Positive cases (%) | Statistics |
|---|---|---|---|
| 1st | 81 (30) | 22 (19.64) | |
| 2nd | 86 (32.57) | 39 (76.78) | **$x2$ = 5.16 p = 0.75** |
| 3rd | 97 (36.74) | 51 (45.53) | |
| **Total** | **264** | **112** | |

**Obstetrics history of pregnant women and its association with positive cases**

The association of gravida and parity of pregnant women with positive cases was found to be statistically insignificant (p >0.05) as shown in table 5.

**Table 5: Obstetrics history of pregnant women and its association with positive cases**

| Gravida | Total No. (%) | Positive cases (%) | Statistics |
|---|---|---|---|
| 1 | 66 (25) | 26 (23.21) | |
| 2 | 59 (22.26) | 21 (18.75) | |
| 3 | 61 (23.10) | 28 (25) | ***x2* = 1.01 p = 0.79** |
| 4 | 78 (29.54) | 37 (33.03) | |
| **Total** | **264** | **112** | |
| **Parity** | | | |
| 0 | 19 (7.19) | 5 (4.46) | |
| 1 | 54 (20.54) | 12 (10.71) | |
| 2 | 55 (20.83) | 35 (31.25) | ***x2* = 9.05 p = 0.05** |
| 3 | 71 (26.89) | 29 (25.89) | |
| 4 | 65 (24.62) | 31 (27.67) | |
| **Total** | **264** | **112** | |

**Types of parasites detected from stool sample**

Figure 2 depicts altogether 8 different types of parasites were detected from stool samples of study population. Among the parasite positive samples, 13% showed presence of Hook worm, followed by 11% A. lumbricoides, 2% E. vermicularis, 20% E. histolytica, 16% G. lamblia, 6% taenia spp., 7% H. nana and 4% cyclospora spp. Likewise, 3% samples contained both Hookworm and Taenia spp., 4% had Hookworm and A. lumbricoides, 2% had A. lumbricoides and Taenia spp., 4% had Hook worm and E. histolytica, 3% had Hook worm and G. lamblia), 4% had G.lamblia + E. histolytica and 1% had T. trichura and Hook worm.

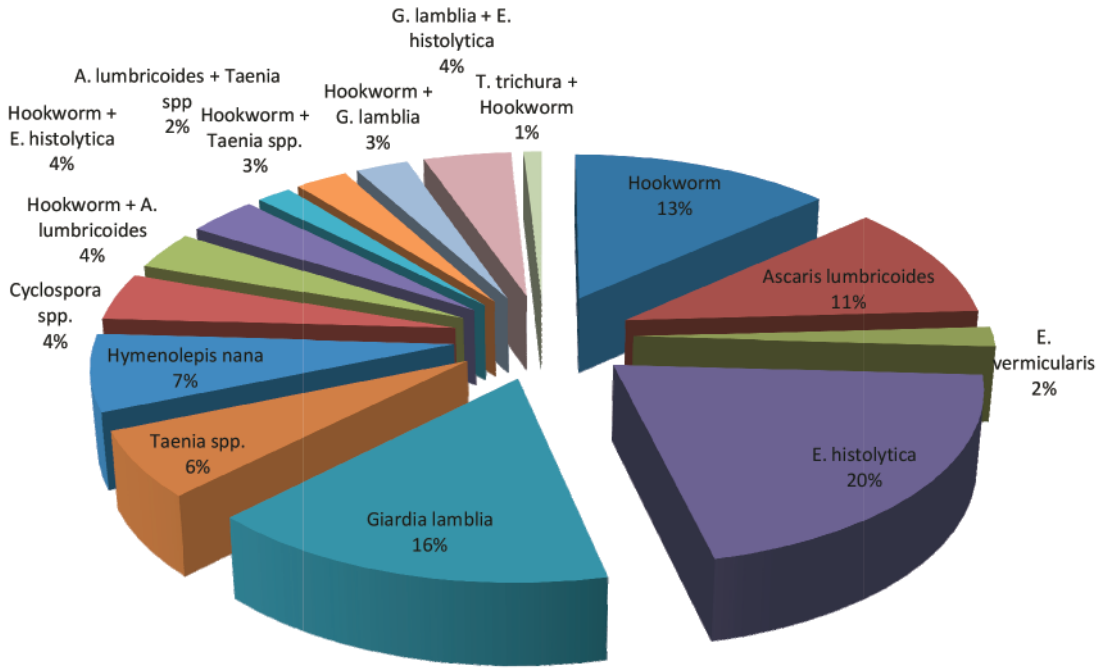


**Figure 2: Types of Parasites detected from Stool sample**

**Pattern of infection**

Figure 3 shows two different types of intestinal parasitic infection among study population. 80% single types of parasites were detected and 20% multiple types of parasites were detected which can cause single and multiple infection.

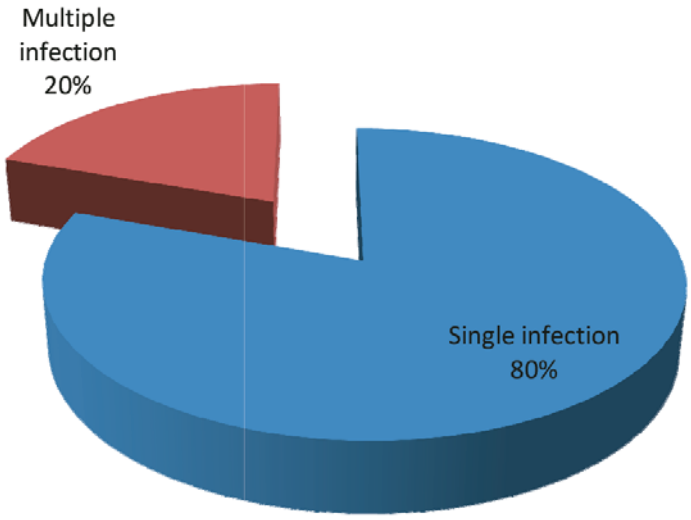


**Figure 3: Pattern of infection**

**Association of trimesterwise study population with single and multiple parasitic infections**

The association of trimesters of pregnant women with single and multiple infections was found to be statistically insignificant ($p > 0.05$) as shown in table 6.

**Table 6: Association of trimesterwise study population with single and multiple parasitic infections**

| Trimesters | Single infection (%) | Multiple infections (%) | Total no. (%) | p-value |
|---|---|---|---|---|
| 1st | 15 (16.66) | 7 (31.81) | 22 (19.64) | p = 0.11 |
| 2nd | 30 (33.33) | 9 (40.90) | 39 (34.82) | |
| 3rd | 45 (50) | 6 (27.27) | 51 (45.53) | |
| **Total** | **90** | **22** | 112 | |

**Association of agewise study population with single and multiple parasitic infections**

Table 7 reveals the association of age of pregnant women with single and multiple infections which was found to be statistically insignificant ($p > 0.05$).

**Table 7: Association of agewise study population with single and multiple parasitic infections**

| Age (years) | Single infection (%) | Multiple infections (%) | Total no. (%) | p- value |
|---|---|---|---|---|
| 15-19 | 18 (20) | 5 (22.72) | 23 (20.53) | |
| 20-24 | 29 (32.22) | 3 (13.63) | 32 (28.57) | |
| 25-29 | 15 (16.66) | 4 (18.18) | 19 (16.96) | p = 0.51 |
| 30-34 | 13 (14.44) | 2 (9.09) | 15 (13.39) | |
| >35 | 15 (16.66) | 8 (36.36) | 23 (20.53) | |
| **Total** | **90** | **22** | **112** | |

**Correlation of variables with intestinal parasites in pregnant women**

The correlation between all the variables with intestinal parasites detected and not detected was found to be statistically significant ($p < 0.05$) except consumption of green leafy vegetables was found statistically difference ($p > 0.05$) as shown in table 8.

**Table 8: Correlation of variables with intestinal parasites in pregnant women**

| Variables | Intestinal parasites detected (n=112) (%) | Intestinal parasites not detected (n=152) (%) | Total (N= 264) | p-value |
|---|---|---|---|---|
| Hand washing before eating with soap and water | | | | |
| Never | 79 (70.53) | 53 (34.86) | 132 (50) | |
| Sometimes | 23 (20.53) | 47 (30.92) | 70 (26.51) | **p < 0.001** |
| Most of the times | 10 (8.92) | 52 (34.21) | 62 (23.48) | |
| **Hand washing after defecation** | | | | |
| Never | 51 (45.53) | 12 (7.89) | 63 (23.86) | |
| Sometimes | 43 (38.39) | 136 (89.47) | 179 (67.80) | **p < 0.001** |
| Most of the times | 18 (16.07) | 4 (2.63) | 22 (8.33) | |
| **Consumption of green leafy vegetables** | | | | |
| Never | 6 (5.35) | 13 (8.55) | 19 (7.19) | |
| Sometimes | 20 (17.85) | 39 (25.65) | 59 (22.34) | **p =0.15** |
| Most of the times | 86 (76.78) | 100 (65.78) | 186 (70.45) | |
| **Use of dirty latrines** | | | | |
| Never | 25 (22.31) | 12 (7.89) | 37 (14.01) | |
| Sometimes | 39 (34.82) | 33 (21.71) | 72 (27.27) | **p < 0.001** |
| Most of the times | 48 (42.85) | 107 (70.39) | 155 (58.71) | |
| **Use of footwear outside home** | | | | |
| Never | 47 (41.96) | 9 (5.92) | 56 (21.21) | |
| Sometimes | 33 (29.46) | 4 (2.63) | 37 (14.01) | **p < 0.001** |
| Most of the times | 32 (28.51) | 139 (91.44) | 171 (64.72) | |

## DISCUSSION

Intestinal parasitosis is one of the most prevalent infectious diseases in women of reproductive age and children in developing countries including Nepal (Nipurte et al. 2020). According to WHO, IPI is considered as a public health problem if its prevalence is greater than 20% (WHO, 2017). The prevalence of intestinal parasitosis among pregnant women in the present study was 42%. Previously, Yesuf et al. in 2019, Hailu et al. in 2020, Nipurte et al. in 2020 have reported 43.8%, 37.3% and 42.67% prevalence. These all findings are almost analogous to the present study. This could be due to the presence of intestinal parasites which is indicative of fecal pollution of soil and domestic water supply due to poor sanitation and improper sewage disposal. Also, it can be attributed to unhygienic practices and lack of awareness of transmission of these intestinal parasites (Nipurte et al. 2020).

In contrast, previous findings reported from Bogota, Colombia (1.2%) (Espinosa et al. 2018), Nepal (35%) (Sapkota and Maharjan, 2018), kwale district of Kenya (25.23%) (Hopkins et al. 2013), Bahirdar, North West Ethiopia (31.5%) (Derso et al. 2016), Gandhi memorial hospital (25.2%) (Gebre, 2012) and Debre Markos, North West Ethiopia (27.4%) (Kumera et al. 2018) have revealed lower prevalence than present study. These variations could be attributable to smaller sample size, the differences in - socio-demographic status, geographical area and cultural practices, implementation of various intervention strategies, study settings, time of the study, and the methods employed for stool examination. The another possible reason might be due to inappropriate handwashing practice, poor shoe wearing habit and difference in existing sanitation facilities and practices.

The result of this study depicts maximum number of parasites detected in age between 20-24 years (28.57%) followed by age group greater than 35 years (20.53%) and 25-29 years (16.95%). Nipurte et al. in 2020 observed the parasites were seen predominantly in the age group 34-40 years (50 %) followed by 26-33 years (44.7%) and 18-25 years (40.4%). Studies conducted by Alli et al. in 2011 and Usip et al. in 2017 also showed similar findings which is not in accordance with the present study. This might be due to the variation in study population's age group size, food habit behaviour and hygiene practice. The association of intestinal parasites detected in relation to age was found to be statistiscally difference (p=0.20).

The present study reports the higher incidence of intestinal parasites in pregnant women was found in Muslim compared to Hindu religion. Maximum

participants were infected from the rural area and those who were unemployed. Also, other related studies in pregnant women carried out by Derso et al. in 2016 and Mahande et al. in 2016 had accounted predominance in rural populations which is in accord with the present study. The prospects might be due to low socioeconomic status, the surrounding environmental factors, level of sanitation and hygiene would have been similar to that of a rural set up which are the major confounding factors for the transmission of intestinal parasitic infection. But, Nipurte et al. in 2020 observed the prevalence of intestinal parasites was almost similar in both rural and urban women contrast to our study (Nipurte et al, 2020).

Education is considered as one of the most basic strategies for health improvement and promotion of quality of life (Mirzaee et al, 2013). The present study showed mostly illiterate participants were infected. Similar findings were also reported by Obiakor-Okeke et al. in 2014 and Hailu et al. in 2020. This might be due to the lack of knowledge, low educational level, unawareness about infections and non-hygienic practices of pregnant mothers which ultimately increase risk of infections. The association of intestinal parasites detected in relation to religion, residence, occupation and education was statistiscally significant ($p<0.05$).

This study reveals the highest number (71.59%) of intestinal parasites detected in those who have symptoms were statistically significant ($p=0.04$). But, the study conducted in Venezuela by Morales et al. in 2006 observed a high prevalence of intestinal parasitosis (more than 70%) those who had no symptoms which are not in harmony with the present study. This might be due to the transversal analysis of pregnant women attending to prenatal control outpatient health care centers in Venezuela which have the larger study population (Morales et al. 2006).

In this study, maximum study participants were infected in 3$^{rd}$ trimester of pregnancy followed by 2$^{nd}$ trimester of pregnancy which was statistically insignificant ($p=0.75$). Since, it is expected to have lower level of haemoglobin in 3$^{rd}$ trimester which is a physiological process in pregnancy. There is no evidence that the cause of infection in 3$^{rd}$ trimester of pregnancy established as to whether it is due to worm infestation or due to physiological cause with superimposed worm infestation (Raut et al. 2016).

The mothers of younger children are expected to come in close contact with their children during their daily activities leading to the more prevalence of infection. The findings of this study depict the maximum number of participants infected with parasites who had four gravida followed by the three, one and two. Higher numbers of respondents were infected those who were multiparous. Similar findngs were also attained in previous studies conducted by Nipurte et al. in 2020 and Alli et al. in 2011. The infection becomes more severe in women who are pregnant for the first time (primigravida) compared with other gravida as reported by Muhangi et al. in 2007. The association of gravida and parity of pregnant women with positive cases was found to be statistically insignificant ($p= 0.79$ and $p= 0.05$) respectively.

Women may even acquire parasitic infections in the process of growing the family's food where insufficiently composted human faeces may be used as fertilizer on vegetable crops (Humphries et al. 1997). In the present study, altogether 8 different types of parasites were detected from stool samples. The most predominant parasites noted during pregnancy were *E. histolytica* (20%), followed by *G. lamblia* (16%) and chased by Hook worm (13%) and *A. lumbricoides* with 11%. Infection with *E. histolytica* is common inhabitants of developing countries and predominantly affects people with poor socio-economic conditions, non-hygienic practices and malnutrition (Braga et al. 2001).

Infections with *E. histolytica, G. lamblia,* hookworm, and *A. lumbricoids* parasites are the most common infection in rural areas, and their transmission is closely associated with socio-economic status, poor sanitation, and absence of adequate safe drinking water supplies (Merid et al. 2001). *G. lamblia* was found to be the second most common protozoan among study participants in current study. The highest prevalence of *Giardia* indicates the poor sanitary and personal hygienic condition of the respondents. Furthermore, the cyst of *G. lamblia* is resistant to the normal level of chlorination, and therefore, it can be easily transmitted through drinking water. The common causes of acute or persisting diarrhoea in people which interferes with intestinal absorption nutrients and growth rate of children (Yadav and Prakash, 2016).

The present study reflects the highest number of

protozoa were detected whereas less number for helminths. A similar study conducted by Hailu et al. in 2020 in West Gojjam Zone, Northwest Ethiopia also observed the prevalence of intestinal protozoa was higher than helminths which are coexisting with the present study. The least number of helminths were detected which might be due to the differences in the distribution of helminths from place to place or from one geographical area to another. The temperature, soil type, rainfall, altitude, and humidity are also the major environmental factors that influence the pre-existence of helminthic infections in one geographical area (De Silva, 2003).

But, a study done in Gondar town, Northwest Ethiopia the presence of protozoa detected was fewer (Alem et al. 2013) which is distinct with this study. The differences might be due to the disparity in the detection method used to identify intestinal parasites. FECT which has higher sensitivity than direct microscopy was used as means of diagnosis in the present study.

The current study depicts Hookworm infection among pregnant women was 13%. Hailu et al. in 2020 reported 18.6% hookworm attacked to pregnant women slightly higher not in accord with this study. The difference might be due to the diversity in shoe-wearing habit and the level exposed to contaminated soil with hookworm larvae that penetrate the human skin. Working bare hands and walking barefoot are the major means of transmission for hookworm infection. But, a similar study conducted by Roberts et al. 2011 in Nepal reported the same trend parallel to our study (Roberts et al. 2011).

The present study depicts 11% *A. lumbricoids* infected the study participants. Nalini et al. 2017 reported the most prevalent ascariasis infestation was 76.5% during pregnancy period incongruent with this study which might be due to larger study population size. Larocque et al. 2005 also accounted ascariasis as the most common infestation in pregnant women. *A. lumbricoids* may cause intestinal obstruction, liver abscess, local irritation, and damage with malabsorption as main cellular related events associated with the infection (Fuseini et al. 2009). *A. lumbricoides* plays an important role in precipitating protein-energy malnutrition in undernourished children (Fuseini et al. 2009).

The other explanations are that the Ascaris ova are also spread by coprophilous animals and can be carried to areas away from defecation sites (Obiamiwe et al. 1991) their eggs resist drying and can survive for long periods in soil. Being coated with mucopolysaccharides ascaris eggs powerfully adheres to different surfaces (Awolaju et al. 2009).

This study also depicted attention to lower prevalence of *H. nana* and *Taenia spp*. which is worldwide parasitic disease with great importance. Lower prevalence was observed in this study which may be due to difficulty in identification of larva. Also, it might be due to rarely transmission occurred from the ingestion of food contaminated with fleas harbouring the cysticercoid larvae. The occurrence of *Taenia* may be due to risk factors associated with eating raw or insufficiently cooked pork, raw vegetables grown in field fertilized with human faeces contaminated with eggs of *T. solium* (Yadav and Prakash, 2016).

This study shows 80% single types of parasites and 20% multiple types of parasites were detected which can cause single and multiple infection. Similar findings were also obtained in the study conducted by Hailu et al. in 2020.

This study reveals maximum number of study participants was infected from single infection in 3$^{rd}$ trimester of pregnancy whereas multiple infections were found in 2$^{nd}$ trimester of pregnancy. The association of trimesters of pregnant women with single and multiple infections was statistically insignificant (p=0.11).This might be due to the fact that parasitic infection could occur at any stage of the three trimesters during pregnancy, but infection during the first trimester is associated with more severe fetal and placental consequences than those occurring later in pregnancy (Muhangi et al. 2007).

The present study established more number of single infections was in age between 20-24 years followed by 15-19 years whereas multiple infections was detected in greater than 35 years. The association of age of pregnant women with single and multiple infections was observed statistically insignificant (p= 0.51). The probable reason is that the health status of young women who are underweight or stunted, those with anemia through its multiple causes or chronic infection will start a pregnancy at great disadvantage of intestinal helminth infection, iron deficiency, and malaria are at increased risk of delivering low birth weight infants. (Steketee et al. 2001)

Hands are the main pathways of germ transmission. The present study shows the intestinal parasites detected in pregnant women who never washed their hands with soap and water before eating was 70.53%. But, women washing their hands most of the times with soap and water before eating were only 8.92%. The correlation between hand washing before eating with soap and water with intestinal parasites detected and not detected was found to be statistically significant (p= 0.00001). The result of this study is almost in accord with the study carried by Nipurte et al. 2020. This prospect may be due to the fact that washing hands before eating a meal is a simple and effective method of infection prevention and protection against germs and illness. In contrast, Raut et al. 2016 found 32.7% respondents had the habit of handwashing with soap and water frequently (Raut et al. 2016) which is distinctive with the present study.

This study showed the pregnant women who washed their hands regularly after defecation had lesser (16.07%) probability of intestinal parasites followed by women (38.39%) who sometimes washed hands and the probability was highest in women (45.53%) who never washed hands after defecation. The reason may be that critical hand washing is preferred as the best washing practices.Other possibility may be due to traditional practice and understanding the importance of cleaning and washing hands with after defecation. The correlation between hand washing after defecation with intestinal parasites detected and not detected was found to be statistically significant (p=0.00001). These findings are similar to the study conducted by Nipurte et al. 2020; Derso et al. 2016 and Mengist et al. 2017.

Pregnancy requires extra nutrients, especially iron, and produces a "physiological anemia" due to hemodilution (Derso et al. 2016). In the present study, the women with high intake of green leafy vegetable were more prone to intestinal parasitic infections which are in accord with the findings of Nipurte et al. in 2020 and Hailu et al. in 2020. Similar findings were also reported in Northwest Ethiopia (Derso et al. 2016) and East Wolega, Ethiopia (Shiferaw et al. 2015). The correlation between consumption of green leafy vegetables with intestinal parasites detected and not detected was statistically insignificant (p=0.15). This might be due to the lack of awareness and the absence of education. Also, it may be due to the pregnant women involved in agriculture and cultivation has limited knowledge about how and when intestinal parasites are transmitted. As a result, eating raw vegetables, open defecation, living in unclean environment, and food with soil during pregnancy are a common phenomenon of infection.

But, in a study conducted by Dutta et al. in 2013 showed that dietary practice of taking green leafy vegetables and fruits had protective effect during pregnancy which is not similar to this study. This depicts that the pregnant women should be advised to have plenty of green leafy vegetables but emphasis should be made on washing it thoroughly before consumption.

In the present study, the occurrence of parasitic infection in pregnant women who used dirty latrine repeatedly was more and reduces the occurrence of infection in women who never used dirty latrines and women who sometimes used dirty latrines. The correlation between use of dirty latrines with intestinal parasites detected and not detected was found to be statistically significant (p=0.00001). These findings are similar to the study conducted by Nipurte et al. 2020. This may be due to lack of cleanliness and poor hygiene practices.

Regarding to the mode of infection of hookworm, the present study evaluated the prevalence of hookworm infection was more in women who never wore footwares (41.96%) followed by those who wore footwares sometimes (29.46%) as compared to those who wore most of the time (28.51%). This might be due to avoiding the personal hygiene like barefoot walking which helps in provoking the infections with soil transmitted helminths like hookworm. In similar type of study, Nipurte et al. in 2020 reported the prevalence of hookworm infection was more in women who never used sandals (7.5%) as compared to those who used sandals frequently (1%). The correlation between use of footwears with intestinal parasites detected and not detected was found to be statistically significant (p=0.00001).

Tesfaye et al. in 2015 and Lorocque et al. in 2005 have also noted positive correlation between barefoot walking and prevalence of hookworm infection which is similar to the present study. This might be due to manipulating the irrigation activity, barefoot and bare hands which leads to parasites like hookworm to enter by skin penetration. Moreover, water for irrigation is not clean and individuals who have the habit of eating food after cleaning their hands with such water have a

possibility to ingest the parasites.

## CONCLUSION

This study concluded that the low hygiene and sanitation habits with lack of awareness about intestinal parasitic infections were the major determinant factors for the moderate prevalence of protozoan infection during pregnancy. *E. histolytica* followed by *G. lamblia* were predominant parasites. The highest number of single type of parasites was identified.

There was positive association of cases with the symptoms of intestinal parasites but negatively associated with trimesters. The relationship of age and trimesters of pregnant women with single and multiple infections was statistically insignificant. Further, the correlation between all the variables with intestinal parasites detected and not detected was statistically significant except for age and consumption of green leafy vegetables. Strengthening the existing water, sanitation and hygiene programs and public health measures like routine deworming of pregnant mothers should be encouraged in community setting to minimize the burden of intestinal parasitic infection.

## LIMITATIONS

This study limits with the small sample size and uses only single stool specimen to assess infection status avoiding the assessment the HIV-status and anemia among study participants. Further, more research is recommended to conduct with a large sample size in this region among pregnant women.

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## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# Screening of Indigenous Yeast From Different Ecological Region of Kathmandu Valley and Its Application in Wine Production

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## ABSTRACT

**Objectives:** The aim of the study was to isolate and screen the potent yeast from the air for implementing new yeast in wine fermentation.

**Methods:** In this study, 35 air samples collected in sterile grape juice in glass jar and left over for four days exposure for the growth of yeast from different locations around the Kathmandu Valley. Yeasts were screened by culturing on selective Ethanol Sulfite Agar (ESA) media at 30°C for 2-3 days in Microbiology Lab of Pinnacle College. Yeast isolates were characterized based on colony morphology, microscopic characteristics, Fermentative capacity, Hydrogen sulfide production. Selected yeast isolates were subjected to ethanol fermentation and tested for alcohol tolerance capacity. Wine quality was assessed by sensory evaluation.

**Results:** Of 35 samples, only 20 yeast isolates were isolated. Among these isolates, the variation in colony characteristics along with oval and ellipsoidal microscopic appearance was observed. All the isolates were able to ferment major sugars such as glucose, fructose and sucrose, but few could not ferment galactose and maltose, while none-fermented lactose and xylose. Here, isolates showing no $H_2S$ (L29, L34) and mild $H_2S$ producer (isolate L31) were subjected to ethanol fermentation. Also, Comparative analysis was made by using commercial standard wine yeast (STAN). Rapid fermentation of grape juice with initial 21 $^0$Brix was observed in L31 isolate, which produced 12.99% v/v alcohol with titratable acidity (TA) 5.25 g/L, followed by L29 strain with 11.99%v/v alcohol and 4.5 g/L TA which were higher than STAN (10.99% alcohol). These isolates specified as Ethanol tolerance up to 13%v/v, while none of them were able to grow at 15% v/v ethanol concentration and 45°C temperature. However, significant growth was observed at pH 3 along with sugar tolerance capacity at 30 $^0$Brix. The wine produced by these isolates was found to be remarkably different among each other. While the sensory analysis of wine led to isolate L31 being congenial to tasters.

**Conclusion:** L31 isolate was found to be efficient and advantageous for wine production indicating its industrial application.

**Key words**: Yeast, fermentation, colony, Brix, titratable acidity (TA)

## INTRODUCTION

Wine, in the simplest terms, is the fermented grape juice, produced as a result of complex biochemical processes by the synergism of many microbial species normally present on the skin of the grapes, which mainly comprises fungi, yeast and bacteria. Despite the existence of diverse microorganisms on the skin of grapes, only a fraction contributes to the fermentation process in winemaking (Barata et al. 2012). Among which *Saccharomyces cerevisiae* plays a crucial role in it, converting sugar present in grape juice into alcohol and carbon dioxide, this process is further improved via different non-saccharomyces yeast genera such as *Hansenula*, *Candida, Brettanomyces, Kloeckera, Pichia, Saccharomycodes, Torulopsis* (Esteve-Zarzoso et al. 1998) provide significant aroma and taste to wine. Similarly, the most commonly involved bacteria in winemaking are lactic acid bacteria, which are responsible for the

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fermentation of malic acid into lactic acid through a process called malolactic fermentation (Nielsen and Richelieu 1999). Generally, the winemaking process starts through the vinification process to the maceration, where grape juice is extracted from grapes, then it is subjected to the fermentation process from which the wines are transferred into oak barrels for aging. After aging for several months, the wines are fined, filtered and bottled, which is later on supplied to the market (Pretorius 2000). In today's global wine market, a large variety of wines are available that have their own distinct flavors and qualities. These differences among the wines are mostly due to the difference in the variety of grapes used, viticulture practices, art of winemaker and the use of innovative technology (Bisson 2002). However, primarily it depends upon the type of *Saccharomyces* yeast strain used, as different strains of the yeast provide a different flavor to the wine and gives uniqueness to the product (Romano et al. 2003). Over the years, much research has been conducted to isolate various wild strains of *Saccharomyces* species for improving ongoing wine fermentation and to obtain the best wine out of it. Nowadays, hundreds of different strains of *Saccharomyces* have been commercialized for winemaking, which is called wine yeast. This commercialization of wine yeast is mostly done by selecting the isolate possessing these characteristics, such as quick initiation of fermentation, tolerance to low $p^H$, high temperature, high sugar and ethanol concentration, low production of hydrogen sulfide, low requirement of nitrogen, production of desirable bouquet and ability to ferment up to dryness (Rainieri and Pretorius 2000). Today, most of the wild strains are genetically modified to enhance their overall characteristics and performance. In spite of the availability of such wine yeast for industrial fermentation, there is still a need of improvement in the existing ones because the potent wine yeast strain has been rarely isolated and exploration of the natural diversity of these yeasts has not yet been accomplished (Wang et al. 2012).This research isolates such indigenous species of *Saccharomyces* present in Kathmandu Valley as it bears different climatic and geographical differences within a small area. It comprises a subtropical to temperate rainforest with a pleasant climate warm in days followed by cool morning and night. The temperature during summer varies from 28-30°C while in winter around 3-10°C and the humidity is approximately 75% with rainfall total monsoon based. The valley is surrounded by four hills; Phulchowki in South-East, Chandragiri in South-West, Shivapuri in North-West and Nagarkot in the North-East with altitudes ranging from 2000- 2831 m (Jha 2016) and the yeast adapted within this region could be different with different fermentative capacity (Spencer 2013).In this study, isolation and characterization of wild-type strains of *Saccharomyces* have been discussed and its uses in winemaking have been interpreted. Hence, this study aids in selecting and improve the wine quality along with its implementation of potent wild yeast isolates of *Saccharomyces*, which will create a strong identity that will eventually facilitate its application in winemaking.

## MATERIALS AND METHODS

**Sample size, site and sample collection:** A total of 35 air samples were examined randomly from different places of Kathmandu valley (12 samples collected from Katmandu, Bhaktapur (11), and Lalitpur (12) respectively).

For sampling, the grape juice of 100 ml approx. was poured in heat resistant and leak proof glass jar of 400 mL and the muslin cloth (15x17-cm length and breadth) wrapped in aluminum foil (to prevent moistening during sterilization) was autoclaved. After sterilization, the glass jar and the muslin cloth were placed into sterile plastic bags before shifting it to the sampling site. After transporting to sampling sites, the lid of the jar was exposed while its mouth was covered with muslin cloth and was left over for four days as an exposure period for the growth of yeast in the sampling site, which was collected and labeled with specific codes

**Isolation and identification of *Saccharomyces*:** For isolating *Saccharomyces*, collected samples were further incubated at 30°C for approximately one week in the lab, from which a loop full of a suspension was streaked onto the selective medium i.e. Ethanol Sulfite Agar (ESA), which was then incubated at 30°C for 2-3 days. The growth of colonies was then sub cultured on the Yeast Extract Peptone D-glucose Agar (YEPDA) for further identification (Kish et al. 1983).

**Culture characteristics and microscopic identification:** Cultural characteristics such as shape, size, color, margin, surface, elevation, consistency and opacity of the single isolated colony were noted along with microscopic identification was performed using a

simple staining method

**Fermentative capacity testing:** Yeast fermentation broth (YFB) containing inverted Durham's tube was used to identify *Saccharomyces* species based on the fermentation of different carbohydrate sources (Glucose, Fructose, Sucrose, Galactose, Lactose, Maltose and Xylose). The YFB media were prepared by the addition of peptone 7.5 g, yeast extract 4.5 g, 1 ml of 1.6 w/v bromothymol blue as an indicator into 1000 ml distilled water which was boiled then 7 mL of each solution was poured into each test tubes along with Durham's tubes, whereas 5% w/v of each carbohydrate solution was prepared separately in 3ml distilled water and was autoclaved. After sterilization, the media was allowed to cool at 40 - 45°C then the different carbohydrate solution was poured into respective tubes along with loop full of suspension of isolate into each medium and incubated at 30°C for 3 days and the result was indicated by change in the color of media from green to either yellow (if acidic) or blue (if alkaline) and the presence of gas in the Durham's tube along with control tubes in each set to monitor the contamination of the medium (Guimaraes et al. 2006).

**Hydrogen sulfide testing:** H2S production test of the isolates was performed by growing yeast isolates on Lead Acetate Agar (LAA) medium (40 g/l glucose, 5 g/l yeast extract, 3g/l peptone, 0.2 g/l ammonium sulfate, 1 g/l lead acetate, 20g/l agar) and the plates were incubated at 30°C for 10 days. The result was indicated positive by observing dark brown colonies of isolates on lead acetate medium and vice versa (Ono et al. 1991).

**Alcohol fermentation:** The isolates producing less or no H2S gas were subjected to the alcohol fermentation which was performed in the glass bottles of 450 ml and 400 ml of freshly pressed grape juice was poured with the sugar concentration around 20° Brix along with initial pH reading of grape juice, which was then autoclave for sterilization, then the suspension of selected isolates equivalent to 2 McFarland was poured and was fermented at 25°C. The reduced sugar concentration was monitored for weeks with one-day gap intervals until concurrent Brix reading was observed. The amount of Ethanol produced was measured by comparing the specific gravity reading of the distillate to the standard chart and the result was interpreted as % v/v (percentage volume by volume). Also, the final PH and the titratable acidity of the grape must be measured (Shrestha et al. 2002; Jacobson 2006).

**Stress tolerance test:** To determine the tolerance capacity of selected isolates at different alcohol concentration and sugar concentrations followed by temperature and PH. The isolates were first grown in YEPD medium with slight modification where sucrose was used instead of glucose and its concentration was maintained at 200 g/l (20 nb° Brix) and the PH was maintained around 3.5 and was supplemented with 80 ml/liter of ethanol and incubated at 30°C for 3 days. The isolates from this medium were then subjected to Yeast Extract Peptone Broth (YEPB) supplemented with 10 g/l yeast extract, 20 g/l peptone for respective tolerance test the concentration of sucrose was chosen as required (Guimaraes et al. 2006).

**Alcohol tolerance test:** The isolates were inoculated into (YEP) broth supplemented with 100 ml/l, 130 ml\l, 150 ml/l ethanol solution respectively was then incubated at 30°C for 3 days and the growth was monitored by measuring the optical density at 540 nm with the use of a colorimeter.

**Temperature tolerance test:** The isolates were inoculated into (YEP) broth, which were incubated in 5°C, 25°C, 35°C, and 45°C respectively for 3 days and the growth was monitored by measuring the optical density at 540 nm with the use of a colorimeter

**pH tolerance test:** The isolates were inoculated into (YEP) broth, where its PH is maintained at 3, 3.5, 4, and 4.5, respectively and incubated at 30°C for 3 days and the growth was monitored by measuring the optical density at 540 nm with the use of colorimetry.

**Sugar tolerance test:** The isolates were inoculated into YEP broth in which sugar concentration was maintained at 10°, 20°, 30°, 40°, and 50° Brix and it was incubated at 30°C for 3 days and the growth was monitored by measuring the optical density at 540 nm with the use of a colorimeter.

**Sensory analysis of wine:** In sensory analysis of selected isolates, a freshly pressed grape juice of approximately 500ml was poured into a glass bottle of 750 ml with sugar concentration was maintained around 24° Brix and initial PH 4.6. The must was supplemented with 50 mg/l potassium metabisulphite and 100 mg/l Ammonium sulfate then suspension of the selected isolates equivalent to 2 McFarland was poured into

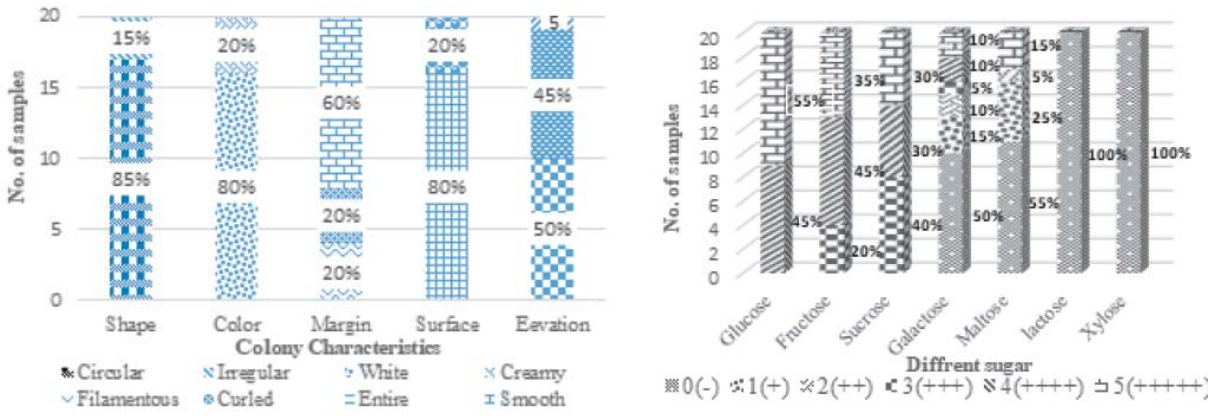
each glass bottle and was allowed to ferment at 25°C for 1month. After a month of fermentation and removing sediments, the clarified wines were subjected to evaluation by 5 experienced tasters including wine quality manager. The sensory attributes (Appearance, Odor, taste and body of a wine) were used to estimate the quality among the tasters, and the divergence was evaluated by the score given by five tasters on each category of sensory attributes using two-way ANOVA table (for repeated values) along with rating based on their perception from 1 to 5 where 1= Not bad, 2= Satisfactory, 3= good, 4= better and 5= excellent (Jackson 2008 & 2009).

**Quality control:** Standard procedure and strict aseptic conditions were maintained to obtain reliable results and sterility of each media from a batch was checked by incubating one media of each batch in an incubator at 37°C for 24 hrs. For comparative analysis, the standard wine producing strain *Saccharomyces cerevisiae* (springer Oenologie, fermentis, Belgium) culture was used.

## RESULTS

Out of 35 air samples, 11(31.42%) from Bhaktapur, 12(34.29%) from Lalitpur and 12(34.29%) from Kathmandu district were examined. The significant growth was observed in 66.67% (8/12) of Kathmandu samples. The culture was positive for 58.33% (7/12) and 45.45% (5 /11) of Lalitpur samples and Bhaktapur samples respectively. On selective ESA media, only 20 samples indicated growth as yeast. These yeast isolates on YEPD agar medium exhibited circular opaque and mucoid characteristics along with umbonate elevation and entire margin, similarly white colony was observed as shown in Fig-1A, which we found similar to the colony characteristics of the standard commercial yeast used in the study. The colony on YEPD agar medium of selected isolates is shown in the figure:2A. Microscopic characteristics of the isolates revealed an oval and ellipsoidal appearance, which resembles the characteristics of *Saccharomyces* species.

All the isolates were able to ferment major sugar, such as glucose, fructose and sucrose, whereas some isolates were unable to ferment maltose and galactose and none of them were able to ferment xylose and lactose as shown in Fig-1B. This result also signifies variance in sugar assimilation properties among the *Saccharomyces* species, which further aid in identification as *Saccharomyces* species as isolates were unable to ferment lactose and xylose sugar.




(A) (B)

**Figure 1: (A) Colony characteristics of the yeast isolates and (B) Fermentative capacity of different sugars by yeast isolates**

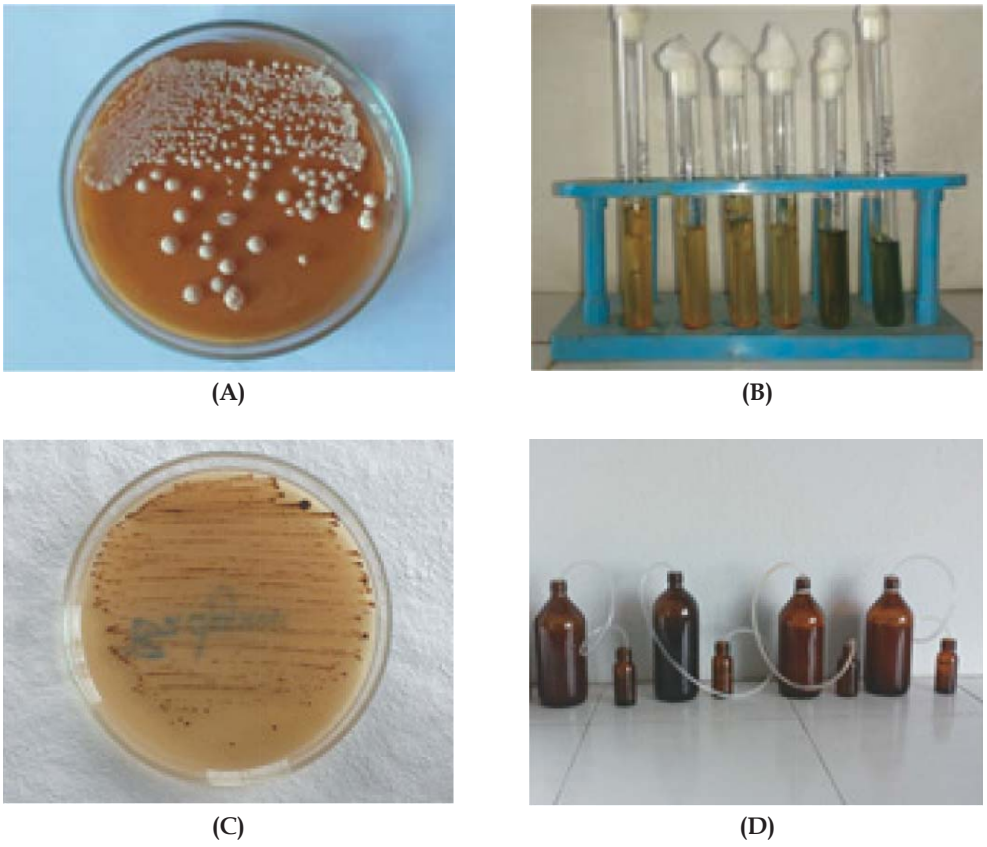


**Figure 2: (A) Colony characteristics; (B) Fermentative Characteristics, (C) Dark black colonies on Lead Acetate Agar, (D) Ethanol fermentation**

In this study, we found that 2 (15%) samples were found to be hydrogen sulfide non-producer i.e. absence, 1 (5%) samples was found to be a mild hydrogen sulfide producer, 8 (35%) samples were found to be moderate hydrogen sulfide producer and 9 (45%) samples were found to be intense hydrogen sulfide producer as shown in Fig-3. Since, H2S can cause rotten egg aroma in the wine, hence only $H_2S$ negative and mild $H_2S$ positive isolates were chosen for further study parameters.

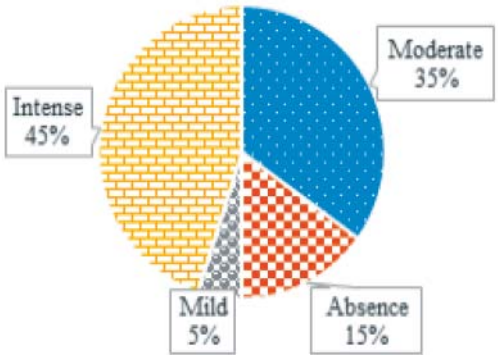
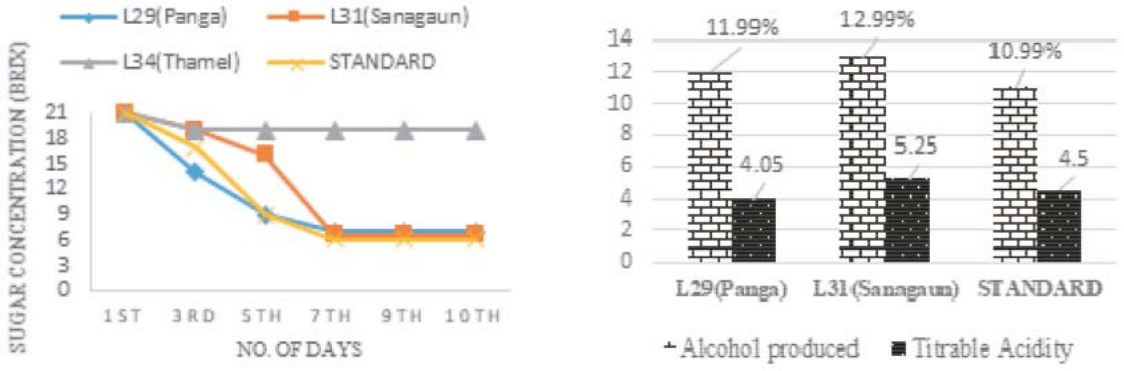



**Figure 3: Comparison of Hydrogen sulfide production**

**Ethanol Fermentation**

Isolates showing no H2S (L29, L34) and mild H2S production (L31) were subjected to ethanol fermentation, where grape juice with initial 21 $^0$Brix was taken as a substrate, and isolate with 2 Mcfarland turbidity was inoculated into the fermentation vessel, and sugar assimilation were noted on alternate day. In this study we observe that, the significant decrease

of 21 $^0$Brix within a 5th day was 9 shown by L29 and STAN, while least was found to be 16 as shown by L31. After the 7th day, $^0$Brix was 7 for L29 & 6.5 for L31 but for STAN's $^0$Brix was 6. Since L34 expressed stuck and sluggish fermentation as shown in Fig-4A So, it was excluded from further processing. Similarly, ethanol production was found to be 12.99% by L31 followed by 11.99% L29 and the least by 10.99% STAN. However, the titratable acidity of grape must be found to be highest by 5.25g/L of L31 followed by 4.5g/L of STAN and the least was found to be 4.05g/L.




**(A)** **(B)**

**Figure 4: (A) Sugar utilization of selected isolates and standard, (B) Comparison of alcohol produced and titratable acidity of selected yeast and standard**

**Stress tolerant test**

**The temperature tolerance test:** The temperature tolerance capacity of the selected yeast showed notable variation. The cardinal temperature of temperature tolerance is at 5°C & 45°C (minimum), 25°C (maximum) and 35°C (optimal). Despite some fluctuation, there was a gradual increment of tolerance capacity leading by L31, which peaked at 25°C by 1.91 (O.D.) followed by L29 1.91 (O.D.) and Standard by 1.67 (O.D.) respectively as shown in the Fig-5A.. After 25°C, there was a rapid decrease in temperature tolerance capacity of the selected strains, including standard, which dropped to null at 45°C by all yeast isolates.

**Sugar tolerance test:** There was a significant increment of sugar tolerance capacity from 10 to 20 $^0$Brix in which L29 showed maximum viable growth of 0.91 (O.D.) followed by 1.86 (O.D.) and 1.76 (O.D.) of L31 and Standard, respectively, as shown in the Fig-5B.. After 20 Brix sugar concentration, there was a sharp decrement of viable growth, which eventually stopped at 40 and 50 Brix with no tolerance capacity from the selected strains along with standard.

**Alcohol tolerance test:** The alcohol tolerance capacity of the selected yeast showed notable variation by the gradual decrease in the difference in viable count by the colorimeter (O.D.) with increment of the alcohol concentration. L31 yeast isolate was leading among selected yeast isolates throughout all alcohol concentration followed by L29 and Standard. The maximum tolerance capacity can be observed at 10% alcohol concentration by 0.17 (O.D.) L31, followed by L29 0.10 (O.D) and by Standard 0.09 (O.D) , which decreased at 13% alcohol concentration to 0.07 (O.D) of L31 and 0.04 (O.D.) of L29 and Standard by 0.02 (O.D). In 15% alcoholic concentration, no growth was observed, L31 showed the tolerance by 0 (O.D) followed by L29 by 0 (O.D) and Standard by 0 (O.D) as shown in the Fig-5C.

**pH tolerance test:** The maximum tolerance capacity and growth of viability was shown by L29 from 0.16 to 1.2(O.D) as the $P^H$ concentration increased from 3 to 4.5 followed by L31 from 0.13 to 1.19(O.D). The least tolerance and growth were shown by Standard from 0.13 to 1.07(O.D) as the $P^H$ concentration increased from 3 to 4.5, as shown in the Fig-5D.

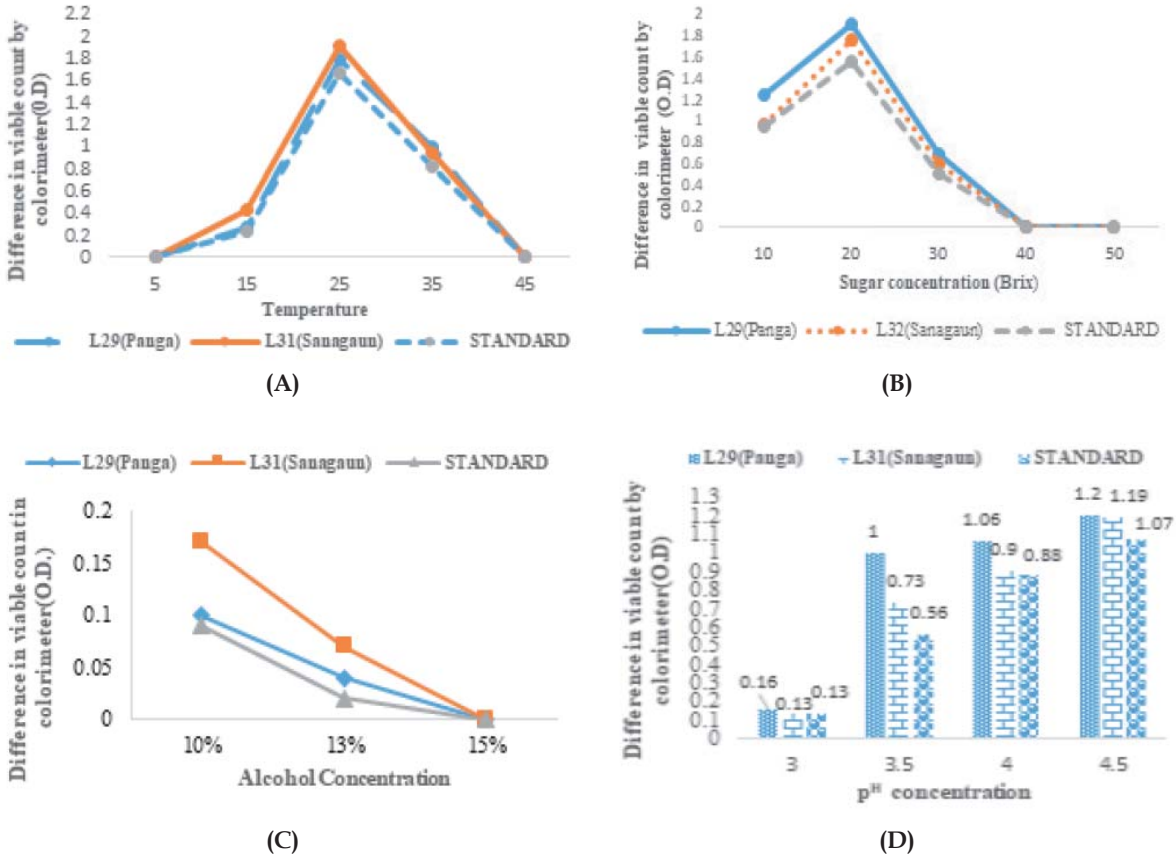



**Figure 5: (A) Temperature Tolerance Capacity, (B) Sugar Tolerance Capacity, (C) Alcohol Tolerance Capacity and (D) pH Tolerance Capacity**

**Sensory analysis of wine:** Sensory analysis was reported for three wines by five testers. According to them, the variance among the wines in their appearance, odor, taste and body was analyzed in which the scoring was manifested that there was a significant difference between the wines and tasters as the value of F-calculated exceeded that of F-tabular at 5% level of significance.

**Table : Two-way ANOVA table designed for repeated value**

| Source | SS | d.f. | m.s | F | $F_{0.10}$ | $F_{0.05}$ | $F_{0.01}$ |
|---|---|---|---|---|---|---|---|
| Total | 152 | 15 | | | | | |
| Wines | 8.4 | 4 | 2.1 | 5.1686 | 2.8064 | 3.84** | 7.006 |
| Tasters | 77.9999 | 2 | 38.9995 | 95.9869 | 3.1131 | 4.46** | 8.649 |
| Interaction | 62.3501 | 1* | 62.3501 | 153.4583 | 3.4579 | 5.32** | 11.259 |
| Error | 3.25 | 8 | 0.4063 | | | | |

**Significance at 5% level*(*SS* =Type III sum of square, *m.s*= Mean square, *d.f*= degree of freedom, F=Test statistic *F)*

**Rating of wine by five testers:** The selected isolated strains were processed for fermentation of grape must for the preparation of wine and named them wine A, B, and C for strains Standard, L31 and L29, respectively. From the sensory analysis conducted among five tasters, 2 from wine A, 3 from wine B and 1 from wine C rated satisfactory as a result, while remaining 3 from wine A, 2 from wine B and 3 from wine C rated good, while 1 taster rated better for wine C. Hence, wine C was found to be comparatively better than wine B and wine C as shown in Fig-6.

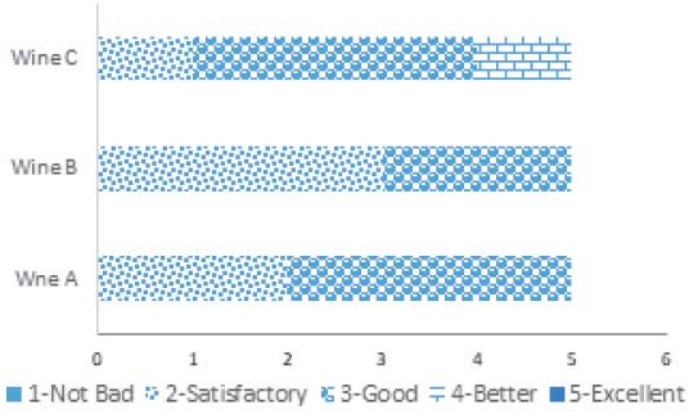



**Figure 6: Comparison of the ratings for three wines by five tasters**

## DISCUSSION

Of 35 samples were examined, only 20 indigenous Saccharomyces species were isolated. Ethanol sulfite agar media were used to isolate selectively for yeast, where 1 from Bhaktapur, 9 from Lalitpur and 10 from Kathmandu was isolated, which may be due to its low occurrence in nature. A similar result was observed by (Ghosh et al. 2013) in which out of 26 ascomycetous isolates only 2 yeast isolates were discovered to be *Saccharomyces* species. Additionally (Martini et al. 1996) has reported the rare isolation of *Saccharomyces* from the grape skin, which was noted to be only 3 isolates among 30 samples.

Colony characteristics (shape, surface, margin and elevation) of the 20 isolates were observed, which expressed similar colony characteristics with standard commercial yeast (STAN). Their colony characteristics were noticed to be circular, smooth and creamy color colonies along with umbonate elevation with an entire margin and were opaque. Similar findings were also observed by (Asyikeen et al. 2013), in which 18 of the yeast isolates were opaque, smooth, regular colonies and creamy in color but other 2 yeast isolates were similar except rough colonies were observed. A rough colony formed by wild Saccharomyces has also been reported by (Kuthan et al. 2003).

In this study, simple staining was performed for microscopic identification of isolates, which demonstrated ellipsoidal or oval-shaped proving the characteristics of Saccharomyces species. Similarly, the fermentative characteristics of 20 isolates expressed variant sugar assimilation profile as all isolates could ferment major sugar, such as glucose, sucrose and fructose, while most of the strains were unable to ferment galactose and maltose and none of the isolates were able to ferment xylose and lactose. From the sugar assimilation profile, the yeast isolates were further identified as *Saccharomyces* species as none of the isolates assimilated lactose and xylose (Guimaraes et al. 2006). Also, (El-Nemr 2001) reported that lactose sugar was unable to be fermented due to the absence of a b-galactosidase enzyme among the Saccharomyces species.

This study observed that among the total 20 yeast isolates, 2 (15%) samples were noted to be hydrogen sulfide non-producer i.e. absence, while rest 18(85%) isolates were hydrogen sulfide producer. Among them, 1 (5%) samples were mild hydrogen sulfide producer, 8 (35%) samples were moderate hydrogen sulfide producers and 9 (45%) samples were intense hydrogen sulfide producer, where the proportion of non- hydrogen sulfide producing wine yeast was rarely present in nature as isolates L29 and L34 implies non-hydrogen sulfide producers. A similar result was discovered by (Asyikeen et al. 2013) where among 16 tentative wild yeast isolates, only 2 isolates were non- hydrogen sulfide producers. Ugliano et al. (2011) reported that the isolate with characteristics of hydrogen sulfide was of no use for wine fermentation as it imparts the off odors described as rotten egg and/ or sewage to wine. So, the isolates with a high profile of producing Hydrogen sulfide i.e. (moderate and intense) were excluded from the study.

During the study, on subjecting the H2S negative isolate L29, L34 and Low H2S isolate L31 to alcohol fermentation discovered significant decrement of 21 Brix within a 5th day, which was 9 shown by L29 and STAN, while least was found to be 16 by isolate

L31. After the 7th day, Brix was noted to be 7 by L29 & 6.5 by L31 but for STAN, Brix was observed to be 6. However, the isolates L34 noted no decrement of sugar by 18 Brix within a 7th day. Therefore, isolate L34 was excluded from further processing, as stuck and sluggish fermentation processes were found to be problematic for wine production (Bisson 1999).

In recent studies, the maximum alcohol produced via specific gravity was noted to be 12.99% by L31 followed by L29 (11.99%) and the least by STAN (10.99%). However, the titratable acidity of grape must was found to be highest in L31 (5.25g/L) followed by STAN (4.5g/L) and the least was observed to be 4.05g/L by L29. A similar study was reported by (Callejon et al. 2010) in which the final alcohol concentration of four wild yeast isolates ranges from 11.9 to 12.6% v/v.

In this study, the temperature tolerance capacity of the selected yeast isolates expressed notable variation. The cardinal temperature of temperature tolerance is at 5°C & 45°C (minimum), 25°C (maximum) and 35°C (optimal). Despite some fluctuation, there was a gradual increment of tolerance capacity leading by L31, which peaked at 25°C by 1.91 (OD) followed by L29 1.91 (OD) and STAN by 1.67 (OD), respectively. After 25°C, rapid decrement of temperature tolerance capacity by all selected isolates including STAN dropped to null reaching at 45C. This result signifies that the appropriate temperature-maintained leads to increased growth, metabolism and survival of yeast during fermentation. A similar result was obtained by (Asyikeen et al. 2013), in which wild yeast isolated from various fruits and other plant materials could not grow at the high temperature of 37°C. (Torija et al. 2003) reported that fermentation of different strains in white must was dynamic at high temperature between intervals of 30-35°C. Additionally, (Bertolini et al. 1991) has reported no growth due to the effect of high temperature on 40°C.

In this research conducted, there was a significant increment of sugar tolerance capacity of selected isolates from 10 to 20 Brix in which significant growth of 0.91 (OD) followed by 1.86 (OD) and 1.76 (OD) of L29, L31, and STAN, respectively. After 20 Brix sugar concentration, there was a sharp decrement of viable growth, which eventually stopped at 40 and 50 Brix with no tolerance capacity from the selected strains along with STAN. Hence, all the isolates showed sugar tolerance capacity at 30 Brix. This result indicated that higher the sugar concentration, greater the inhibitory effect due to osmotic stress. A similar result was reported (Bertolini et al. 1991) that the strains they isolated from Brazilian alcohol factories were potential enough to ferment up to 30% of sucrose readily. However, the sugar concentration of 200 g/L to 300 g/L decreased Saccharomyces cerevisiae growth rate as reported by (Charoenchai et al. 1998). Which signifies that the greater the sugar concentration greater the inhibitory effect on the yeast cell.

This study showed that the alcohol tolerance capacity of the selected yeast isolates revealed notable variation by the gradual decrement in a difference in the viable count by the colorimeter (OD) with an increment of the alcohol concentration. L31 yeast isolate was leading among selected yeast isolates throughout all alcohol concentration followed by L29 and STAN. The research conducted showed that the maximum tolerance capacity was observed at 10% alcohol concentration by L31 0.17 (OD), followed by L29 0.10 (OD) and by STAN 0.09 (OD) , which decreased at 13% alcohol concentration to 0.08 (OD) of L31 and 0.04 (OD) of L29 and STAN by 0.02 (OD). In 15% alcoholic concentration, all the yeast isolates, including standard manifested no visible growth i.e. 0 (OD). This indicates that higher ethanol concentrations minimum the growth of yeast cells, which unfortunately cease the fermentation process. Similarly, higher ethanol produced during fermentation leads to inhibitory effect was also reported by (Wayman and Parekh 1990) In contrast, most of the ethanol-resistant yeast isolation has been reported by (Umeh 2016). While in this study selected yeast isolates along with standard commercial yeast showed no ethanol tolerance above 13% v/v. The study noticed that the maximum tolerance capacity and growth of viability was shown by L29 from 0.16 to 1.2 as the pH concentration increased from 3 to 4.5 followed by L31 from 0.13 to 1.19. The least tolerance and growth were shown by STAN from 0.13 to 1.07 as the pH concentration increased from 3 to 4.5. This study implies that the best pH tolerance capacity due to the tolerance of H+ ions excreted by yeast and eventually drops of the pH of the media. A similar report was reported by (Charoenchai et al. 1998) reported that the pH range between 3 and 4 does not affect the growth rate and cell biomass of wine yeast. This indicates that yeast isolates in this study showed tolerance capacity at

pH concentration 3.

In this study, the wine produced by the selected isolates was noted to be significantly different from each other, which signifies that the flavor profile of the particular wine is influenced by the different types of yeast used (Romano et al. 2008). In addition, also by analyzing the ratings given to particular wine by five experienced tasters revealed that the highest vote was manifested to be in the wine produced by L31 (i.e. 3 tasters voted for good, 1 voted for the satisfactory and 1 voted for better) which is then followed by STAN (i.e. 3 tasters voted for good and 2 voted for satisfactory) While in the wine produce by L29 3 tasters voted for satisfactory and 2 voted for good. This result signifies that the wine produced by L31 was found to be mostly likable among the tasters.

## CONCLUSION

Indigenous yeast isolated from the air samples in Kathmandu were more potential than a standard industrial yeast when compared the physiological characteristics including growth profile, appearance, fermentative capacity, hydrogen sulfide, tolerance capacity (such as temperature, sugar, alcohol and pH), alcoholic fermentation and sensory analysis. All yeast isolates fermented sugars such as glucose, sucrose and fructose, but only a few fermented galactose and maltose and none-fermented lactose and xylose sugars enabling yeast identification. From the total yeast isolates, only 2 (15%) yeasts are hydrogen sulfide non-producer, which is lowly compared to the H2S producer i.e. 18(85%). The selected yeast isolates are capable of producing high ethanol concentration with significant titratable acidity compared to commercial yeast which i.e. 11.99% and 4.5 g/L for L29, 12.99%, and 5.25 g/L for L31 and 10.99%, and 4.05 g/L for (STAN) commercial yeast, respectively. These isolates were able to tolerate up to 13% ethanol concentration along with visible growth at pH 3 and sugar concentration of 30 Brix with no thermotolerant capacity at 45°C. Similarly, the sensory analysis revealed the diversity of wine produced and wine produced by the isolate L31 seems most likable among the tasters. Hence, it signifies that the yeast isolates L29 and L31 are superior to commercial strain, which enables them for industrial application.

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## CONFLICT OF INTEREST

The authors declared that they have no competing interests

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# Field evaluation of native *B. thuringiensis* isolates against aphids (*Aphis fabae*)

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## ABSTRACT

**Objectives**: The purpose of this study was to evaluate the aphicidal activity of native *Bacillus thuringiensis* (Bt) strains.

**Methods**: Soil samples of Provinces 2 and 3 of Nepal were collected randomly for isolation of Bt by acetate selection method. Bt were identified by observing insecticidal crystal proteins (ICPs) by Commassie Brilliant Blue (CBB) staining technique. Aphicidal activity of 12 *B. thuringiensis* isolates was evaluated by two processes. The preliminary screening was done by spraying the suspension containing the spore and ICPs mixture in *Phaseolus* species heavily infested with black aphids (*Aphis fabae*) in fields. The second process (selective bioassay) was done by counting the number of aphids (nymphs, instar, winged, wingless) before and after spraying 5ml of suspension containing the spore and ICPs mixture on the leaf or on the beans pods surface infested by Aphids. The mortality percentage of Aphids after treatment was calculated on the 4$^{th}$ day, by counting the live aphids and the result was recorded.

**Results:** Preliminary screening for aphicidal activity revealed that 4 isolates ML5(1), CW1(1), SN2(1) and MP2(1) producing spherical crystal protein, showed 100% mortality against nymphs, instar, winged and wingless Aphids. Isolates were effective in controlling the Aphid (*Aphis fabae*) within 4 days and the part of the plant that was sprayed becomes free of Aphids. Selective bioassay of native isolate MP3(3) was most effective in killing 95.83% of aphids followed by CW2(1), 85.71%, ML5(1), 77.34%, SN3(1), 72.72%, CW1(1), 70.21%.

**Conclusion:** This study revealed that indigenous *Bacillus thuringiensis* of Terai region of Nepal are effective in controlling Aphids.

**Keywords:** Aphicidal, ICPs, screening, *Bacillus thuringiensis*, Nepal,

## INTRODUCTION

Insects are the major pests of crops. Among insect Aphids are plant lice, sap-sucking insects or phloem feeder, virus vector and enhancer of sooty mold. Aphids vary in their color but most popular ones are green, black, white and yellow. About 5000 species of aphids are known, all of which belong to Phylum- Arthropoda, Class- Insecta, Order- Hemiptera, Family- Aphididae, and Genus- *Aphis*. Around 400 species are serious pests for agricultural and forest plants as well as an annoyance for gardeners (Aphid, 2020). Aphid infestation decline and causes extermination of major cash crop production in Nepal. Mustard Aphid declined rapeseed oil production in Chitwan (Kafle and Jaishi, 2020). Similarly the cultivation of *vicia faba* in Bhaktapur municipality was declined due to poor efficiency of existing Aphid management practices (Subedi, 2015).

Biopesticides appear to be ecologically safer then commercial chemical pesticides because they do not accumulate in the food chain. Biopesticides often are effective in very small quantities and often decompose

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quickly, thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides (Ammouneh *et al.*, 2011). When used as a component of Integrated Pest Management (IPM) programs, biopesticides can greatly decrease the use of conventional pesticides, while crop yields remain high. Biopesticides falls in major three categories-microbial pesticides, plant incorporated protectants and biochemical pesticides (Çetinkaya, 2002; Leahy *et al.*, 2014; Damalas, 2018). Biopesticide use at a global scale is increasing by almost 10% every year (Damalas, 2018). Various types of microorganism that can be used as biopesticides are bacteria, fungi and viruses. Almost 90% of the microbial biopesticides currently available in the market are derived from only one entomopathogenic bacterium i.e., *Bacillus thuringiensis* (Bt) (Leahy *et al.*, 2014; Damalas, 2018).

*Bacillus thuringiensis* is a bacterium known for producing protein crystals with pesticidal properties. *Bacillus thuringiensis* are Gram positive, rod shaped, spore forming, soil dwellers. During sporulation it produces insecticidal parasporal crystal proteins that are toxic against many insect pests. When orally ingested by insects, this crystal protein is solubilized in the mid gut of the insect and the insect dies within 6-12 hours (Schünemann, Knaak and Fiuza, 2014). *Bacillus thuringiensis* biopesticide is called as Bt. Bt has been used commercially in the biological control of insect pests for the last 4 decades. These toxins are widely sought for controlling agricultural pests due to both their specificity and their applicability.

This study aims at isolating *Bacillus thuringiensis* from soil for controlling insect pest.

## MATERIALS AND METHODS

### Sample collection

Soil samples (50) were randomly collected from tropical regions of 2 provinces of Nepal (Province No: 2 Parsa, Bara, Rautahat, Sarlahi, Mahottari and Dhanusa and Province No: 3 Sindhuli, Chitwan and Makwanpur). Soil weighing about 10 grams was obtained from 5cm depth in a plastic bag (Soares-da-Silva *et al.*, 2017). Collected samples were stored at $4^0$C before processing.

### Isolation of *Bacillus thuringiensis*

Isolation was carried out using the acetate selection method as described by (Travers, Russells, Martin, Phyllis, and Reichelderfer, 1987). The Nutrient broth (NB) was acetated by using 0.25M sodium acetate for selective enrichment of *Bacillus thuringiensis*. To the sterile 9 ml enriched NB broth media 1gram of soil sample was added and incubated overnight at $35^0$C. After incubation the broth was heated at $100^0$C for 5 minutes as described by (Apaydin, Ozgur, Yenidunya, A. Fazil, Harsa, Sebnem and Gunes, 2005) with slight modification. Following heat treatment, the sample was serially diluted in dilution blank and the suspension was inoculated on Nutrient agar plate (NA) by spread plate technique and incubated at $35^0$C for 24 hours. The isolated colonies were further sub-cultured in Nutrient agar (NA) to obtain the pure culture.

### Phenotypic characterization

Coomassie Brilliant Blue staining technique was performed to detect the presence of crystal protein production in the cells after incubation of culture for 72 hours in a Nutrient broth to distinguish from other *Bacillus* spp as described by (Rampersad, Khan and Ammons, 2002). Physio chemical characterization was done by biochemical tests like, Catalase test, Oxidase test, Indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, Citrate test, Starch hydrolysis test, Gelatin hydrolysis test, beta-haemolysis test, motility test, sucrose, fructose, mannitol, lactose fermentation tests and lecithinase test in the respective biochemical test media and the result was recorded after 24 hours incubation. The identified isolates were preserved in nutrient agar media by adding 60% glycerol.

### Formulation of bioinsecticide

Formulation of bioinsecticide was performed by submerged fermentation (SmF) /liquid fermentation (LF) technique as described by (Ralte, Nachimuthu, & Guruswami, 2016) with slight modification by inoculating the isolates in a 250ml conical flask containing sterilized Luria-Bertani (LB) broth (Tryptone 10g/lt, NaCl 10g/lt and yeast extract 5g/lt). The conical flask was placed in a shaker water bath at $35^0$C for 90hrs until the colony forming unit (cfu/ml) was greater than $10^9$ and the bioactive ICPs (Insecticidal Crystal Proteins) was maximum when observed by light microscopy (Ammouneh *et al.*, 2011). The formulated biopesticide was used to control the Aphids.

### Rearing of aphids

For rearing of Aphid natural environment was used (Madanat, Hanna M, Antary Tawfiq M, Al, Zarqa, Musa,H, 2016). Native host *Phaseolus* spp plant was planted in the garden. Using compost as a fertilizer the

plant was allowed to grow till maturation producing lots of flowers and pods and it was allowed to infest by aphids naturally (Abderrahmane, 2015). The Aphid culture was maintained on *Phaseolus* spp plant during the study period. The plants were irrigated regularly and fertilized by using compost. No pesticides or any other chemicals were used during its growth (Photograph 2a).

**Bioassay**

The insecticidal property of the isolated *B. thuringiensis* against aphids was evaluated by two processes preliminary screening and selective bioassay.

**Preliminary screening for aphicidal activity in the field**

Preliminary screening was performed by foliar spraying the suspension containing the spore and the ICPs mixture (crude mixture) of randomly selected 4 isolates (ML5(1), CW1(1), SN2(1) and MP2(1)) (El-Kersh *et al.*, 2016) in *Phaseolus* species heavily infested with black aphids (*Aphis fabae*) in field. The aphicidal activity was observed in nymphs, instars, wingless, and winged aphids. The mixture (spores and ICPs) was formulated as mentioned above. Crude suspension 50ml was used as bio insecticide without dilution and sprayed by use of manual sprayer in a heavily infested plant part (Abderrahmane, 2015). The process was duplicated with each isolates. The sprayed part was observed for 4 days.

**Selective bioassay**

The second process was done by counting the number of aphids (nymphs, instar, winged, wingless) present on the leaf or on the beans/ pods surface before and after spraying. The spray suspension for selective bioassay was prepared by centrifuging the fermented broth at 5000rpm for 10minutes and the sediment was serially diluted to obtain $10^9$ cfu /ml in saline solution (to remove soluble exotoxins, bacterial cultures were centrifuged at 5000rmp) (Heidari and Zeinali, 2019). The $10^9$ cfu/ml concentration of biopesticides of 12 isolates of 5ml was spread onto the infested leaves and beans pods surface containing the counted aphids, the mortality percentage was calculated by counting the live aphids on the 4$^{th}$days and the result was recorded (Lobo *et al.*, 2018). The infested leaves were kept fresh till the 4$^{th}$ day by spraying water. Mortality was calculated by using the following formula.

$$Mortality\ P\% = \frac{Total\ No.of\ Aphids - No.of\ live\ Aphids}{Total\ No.of\ Aphids} \times 100\%$$

## RESULTS

***Bacillus thuringiensis* isolation and identification**

From the randomly collected 50 soil samples, 84 isolates of *Bacillus thuringiensis* (Bt) were obtained. The colony morphology revealed that 7 different types of colony morphologies were produced by the Bt strains Table 1. Fried egg type of colony was present in all the soil samples collected Photograph 1. The light microscopic morphology revealed that they were Gram positive, spore producing and their vegetative size varied. Presence of ICPs and their shapes was confirmed by Coomassie Brilliant Blue staining technique by light microscopy. Five different crystal morphology were observed: Amorphous (17.85%), Rod (53.57%), Spherical (10.71%), Ovoid (8.3%) and Cap headed (9.5%) Fig 1.

**Table 1: Types of Colony Morphology and the Number of Isolates**

| S.N. | Sample size | Type of colony Morphology | Colony code | No. of isolates | Percentage |
|---|---|---|---|---|---|
| 1 | | White, raised wavy (fried egg type) | A | 50 | 100% |
| 2 | | White, flat, irregular | B | 23 | 46% |
| 3 | | Yellow, raised, smooth | C | 1 | 2% |
| 4 | 50 | White, raised, round, smooth, mucoid | D | 1 | 2% |
| 5 | | Shiny(watery type),raised, round | E | 1 | 2% |
| 6 | | White rhizoid type | F | 1 | 2% |
| 7 | | White membraneouse slightly raised center | G | 7 | 14% |
| | Total | | | 84 | |

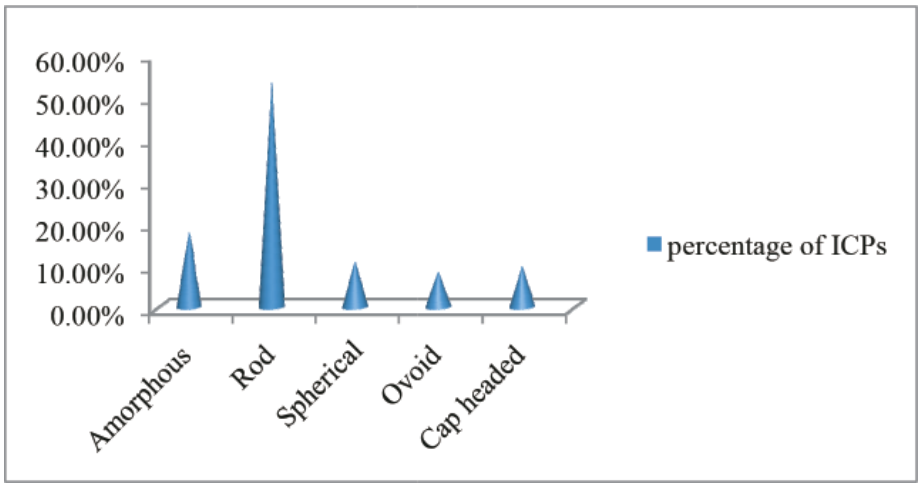


**Figure 1: Types of ICPs and their frequency.**

**Photograph 1: Isolates obtained from the sample ML5(1) by spread plate technique. Note only 2 colony morphology was observed in this sample, creamy white fried egg & flat pale yellow.**

The dominant type of ICPs was rod shaped. Based on the biochemical characteristics, all the isolates were positive for Catalase, Oxidase, Starch hydrolysis, Gelatin hydrolysis, beta - hemolysis, lecithinase and sucrose, fructose, mannitol, and lactose fermentation tests but showed variable reaction in Indole, MR, VP, Motility and Citrate tests.

**Bioassay**

**Preliminary screening:** Preliminary screening for aphicidal activity of the 4 isolates ML5(1), CW1(1), SN2(1) and MP2(1) of native *Bacillus thuringiensis* (Bt) revealed 100% mortality, against nymphs, instar, winged and wingless aphids. Isolates were effective in controlling the aphid within 4 days the plant part that was sprayed became free of aphids Photograph 2. These four isolates produced spherical shaped crystal protein.

(A) (B)

**Photograph 2: Preliminary screening of insecticidal property of native isolates a) heavily infested *Phaseolus* plant before spraying. b) *Phaseolus* plant free of *Aphis fabae* on 4th day.**

**Selective bioassay:** The mortality percentage of aphids after treatment (spraying 5ml of diluted pellets with 109cfu/ml with $10^9$cfu/ml of the suspension containing spores and the ICPs) on the leaf and pods surface was calculated on the 4th day as shown in Table: 2.

**Table 2: Effectiveness of each Bt isolates in terms of Aphid Mortality percentage**

| SN | Isolates | Types of crystal | Mortality % |
|---|---|---|---|
| 1 | P1(1) | Spherical | 56.00 |
| 2 | CW3(1) | Spherical | 48.14 |
| 3 | CW1(1) | Spherical | 70.21 |
| 4 | SN3(1) | Spherical | 72.72 |
| 5 | ML5(1) | Spherical | 77.34 |
| 6 | CW2(1) | Spherical | 85.71 |
| 7 | MP3(3) | Short rods | 95.83 |
| 8 | SN3(3) | Short rods | 43.80 |
| 9 | CW3(3) | Short rods | 67.64 |
| 10 | D2(2) | Long rod | 39.65 |
| 11 | CW2(2) | Long rod | 43.90 |
| 12 | D3(2) | Long rod | 42.15 |

Native isolate MP3(3) was most effective in killing 95.83% of aphids followed by CW2(1), 85.71%, ML5(1) 77.34%, SN3(1) 72.72%, CW1(1) 70.21%. In an average 40%, mortality rate was observed in other isolates.

## DISCUSSION

All the 50 soil samples processed for the isolation of Bt showed the presence of Bt strain and a total of 84 Bt isolates were obtained. The isolates were confirmed as Bt by detecting the crystal protein by Coomassie brilliant blue staining (CBBS) technique. Coomassie brilliant blue R-250 (CBB) is a popular dye used for detection of proteins. Crystal protein of Bt stained by CBB appear as dark blue color and the spore remain unstained and the vegetative cell took up the light blue stain as describe by (Rampersad et al 2002). In this study, the numbers of isolates are greater than the number of soil samples processed for isolation Table 1. These findings differ from earlier reports by (Ralte, Nachimuthu and Guruswami, 2016) who isolated 29 Bt isolates from 55 soil samples. This may be due to focusing on only one type of colony morphology either to fried egg or white glossy or rough creamy etc, neglecting other types of colony morphology which may also harbor crystal protein producing gene. In this research, Bt with diverse type of colony morphology was observed, and produced ICPs. The isolates produced 7 different types of colony morphology indicating the presence of

different strains of Bt (Table 1). The dominant colony type was fried egg, which was present in all the 50 soil samples (frequency 100%), followed by flat white type of colony (frequency 46%). In this research, enrichment was performed by acetate selection method, followed by isolation in NA (composition) as described by (Travers, Russells, Martin, Phyllis, and Reichelderfer, 1987).On an average, only 3 to 4 different colony morphologies developed in the agar media by spread plate technique (Photograph 1). All of which demonstrated the presence of ICPs by CBB staining. In case of others research the isolation technique as well as the media used is different, and they focus on particular colony morphology so the isolates are less in numbers. Other colony morphology, colony codes C, D, E, F and G are less commonly isolated during this research. This may be due to, the isolates have gained the plasmid that carry cry gene from the prevalent strains and appear as positive Bt strain (Table 1). As the ICPs producing gene is located in the plasmid and in the chromosome (Reyaz *et al.*, 2013). The plasmid might have been transmitted to the same type of strain by conjugation or the selection process, temperature or media used may have been favorable for the growth of such types of isolates. Diversity, distribution and abundance of *cry* gene types are dependent on the geographical area where *B. thuringiensis* strains were collected as well as the cultural condition provided may enhance in the isolation of organism with different ICPs producing isolates (Tuba, 2002). Even though the colony morphology remained same, some isolates produced different shapes of ICPs indicating that different *cry* gene may present and expressed in a particular environment. The plasmid may harbor different types of genes in a bacterial cell or different plasmid harbors different genes. More than one plasmid is present in Bt. The plasmids may contain different *cry* gene responsible for producing different types of crystal protein (Rangeshwaran *et al.*, 2014).

Randomly selected 4 isolates (ML5(1), CW1(1), MP2(1) and SN2(1)) producing spherical shaped ICPs were used for screening aphicidal activity by preliminary bioassay against nymphs, instar, winged and wingless Aphids by spraying about 50ml mixture containing endospore count >$10^9$ cfu/ml and ICPs, on a heavily infested part of the plant (Photograph 2) within 4 days cent percent mortality was observed on *Aphis fabae.* The result was consistent with (Palma *et al.*, 2014) cry protein (cry2A, cry3A, cry11A) of Bt show toxicity against hemiptera. So four isolates producing spherical ICPs harbors either of these cry protein producing genes and thus are able to show 100% mortality against *Aphis fabae.* In this research, the concentration of ICPs was not determined but presence of excess ICPs i.e the crystal shapes was observed by light microscopy and the spore count present in the broth was □$10^9$cfu/ml as determined by endospores count method in NA. Aphids take up the nutrients for their survival by suckling the sap from the plants. When the aphicidal mixture (spore and ICPs) is present in higher dose on the surface of the leaves and on stems, the mixture is also absorbed by the plant tissues. While suckling the nutrients, the Aphids consumes the aphicidal mixture along with the nutrients causing toxicity to Aphids. According to (Elatti *et al.*, 2010) toxins should be circulated throughout the phloem to control suckling insects like Aphids., Thus it ensures effective control of aphids. Thus the study suggests high dose and excess volume of aphicidal concentration (biomass, Bt spore and the ICPs) must be used to control Aphid pests in the field. In this bioassay, bacterial culture suspension as a whole was used as an insecticides, whole of the bacterial culture suspensions may contain in addition to cry and cyt protein other secreted toxins like vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip)(Schnepf *et al.*, 1998) that are secreted into the medium and have insecticidal properties against aphids. The part of phaseolus plant which was heavily infested by Aphids was observed to have dried out after their mortality. The sprayed biopesticide mixture was sucked by the aphids, after ingestion of the of mixture, Aphids got paralyzed, were not able to move to other parts of plants for feeding, so the infested part dried out and the aphids were turned to black powder on the surface of infected plant part. The infested plant part dried out because of excess sucking of sap by the paralyzed aphid. According to the observation of this study, crude extract, mixture of proteins (cry, cyt, Vip, sip) present in the suspension kills aphids within 4 days but spraying only once does not make the plants free of aphids for the whole season. So repeated spraying in the infested parts should be carried out. For effective control of Aphids Bt gene should be incorporated in the plant so the plant itself can synthesize the ICPs to control the Aphids it would be much better to incorporate a cry gene showing abroad spectrum of activity towards insect pests. Selective bioassay performed by using 12 native isolates showed a different mortality rate (Table

2). The most effective native isolate MP3(3) showed 95.83% of aphicidal activity followed by CW2(1), 85.71%. In an average, 12 isolates were effective in controlling the aphids. Based on the toxicity test, the type of *cry* gene present in the isolates may be the *cry1I, cry2, cry3, cry10* and c*ry11*. According to Ibrahim et al (2010) based on the insecticidal toxicity test the Bt are categorized as Group 1—lepidopteran (*cry1, cry9* and *cry15*); Group 2—lepidopteran and dipteran (*cry2*); Group 3—coleopteran (*cry3, cry7* and *cry8*); Group 4—dipteran (*cry4, cry10, cry11, cry16, cry17, cry19* and *cry20*); Group 5—lepidopteran and coleopteran (*cry1I*); and Group 6—nematodes (*cry6*). The *cry1I, cry2, cry3, cry10* and *cry11* toxins (73–82 kDa) are unique because they appear to be natural truncations of the larger Cry1 and Cry4 proteins (130–140 kDa). *Aphis fabae* belong to Order Hemiptera, the native isolates showing insecticidal activity towards this Aphids may contain *cry1I, cry2, cry3, cry10* and *cry11* or other types of genes.

Less effectiveness by selective bioassay may be due to the absence of other exotoxins like (Vip), (Sip) which are removed by centrifugation. So only the ICPs or cry proteins is less effective in killing the Aphids.

Based on the morphology of ICPs, the indigenous Bt stains can be related to the type of *cry* gene present in it. Spherical and ovoid related to *cry1*or *cry3* or *cry8* or *cry9*,(Tuba 2002; Zonthansanga et al 2016). Rod shaped ICPs may be related to rectangular type, that relates to *cry1* gene (Tuba, 2002). The reference strain used during the study *Bacillus thuringiensis* var *Kurstaki*, serotype 3a, 3b, 3c, Strain DOR Bt-1 also produced spherical type of crystal protein (Cry1).

## CONCLUSION

This study reveals that indigenous *Bacillus thuringiensis* isolated from soil of Terai region of Nepal exists in variable diversity. The native Bt isolates with spherical and rod shaped ICPs are effective in controlling Aphids.

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# Bacteriological Profile and Antibiotic Susceptibility Pattern of Isolates of Wound Infection In Children Visiting Kanti Children Hospital

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## ABSTRACT

**Objectives**: The objectives of this study was to isolate and identify the bacterial etiological agent of wound infection and explore the status of methicillin-resistant *Staphylococcus aureus* ( MRSA), multidrug Resistant (MDR) and extended spectrum β-lactamase (ESBL) producers' strains in clinical specimens and to find the antibiotic susceptibility pattern.

**Methods**: A prospective cross sectional study design was conducted from period of February 2014 to October 2014 at Kanti Children Hospital, Kathmandu. The organisms were isolated and identified from pus sample by standard microbiological methods. Antimicrobial susceptibility test was performed by modified the Kirby Bauer disc diffusion method to evaluate the status of MRSA and MDR. ESBL detection was performed by the combined disc diffusion method.

**Results**: Out of 365 specimens collected between the age group below < 2 to 15 years, 210 (57.73%) samples from male patients and 155 (42.47%) from female patients. In the total samples processed, Gram-positive organisms were found to be more prevalent in which *Staphylococcus aureus* accounts for 135(47.20%), followed by *P. aeruginosa 62 (21.67%), E. coli 29 (10.20%), K. pneumoniae* 27 (9.44%), *Acinetobacter* spp. 20 (6.70%), *P. vulgaris* 7 (2.44%) and CoNS 6 (2.10%). Among the *S. aureus* isolates, 29 (21.48%) were found MRSA. Of the total Gram-negative organisms isolated, 74 (51.03%) were MDR and 14 (100%) ESBL producer, (P<0.01). *S. aureus* was found to be the most important and leading cause of wound infection in this study.

**Conclusion**: Thus, routine antibiotic susceptibility testing is recommended for empirical drug therapy and proper management of disease.

**Key words**: Bacteriological profile, MRSA, MDR, ESBL, wound infection.

## INTRODUCTION

Wound infection is becoming a serious burden in surgical procedure hospitals. The prevalence of wound infections has been reported to be 40% in developing countries. The emergence of resistant strains has increased the morbidity and mortality associated with wound infections (Goswami et al. 2011; Kassam et al. 2017)

Wounds break the continuity of the skin and allow the organism to gain access to the tissues. The outcome of wound infection depends on the interaction of complex host and microbial factors (Shittu et al. 2003). According to Bryan (1983), a wound can be considered infected if purulent materials drain from it even if a culture is found negative.

The emergence of resistant strains has increased the morbidity and mortality associated with wound infections (Goswami et al. 2011**).** The predominant isolates of wound bacteria are multidrug-resistant nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and enteric bacteria such as extended spectrum β-lactamase (ESBL) producing *E. coli* and *Klebsiella* spp. Of the various research

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conducted, the most prevalence organism involve in wound infections are *S. aureus*, *Pseudomonas* spp, *E. coli*. (Zafar et al. 2008**).**

The previous studied stated that the incidence of postoperative wound infection in children varies significantly, from 1.6 to 18.7%, and even up to 27% for contaminated operations and up to 30%–40% for dirty-infected operations (Varik et al. 2011). The risk of surgical wound infection is based on the susceptibility of a surgical wound to microbial contamination (Raahave et al. 1986). The rate of surgical wound infection was found to be 4% in Tribhuwan University Teaching Hospital, Nepal (Banjara et al. 2002). Similarly, in case of post-operative cesarean wound infection rate in Patan Hospital was found to be 2.6% (Pandit et al. 2002). *S. aureus* is responsible for large variety of infections and predominates in surgical wound infections with prevalence rate ranging from 4.6% - 54.4% (Batabyal et al 2012). Overall prevalence of MRSA in clinical samples of hospitals located in Kathmandu Valley, Nepal was 62% (Mukhiya et al. 2013).

This study focused to find the pattern of bacterial isolates from infected wounds and their antimicrobial susceptibility pattern. Therefore, the acquired data of the causative agents of infected wounds from this study would be helpful in the selection of empiric antimicrobial therapy and infection control measures. The other objective of this study was to isolate bacteria from infected wounds from different wards of the hospital.

Hence, this study gives the comprehensible image of current scenario of wound infection and their causative microorganisms in the study site. Moreover, antibiotic susceptibility testing will help understand the current susceptibility pattern of isolates toward the commonly used antibiotics and the corrects choice of antibiotics with respect to causative agents.

## MATERIALS AND METHODS

**Study design:** A Prospective hospital based cross sectional study design was used.

**Sample size**: A total of 365 pus sample were collected from clinically defined children being affected by the wound infection.

**Sampling methodology:** Stratified a random sampling method was used for collecting data and pus sample.

**Population:** Populations for the study were patients visiting Kanti Children Hospital. The target patients were younger children up to 15 years of age with clinically diagnosed bacterial wound infection**.**

**Ethical consent:** Ethical approval was obtained from the Institutional Review Committee (IRC), Kanti Children Hospital, Maharajgunj, Kathmandu. After briefly informing the participant about the objectives of the study, verbal and written consent were also taken from all participants.

**Sample collection:** Wound samples were collected using sterile cotton swabs (fresh pus) but small screw-capped bottle a firmly stopper tube or syringe or a sealed capillary tube, and the patients name, age and gender were clearly written (Koneman et al. 2005). The sample was taken to the laboratory for further analysis without any delay. In case of delay, the samples were refrigerated at 4°C.

**Macroscopic examination:** Macroscopic examination of the collected pus samples was conducted by observing its color and appearance and reported accordingly. However, data of macroscopic examination were not included in this study.

**Culture:** The samples were inoculated on Blood agar and MacConkey agar and incubated aerobically at $37^{O}$C for 24hrs. Positive growth was identified by Gram staining, colony characteristics, hemolytic pattern in BA and standard biochemical tests. (Koneman et al. 2005; Cheesbrough 2000).

**Identification of isolates:** The isolated colony from plates showing positive growth was further preceded for identification. Plate showing no growth, mixed growth and bacterial growth of insignificant number was excluded from the study. The isolates were identified by standard microbiological methods as described by Collee et al. (1999). A single distinct colony from BA, MA for both Gram-negative and Gram-positive bacteria was picked up and inoculated on NA. It was incubated at 37°C for 24 hrs. After overnight incubation, the culture was used to perform biochemical tests and antibiotic susceptibility test.

**Antibiotics susceptibility test:** Antimicrobial susceptibility testing of the pus isolates were done by modified the Kirby-Bauer M2-A9 disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI 2014) using Mueller Hinton agar (MHA). For *Staphylococcus* MHA incorporated

with 0.2% NaCl was used.

**Screening of Methicillin-Resistant S. *aureus* (MRSA):** Screening of MRSA was based on the disc diffusion method using cefoxitin (30mcg). The diameter of the zone of inhibition of ≤ 21 mm was considered as methicillin resistant (MRSA), whereas diameter of the zone of inhibition ≥ 22 mm was considered as methicillin susceptible (MSSA) (CDC 2014) .

**Screening of MDR:** The organisms' showing resistant to more than three classes of antibiotics were taken as Multi-drug resistant isolates (Magiorakos et al. 2010 and CDC 2006). In this study, four classes of antibiotics were chosen.

**Detection of ESBLs**

**Screening of ESBL producers:** Bacterial isolates were first tested with at least one of the five antibiotics i.e., Ceftriaxone (30 µg) according to the CLSI screening criteria. The isolates were suspected to produce ESBL if the zone of inhibition was ≤25 mm. Ceftriaxone (30 µg) was included in the initial line of AST for screening.

**Confirmation of ESBLs**

**The phenotypic combination disc method:** The suspected ESBL isolates were tested for confirmation by using the Combination Disc Method, using Cefotaxime (10 µg) and cefotaxime (10 µg) plus clavulanate (1 µg), Cefotaxime (30 µg) and Cefotaxime (30 µg) plus clavulanate (10 µg) and ceftazidime (30 µg) and ceftazidime (30 µg) plus clavulanate (10 µg). An increase in zone diameter of ≥ 5 mm in the presence of clavulanate from any or all of the set was confirmed as ESBL producers.

**Quality control:** The quality of each agar plate prepared was maintained by incubating one plate of each batch in the incubator. Control strains of *E. coli* ATCC 25922 and *S. aureus* 25923 were used for the identification test and for the standardization of Kirby-Bauer test and for correct interpretation of the diameter of inhibition zones. The quality of sensitivity test was maintained by maintaining the thickness of MHA to 4 mm and the pH of 7.2-7.4.

Purity plate for each biochemical test was maintained to ensure pure culture, inocula used and to assess that the biochemical tests were undertaken in aseptic conditions.

**Statistical analysis:** All the data obtained were statistically analyzed using Statistical Package for Social Science (SPSS) version 17 software packages. The chi-square, one way ANOVA was used according to should determine a significant association between different factors for the causation of wound infections.

## RESULTS

Out of 365 pus sample collected and processed, where 38 (10.42%) samples were from surgical wound infection patients, 115 (31.50%) from burn ward and 212 (58.08%) from OPD.

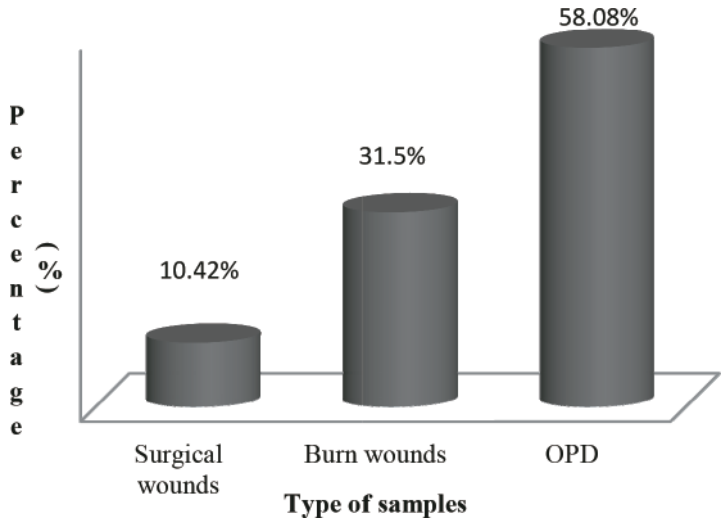


**Figure 1: Types and distributions of Samples**

Among the total pus sample, 248 (67.95%) were collected by pus aspirates and 117 (32.05%) pus by swabs. Among the collected pus samples, 210 (57.73%) were from male patients and 155 (42.47%) from female patients.

The maximum 144 (39.45%) pus samples were collected from patients of the age group of 2-5years, (male= 76 and female= 68), followed by the age group <2 years with 136 (37.26%) samples (male = 84 and female= 52) and the least samples were 15 (4.11) from the age group of more than 10.

Among the 365 samples collected, 286 (78.25%) samples showed growth and 94 (21.64%) did not detect any growth. Similarly, Out of 286 isolates, Gram-negative bacteria were predominating constituting 145 (50.70%) and Gram-positive bacteria constituted 141 (49.30%). Among total bacterial isolates, S *aureus* was predominant species with 135 (47.20%), followed by *P. aeruginosa 62 (21.67%), E. coli 29 (10.20%), K. pneumoniae* 27 (9.44%), *Acinetobacter* spp. 20 (6.70%), *P. vulgaris* 7 (2.44%) and CONS 6 (2.10%) in number.

**Table 1: Types of bacterial isolates in wound specimens**

| S.N. | Name of Organisms | No. (%) |
|---|---|---|
| 1 | *Acinetobacter* spp. | 20 (6.70) |
| 2 | *Escherichia coli* | 29 (10.20) |
| 3 | *Klebsiella pneumoniae* | 27 (9.44) |
| 4 | *Proteus vulgaris* | 7 (2.44) |
| 5 | *Pseudomonas aeruginosa* | 62 (21.67) |
| 6 | CoNS | 6 (2.10) |
| 7 | *Staphylococcus aureus* | 135 (47.20) |
| | **Total** | **286 (100)** |

Among 145 Gram-negative bacteria, *P. aeruginosa* accounted for 62 (42.76%) followed by *K. pneumoniae* 27 (18.62%), *E. coli* 29 *(13.79), Acinetobacter* spp. 20 (13.79%), *P. vulgaris* 7 (4.83%).

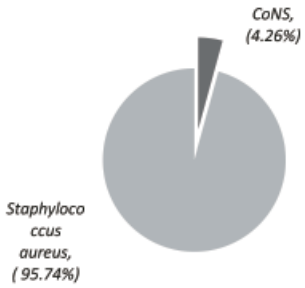


**Figure 2: Types of gram–positive bacteria isolates in wound specimens**

Similarly, among 141 Gram-positive bacteria isolates, *S. aureus* was present in the highest number i.e. 135 (95.74%) followed by CONS i.e. 6 (4.26%).

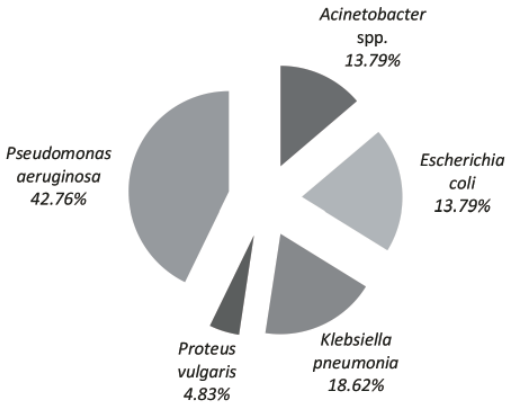


**Figure 3: Types of Gram negative bacterial isolates in wound specimens**

**Antibiotic sensitivity pattern of Isolates**

Piperacillin and Tazobactam were the most effective drug showed sensitivity 85% followed by Chloramphenicol (75%) whereas Cotrimoxazole, Amoxiclave was less effective against *Acinetobacter* spp.

Similarly, ciprofloxacin, Gentamycin and amoxicillin and Chloramphenicol were the most effective drug of choice and Penicillin was the least effective toward CONS isolates.

However, Cefixime, Nitrofurantoin, Amoxiclave and Nalidixic acid was the most effective for *E. coli* and least was Amikacin.

Nalidixic acids, Cefixime, Norfloxacin were effective antibiotics and Amoxiclave was least effective antibiotics of choice for *K. pneumoniae.*

Similarly, Chloramphenicol (100%), Piperacillin (71.43%), Ciprofloxacin (71.43%), Nitrofurantoin (71.43%) were the most effective antibiotics for *Proteus vulgaris* and Norfloxacin was least effective. The sensitivity pattern was statistically found significant with $p<0.01$.

Piperacillin and Tazobactam (82.26%), Clindamycin (87.10%), Amikacin and Imipenem (76.74%) was the most effective antibiotics, whereas, Ceftazidime and

Gentamycin was least effective for *P. aeruginosa*. The sensitivity pattern was statistically found significant with $p<0.001$.

Likewise, Cotrimoxazole (83.70%), Amikacin (74.81%) was the most effective antibiotics and Penicillin (20%) was least effective antibiotics for *S. aureus* ($p<0.001$).

**MRSA DISTRIBUTIONS**

Among *S. aureus* 135(47.20%) isolates, 106 (78.52%) were Methicillin-Sensitive S. *aureus* (MSSA) and 29 (21.48%) were Methicillin-Resistant S. *aureus* (MRSA).

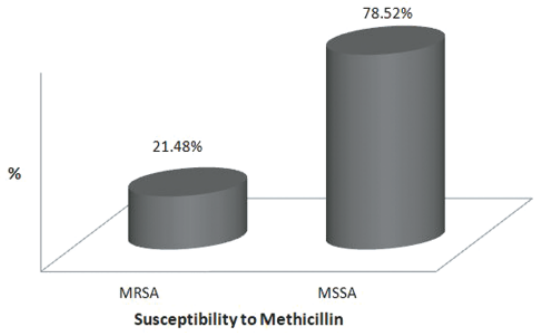


**Figure 4: Distribution of MRSA and MSSA among *S. aureus* isolates**

**Distribution of MRSA based on the Age group, Gender wise, patient type, Department wise:**

The highest rate of infection by MRSA isolates was found to be 13 (44.8%) in the age group 2-5 years, followed by age group <2 years 9 (31.08%), 10-15 years 6 (20.69%), 5-10 years 1 (3.45%) respectively. The rate of MRSA infection was found to be highest among pediatrics than in adults.

Similarly, the rate of infection due to MRSA was higher in males 22 (65.52%) than in females 10 (34.48%).

Moreover, inpatient 17 (58.62%) were more susceptible by MRSA than outpatient 65 (61.32%)

Similarly, among the 29 (21.48%) MRSA isolates, the highest MRSA isolates were from 16 (55.17%) OPD and lowest isolates were 6 (20.69%) from Surgical ward.

**MDR DISTRIBUTION**

**The distribution of MDR among Gram-negative isolates:**

Among the 286 culture positive isolates, 145 were Gram-negative organisms. In total Gram-negative isolates, 74 (51.03%) were multidrug resistant (MDR).

MDR isolates were found to be most prevalent among the age group below 1 years 30 (40.45%), followed by age group 2-5 years 27 (36.49%), 6-10 years 16 (21.61%), 11-15years 1(1.35%), respectively.

Similarly, Gram-negative MDR isolates were found to be most prevalent in females 32(43.24%) than in male patients 42 (56.76%).

Likewise, the distribution of MDR was high in the Outpatient 32 (43.24%) than Inpatient 42 (56.76%).

Among 74 MDR isolates, the highest distribution was found to be from OPD 37 (50.00%), followed by burn ward 30 (40.54%) and least from surgical ward 7(9.46%).

Out of 145 gram negatives isolates, MDR isolates accounted for 74 (51.03%) in which a higher number of *Pseudomonas aeruginosa* isolates, 23 (31.08%) were multidrug resistant, followed by *Escherichia coli* 22 (29.73%) and *K. pneumoniae* 18 (24.32%), *Acinetobacter* spp. (9.46%) and *Proteus vulgaris* 4(5.41%)

**Distributions of ESBL Producers**

Among the isolates, 72 were ESBL suspected, in which14 (19.44%) were found to be ESBL positive and 58 (80.56%) were found to be negative by confirmatory tests. In confirmation test, two combination

Disks were used, namely the ceftazidime clavulanate and Cefotaxime-clavulanate Among the 14 ESBL positive isolates, most predominant was found to be *E. coli* with 8/19 (42.11%) followed by *K. Pneumonia*, *P. aeruginosa* and *Proteus vulgaris* (P<0.01).

**Table 2: ESBL production from various isolates**

| Organism | No. Of ESBL suspected | ESBL confirmatory Test | | | |
|---|---|---|---|---|---|
| | | Positive | % | Negative | % |
| ***Acenetobacter* spp.** | 7 | 1 | 14.29 | 6 | 85.71 |
| ***E. coli*** | 19 | 8 | 42.11 | 11 | 57.89 |
| ***Proteus vulgaris*** | 2 | 0 | 0 | 2 | 100 |
| ***K. pneumoniae*** | 14 | 3 | 21.43 | 11 | 78.57 |
| ***P. aeruginosa*** | 30 | 2 | 6.67 | 28 | 93.33 |
| **Total** | **72** | **14** | **19.44** | **58** | **80.56** |

**Table 3: Pattern of ESBL production using 2 combination disks**

| Combination disk used | Criteria for confirmation | No. of suspected ESBL producer | No. of confirmed cases | Total confirmed cases | Negative after confirmation |
|---|---|---|---|---|---|
| **CTX: CTX+CV** | Increase in zone size of ≥ 5mm with ≥ 1 combination disks | 72 | 12 | 14 | 58 |
| **CAZ, CAZ+CV** | | | 14 | | |

Note: CPD-Cefpodoxime, CAZ-Ceftazidime, CTX-Cefotaxime, CV-Clavulanate

## DISCUSSION

In this study, out of 365 wound samples collected, 286 (78.25%) were found to be culture positive and only 94 (21.64%) showed no growth.

This study is comparable to the study by Nepal et al. (2008) and Shrestha (2009) in which 80.6% and 72.4% showed bacterial growth. This might be due to similar culture medium used and growth environment condition for isolation of organisms.

In this study, the higher number of growth was observed in in-patients 17 (58.62%) than in out-patients. This may be due to weak in immune status after wound infection and acquirement of nosocomial infection facilitates the wound infection. Similar results reported by Mahat et al. (2017) the higher number of growth positive cases in in-patients

The occurrence of higher number of male patients 57.73% than female patients from the collected of pus samples may be due to the involvement of higher outdoor activities like playing as compared to females and more chances of getting accidents during outdoor activities. This finding was supported by similar type of study conducted by Mahat et al. (2017) and KC et al (2013).

In this study, Gram-negative rods (50.70%) were the predominant and leading cause of wound infections. In similar study conducted by Yakha et al. (2014) and Mahat et al. (2017) Gram negative bacteria were found predominant. Gram negative bacteria isolation was higher, as they are more common aerobes and facultative anaerobes in abscesses and skin wound. Gram negative bacteria increases in the cases of Hospital acquired infections (HAIs). This might be due to high resistances to antibiotics showed by Gram negative bacteria compared to Gram-positive isolates, and therefore their persistence in infected wounds. Furthermore, chronic wounds were infected by multiple Gram-negative rods. The multiple bacterial infections in this case might be due to impaired immune responses. But Maharjan et al (2020) Khanam et al.(2018) and Pandey et al. (2017) showed high isolation rate of gram positive bacteria.

In this study, *S. aureus* accounted for 135 (47.20%) followed by *P. aeruginosa 62 (21.67%), E. coli 29 (10.20%), K. pneumoniae* 27 (9.44%), *Acinetobacter* spp. 20 (6.70%),

*P. vulgaris* 7 (2.44%) and CONS 6 (2.10%).

*S. aureus* was the leading isolates of wound infection. High rate of isolation may be due to its presence of normal flora nasal cavity and skin of the individuals and unhygienic behavior like contact of wound site with the hand contaminated with the nasal discharge may be the possible reason. Similar type of results was found by Maharjan et al. (2020). Nazeer et al. (2014) found similar results in which the most predominant isolate from a wound infection was *Staphylococcus aureus* (37.12%) followed by *Klebsiella* species (20.45%).

The study reported by Karkee (2008) also supported the finding of this study in which the most common bacterial isolates (46.58%) were *S. aureus. E. coli* (12.38%) emerged as the next common organism causing wound infection followed by CONS (11.40%) and *P. aeruginosa* (7.49%). The least common bacteria isolated were *C. freundii* (0.65%). In Saudi Arabia, Abussaud (1996) isolated *S. aureus* (35%), *P. aeruginosa* (25%) and *Klebsiella* spp. (10%) as the major causative agents.

However, different studies have shown *P. aeruginosa* as the leading cause of burn wound infections. A study by Mousa (1997) to assess the rate of burn wound infection by aerobic bacteria and found that 19.1% of the wound infection was caused by *P. aeruginosa*. Similar studies on burn wound infection by Nasser et al. (2003) showed that *P. aeruginosa* (21.6%) as the most common isolate. This result supports the findings of this study in which the prevalence rate of *P. aeruginosa* was found to be 62 (21.67%). In contrast to this finding, studies carried out by Ranjan et al. (2011) and Mahat et al. (2017) also showed *Pseudomonas spp.* was the most prevalent bacteria among the total cases with 27.8% and 29.6% and 34.55% respectively. This might be due to chances of nosocomial infection by S. *aueus and P. aeroginosa* both in in-patients and out-patients

In a study by Shah et al. (1997) at TU Teaching Hospital, 11.43% of CONS was reported from pus sample, which was far-away to this finding. Shampa et al. (2006), in their study to determine the prevalence of *P. aeruginosa* and its antimicrobial sensitivity pattern found 32% prevalence rate of *P. aeruginosa* of all pathogens isolated. This study agreed with the study done in Africa by Oguntibeja et al. (2004) where 33% of the isolates were *P. aeruginosa* in post-operative wound infections. Similar results were found in the study by Anbumani et al. (2006). Giacometti et al. (2000) studied microbiology of wound infection in Italy and found that the prevalence of *S. aureus* (28.2%), *P. aeruginosa* (25.2%), *E. coli* (7.8%), which gave a different result than our study.

Cotrimoxazole, Amikacin, Ciprofloxacin, Gentamycin, Amoxycillin were effective drug of choice for Gram-positive. In contrast to this study, Shrestha (2009) found Chloramphenicol as the most effective drug with susceptibility (85.58%) followed by ceftriaxone (77.88%), Amikacin (72.12%) and Ofloxacin (67.13%) against Gram-positive isolates least was found Cloxacillin (20.19%) and Cotrimoxazole (40.38%). In this study, Chloramphenicol, Piperacillin, Nitrofurantoin, Amoxiclave and Nalidixic acid were found to be the most effective drug for treatment for Gram-negative isolates. In contrast to this study, Shrestha (2009) reported that against Gram-negative isolates from a wound infection, Amikacin was found to be the most effective drug (55.84%) followed by Ofloxacin (51.95%), Ciprofloxacin (45.45%) and Ceftriaxone (40.26%) and the least effective was found to be Cloxacillin (16.88%). Similarly, sensitivity pattern of Gram-negative bacteria to Amikacin observed in this study was similar to study by Agnihotri et al. (2004), in which Amikacin was found to be the most effective antibiotic for Gram-negative bacteria.

In this study, among 286 culture positive isolates, 135(47.20%) were *S. aureus* and 29 (21.48%) were Methicillin-Resistant *S. aureus* (MRSA). This finding in this study was supported by research conducted by Kshetry (2014) in which of the 149 culture positive isolates, 83(55.7%) were *S. aureus*. Out of total *S. aureus* isolates, 27(32.5%) were MRSA.

Among 286 culture positive isolates, 145 were Gram-negative organisms in which 74 (51.03%) were multidrug resistant (MDR). The rate of MDR was found to be higher 30 (40.45%) in the age group < 2 years. Higher number 23 (31.08%) of MDR was found to be *Pseudomonas aeruginosa* followed by *Escherichia coli* 22 (29.73%) and *K. pneumoniae* 18 (24.32%), *Acinetobacter* spp. (9.46%) and *Proteus vulgaris* 4(5.41%). Outcome of prevalence of MDR depends on various factors, MDR criterion being the chief one followed by the types of antibiotics used in antibiogram and study population.

Bhandari (2016) reported similar results in which 138 isolates were evaluated, MDR isolates accounted for 100/138 (72.45%). A relatively lower prevalence of

MDR, the prevalence of 56.09% and 35.2% were found in the study by Tuladhar et al. (2003), whereas 80% MDR has been reported in the study by Menon et al. (2006) and 81.1% MDR has been reported in the study carried by Waikhan and Devis (2012). Similarly, in contrast to this study , Bhandari (2014) Pokhrel et al. (2006) reported that the high number of *E. coli* isolates 64 (72.7%) as multidrug resistant followed by *Pseudomonas aeruginosa* 11 (91.7%) and *K. pneumoniae* 6(75%). Outcome of prevalence of MDR depends on various factors, MDR criterion being the chief one followed by the types of antibiotics used in antibiogram and study population. The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used (Levy, 1991).

Among the 14 ESBL positive isolates, the majority consist *of E. coli* with 8/19 (42.11%) followed by *K. Pneumonia*, *P. aeruginosa* and *Proteus vulgaris*. Of the total primary screened *E. coli* with 8/19 (42.11%) were ESBL positive.

This study was supported by Bhandari (2016), Out of 68 screened positive samples, 44 (64.7%) were found to be ESBL positive isolates. A similar result was obtained in the study by Poudyal (2010), which showed 62.7% positive after confirmation test. *E. coli* 34/44 (77.3%) was found to be most predominant ESBL positive isolates followed by *K. pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter* spp each with 2/44 (4.5%) isolates. The pattern of *E. coli* to be the most predominant ESBL positive isolates was found in various other studies conducted by Poudyal (2010) and Baral (2011).

We observed 14 (19.44%) of ESBL among total 72 isolates. A previous study by Ashrafian et al. (2012), Srisangkaew, and Vorachit (2003) found 32.7% and 40% ESBL, respectively, whereas various other similar studies such as research performed by Singh (2013) observed 49% ESBL producers, Rijal (2010) observed 28.88% and Pokhrel et al. (2006) observed 16% ESBL producers; however, the global prevalence of ESBL producing organisms presently varies from <1%-74% (Thokar et al. 2010). The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. These differences may be due to geographical variations, local antibiotic prescribing habits (Pokhrel et al. 2006).

## CONCLUSIONS

*S. aureus* was the most important and leading cause of wound infection that was the organism of emphasis of this study. Antibiotic susceptibility testing of all isolates was performed. Antibiotic susceptibility testing of all isolates showed that Piperacillin, Nitrofurantoin, Amoxyclav and Nalidixic acid, imipenem was the most effective drugs for Gram-negative bacteria and vancomycin, Cotrimoxazole, Amikacin, Ciprofloxacin, Gentamycin, Amoxycillin and chloramphenicol was the most effective drug for Gram-positive organisms. In case of Gram-negative isolates MDR pattern observed which accounts for 74/145 (51.03%). Similarly, ESBL was primarily screened by Ceftriaxone/Ceftazidime (CAZ) and confirmation by the combined disk method, confirmed ESBL producers were found to be 14 (19.44%). *E. coli* is leading ESBL Isolates. Thus, routine antibiotic susceptibility testing is recommended for empirical drug therapy.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Characterization of β-Galactosidase from Lactose Utilizing Yeast Isolated from the Diary Sample

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## ABSTRACT

**Objectives:** The objective of the study was to isolate lactose positive yeasts from dairy samples collected from local markets of Kathmandu, to extract crude β-galactosidase from the lactose positive yeast and to characterize the enzyme for optimum time duration, pH, temperature, Michaelis-Menten constant ($K_m$) and maximum activity ($V_{max}$).

**Methods:** Four lactose positive yeast strains were isolated from dairy samples collected from local market of Kathmandu by pour plate method. Single strain having maximum lactose positive activity was selected for the study. The mass culture of the lactose positive yeast strain was lysed by 2% Chloroform and the yeast cell lysate containing β-galactosidase (i.e. crude enzyme extract) was characterized by using ONPG (Ortho-Nitrophenyl-β-D-galactopyranoside) as substrate. ONPG is a colorless substrate for the enzyme assay which is hydrolyzed by the enzyme into yellow colored product ONP (Ortho-Nitrophenol). The concentration of product formed was monitored spectrophotometrically at 420 nm to determine the enzyme activity and to characterize the enzyme.

**Results:** The enzyme had wide range of working temperature from 0-50°C, with optimal temperature of 37°C. However, greater than 50% hydrolyzing ability was maintained in the range of 14-40°C. Optimum time of reaction was 70 min. The enzyme had maximum activity in the near neutral pH of 6.8. Michaelis-Menten constant of the enzyme was found to be 2.23 mM of ONPG and $V_{max}$ was 58.82 nmol/min/ml. Enzyme activity was 27.88 nmol/min/ml, Specific enzyme activity was 59.97 nmol/min/mg and total enzyme activity was 3346.33 nmol/min.

**Conclusion:** The activity over a wide range of temperature 0-50°C with low $K_m$ value shows that the enzyme has a commercial application in clearance of lactose pollution in waste water in different environmental conditions.

**Keywords:** Yeast, β-galactosidase, enzyme activity, ONPG, Km

## INTRODUCTION

β-galactosidase (E.C.3.2.2.1.23), also called beta-gal or β-gal, is a hydrolase enzyme that catalyses hydrolysis of beta-galactosides into monosaccharides. It is an exo-glycosidase which hydrolyses the β-glycosidic bond formed between a galactose and its organic moiety. Substrates for different β-galactosidases include ganglioside GM1, lactosylceramides, lactose and various glycoproteins. It may also cleave fucosides and arabinosides with much lower efficiency. β -galactosidase catalyzes the hydrolysis of lactose, abundant disaccharide in milk to monosaccharides; glucose and galactose (Husain, 2010). β-galactosidase hydrolyses the β-1,4-D-galactosidic linkage of lactose, as well as those of related chromogens, O-nitro-phenyl-β-D-galactopyranoside (oNPG), p-nitrophenyl-β-D

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galactopyranoside (PNPG) and 6-bromo-2-naphthyl-galacto pyranoside (BNG) (Mahoney, 1998).

Glycoside hydrolases ($GH_s$) including β-galactosidase constitute 113 protein families (Cantarel et al., 2009). Lactose degrading β-galactosidases (lactases) are classified as GH2 family and others are classified as GH42 family which shows activity towards β-galactosyl linkages in oligosaccharides (Hinz et al., 2004; Shipkowski et., 2006). Lactase is often confused as an alternative name for β-galactosidase, but it is actually simply a sub-class of β-galactosidase.

Some β-galactosidases may also have an activity of transferring one or more, D-galactosyl units onto lactose (Rabiu et al., 2001). This enzymatic property is called transgalactosylation which is potential for the production of GOS (Galacto-oligosaccharides) (Miller and Whistler, 2000).

β-galactosidase is an important enzyme in food, pharmaceutical industries and also in waste management. β-galactosidase can be used to avoid problems of lactose intolerance in individuals who are deficient in lactase. Lactose intolerance is a common problem prevalent in more than half of the world's population (Amir and Whorwell, 2009; Johnson et al., 1993; Suarez and Savaiano, 1997). High lactose content in non-fermented, sweetened, condensed and frozen dairy products such as ice-cream and condensed milk can lead to excessive lactose crystallization resulting in products with mealy, sandy or gritty texture (El-Kader et al., 2012). Thus β-galactosidase can be used to avoid the crystallization of lactose in sweetened, condensed and frozen dairy products. This helps to improve sweetness and to increase the solubility of milk products (Kara, 2004). It can be used to manage biodegradable wastes of food, dairy and beverage industries. Lactose is one of the major components of whey in cheese industry that is non-friendly in environment. Whey is the relatively clear supernatant that remains after the coagulated casein is separated from the milk for cheese making. Its lactose content is 4.5 to 5% (Nahvi and Moeini, 2004). GOS are non-digestible oligosaccharides comprised of 2-20 molecules of galactose and 1 molecule of glucose (Miller and Whistler, 2000). GOS are recognized as prebiotics since, they can stimulate the proliferation of lactic acid bacteria and *Bifidobacteria* in human intestine (Sako et al., 1999) [15]. So, much attention has been given to the production of GOS, especially *via* enzymatic transgalactosylation, since the chemical synthesis is very tedious (Sears and Wong, 2001).

β-galactosidases are widely distributed in numerous biological systems e.g. Plant and animal tissues, microorganisms (Fungi-yeasts and molds, actinomycetes) (Zadow, 1992). Compared to the animal and plant sources of the enzyme, microorganisms produce enzyme at higher yields and thus lower the price of commercial β-galactosidase production (Santos et al., 1998). Thus, for large scale commercial production of the enzyme much work is focused on microbial production. The characteristics of the enzyme vary depending on its source. In general, β-galactosidase from yeasts and bacteria have optimum pH near neutral range (6.5-7) whereas the enzyme from molds act better at acidic range (3-5) (Wierzbicki and Kosikowski, 1973).

The most studied β-galactosidase is the one produced by *Escherichia coli* but due to possible toxic factor associated with the coliform, the crude isolate of β-galactosidase is unlikely to be used in food processes (Santos et al., 1998). Therefore, β-galactosidases used in industrial scale for the production of milk and dairy products are isolated from microorganisms with GRAS status (generally recognized as safe). The commercial β-galactosidase are generally extracted from yeasts (*K. lactis and K. fragalis*) and fungi (*Aspergillus niger and A. oryzae*) (Harju 1987).

So far, no studies have been made on β-galactosidase extracted from lactose utilizing yeasts isolated from dairy samples in Nepal. Hence, we aimed to characterize β-galactosidase extracted from lactose utilizing yeasts isolated from dairy samples collected from local market of Kathmandu valley and evaluated the potential applications of enzymes in clearance of lactose pollution in waste water.

## MATERIALS AND METHODS

**Isolation:** Eight different dairy samples were collected from different dairies in Kathmandu valley (4 yoghurt samples, 2 milk samples and 2 cotton cheese/farmer cheeses/paneer samples). 1gm of each dairy sample was taken for serial dilution upto $10^{-5}$. 100µl of dilutions ($10^{-2}$ $10^{-4}$, $10^{-5}$) were spread on YGCA Plates (5 gm Yeast extract, 20 gm Glucose, 0.1 gm Chloramphenicol in 1 liter of distilled water) and incubated at 25°C for 72 hrs. Yeast colonies with distinct morphological differences were selected and then purified by streaking on PDA plates and stored at 4°C (Nahvi and Moeini, 2004).

**Sugar assimilation test:** Sugars used were Glucose, Fructose, Galactose, Sucrose, Lactose, Maltose, Raffinose, Xylose, Mannitol, and Dextrose. Sugar assimilation test was done using basal fermentative medium YEP Broth containing chloramphenicol (4.5 gm Yeast extract, 7.5 gm Peptone, 20 gm sugar (Raffinose 40 gm), Chloramphenicol 0.1 gm per liter). The final working solution was made by addition of 4 ml of Bromothymol blue stock solution (50 mg/75 ml in distilled water) to 100 ml of basal fermentative medium. After sterilization by autoclaving, chloramphenicol was added to the basal media. 10 ml of basal media was dispensed into sterile test tubes and test tubes were inoculated with yeast isolates and incubated at 25°C for 72 hrs. Test tubes were inspected at frequent intervals and positive sugar assimilation results were indicated by change in color of indicator from green to yellow. Control tubes were used in sets to monitor contamination. Lactose positive yeast isolates were identified from this test and these isolates were subjected to further study and mass culture (Nahvi and Moeini, 2004).

**Study of colonial and microscopic morphology:** Colonial morphology of lactose positive yeast isolates on PDA plates such as color, pigmentation, consistency, elevation, shape, size, margin, opacity was studied. The microscopic morphology of the lactose positive yeast isolates was studied after gram staining (Barnett et al., 1990; Nahvi and Moeini, 2004).

**Mass culture:** Mass culture of lactose positive yeast isolates was done in enrichment media YEP broth containing chloramphenicol (10 gm Yeast extract, 20 gm Peptone, 20 gm Lactose, 0.1 gm Chloramphenicol per liter of distilled water). Yeast isolates were inoculated into 1000ml of sterile broth (pH 7.0) in sterile condition in a conical flask. The flask was then incubated in water bath shaker at 30°C for 48 hours (Nahvi and Moeini, 2004).

**Enzyme extraction:** 1000 ml YEP broth was centrifuged at 7000 rpm for 10 minutes and resulting pellet of yeast cells was first washed with 20 ml phosphate buffer (pH 7.0, 0.1M) and then re-suspended in 120 ml of phosphate buffer (pH 7.0, 0.1M). Chloroform (2% v/v) was added to lyse the yeast cells and incubated overnight at 37°C. Yeast lysate was then centrifuged at 10,000 rpm for 20 minutes. Supernatant was then collected. Supernatant is the crude enzyme extract (Nahvi and Moeini, 2004).

**Determination of protein concentration:** Protein concentration was determined by Bradford method. In 6 test tubes working BSA solution (1mg/ml) 10, 20, 40, 60, 80, 100µl was taken respectively. Volume was maintained to 200µl by addition of $PO_4$ buffer (pH 7.0, 0.01M). 2300 µl of Bradford reagent was added to each test-tubes and the final volume was 2500µl. Absorbance was taken at 595 nm against the blank (200 µl $PO_4$ buffer and 2300µl Bradford reagent) after 5min of incubation. Calibration curve was made for protein concentration Vs. absorbance. 40µl of test sample (β-galactosidase extract) was taken in a test tube and volume was maintained to 200µl by addition of $PO_4$ buffer. 2300 µl of Bradford reagent was added and after 5min of incubation absorbance was taken at 595 nm. The concentration of the test sample was determined by comparing the absorbance of the sample with the calibration curve (Bradford, 1976).

**Preparation of enzyme substrate:** (ONPG) Ortho-Nitrophenyl-β-D-galactopyranoside (0.01 molesL$^{-1}$) prepared in a phosphate buffer of pH 6.8 was used as substrate for the β-galactosidase assay (Zhou and Chen, 2001).

**Determination of enzyme activity:** 750µl of substrate solution (ONPG) with pH 6.8 and 250 µl of crude enzyme extract were added in a test tube. 2ml of phosphate buffer (pH 6.8, 0.1 M) was added to the test tube and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250 µl $Na_2CO_3$ (0.5 moles L$^{-1}$). Reaction progress was determined spectrophotometrically at 420 nm against the blank (750 µl ONPG, 2250 µl phosphate buffer of pH 6.8) (Fernandes et al., 2002).

Enzyme activity was calculated by accordingly.

$$\text{Enzyme activity} = \frac{0D420\times \text{Reaction Volume}}{0.0045\times \text{Enzyme volume}\times \text{Time}}$$

$$\text{Specific enzyme activity} = \frac{0D420\times \text{Reaction Volume}}{0.0045\times \text{Enzyme volume}\times \text{Time}\times \text{protein concentration}}$$

$$\text{Total enzyme activity} = \frac{0D420\times \text{Reaction Volume}\times \text{total protein}}{0.0045\times \text{Enzyme volume}\times \text{Time}\times \text{protein concentration}}$$

**Determination of optimum time duration:** 10 test tubes were filled with 750 µl of 0.01M ONPG prepared in phosphate buffer (pH 6.8, 0.1M) .To every test tubes 250µl of crude enzyme extract and 2ml of phosphate buffer (pH 6.8, 0.1M) was added to the test tube and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250µl of 0.5M $Na_2CO_3$ at every 30 minutes interval and the reaction progress was determined spectrophotometrically at 420nm against

the blank (750 µl ONPG, 2250 µl phosphate buffer of pH 6.8) (Fernandes et al., 2002).

**Determination of optimum temperature:** 8 test tubes were filled with 750µl of 0.01M ONPG prepared in phosphate buffer (pH 6.8, 0.1M) .To every test tubes 250µl of crude enzyme extract and 2ml of phosphate buffer (pH 6.8, 0.1M) was added to the test tube and then incubated separately at different temperatures of 0°C, 14°C, 22°C, 27°C, 37°C, 40°C, 45°C and 50°C for 70 minutes. The reaction was quenched by adding 250µl of 0.5M $Na_2CO_3$ and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 µl ONPG, 2250 µl phosphate buffer of pH 6.8) (Zhou and Chen, 2001).

**Determination of optimum pH:** Eight test tubes were filled with 750 µl of 0.01M ONPG prepared in phosphate buffer of pH 5.5, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and 7.12. To every test tube 250 µl of crude enzyme extract and 2ml of phosphate buffer of respective pH was added and then incubated at 37°C for 70 minutes. The reaction was quenched by adding 250 µl of 0.5M $Na_2CO_3$ and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 µl ONPG, 2250 µl phosphate buffer of pH 6.8) (Zhou and Chen, 2001).

**Determination of $K_m$ value:** Nine test tubes were filled with 50, 100, 150, 200, 250, 300, 350, 400 and 450 µl of 0.01M ONPG prepared in phosphate buffer of pH 6.8. Final volume was made 1750 µl by addition of phosphate buffer of pH 6.8. 250 µl of enzyme was added to each test tubes and incubated at 37°C for 70 min. The reaction was quenched by adding 250 µl of 0.5M $Na_2CO_3$ and the reaction progress was determined spectrophotometrically at 420 nm against the blank (750 µl ONPG, 1250 µl phosphate buffer of pH 6.8) (Fernandes et al., 2002).

## RESULTS

**Isolation of lactose positive yeast strains:** Four lactose positive yeast isolates were obtained from the dairy samples. Isolate 1 showing the highest lactose positive activity was taken for further study.

**Protein concentration:** The protein concentration in extracted and purified enzyme extracts was found to be 0.465 µg/µl (mg/ml) using Bradford assay compared with standard BSA solution.

**Effect of incubation time, temperature and pH on enzyme activity:** The enzyme has an optimum incubation time of 70 min and optimum temperature at 37°C. Whereas the optimum pH of enzyme was found to be slightly acidic at 6.8 (Figure 1, Figure 2 and Figure 3).

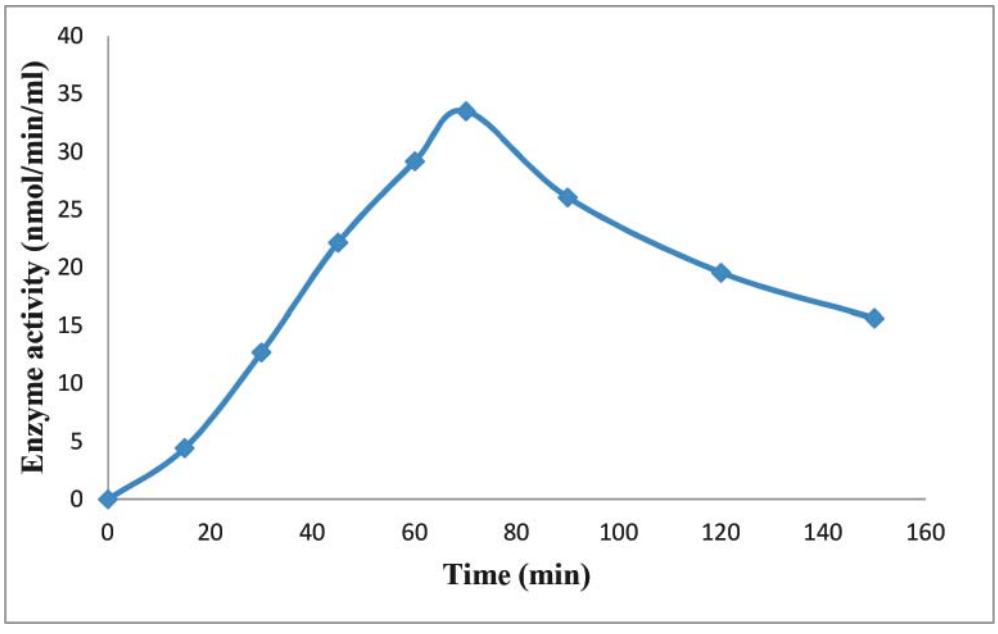


**Figure 1: Effect of incubation time on enzyme activity**

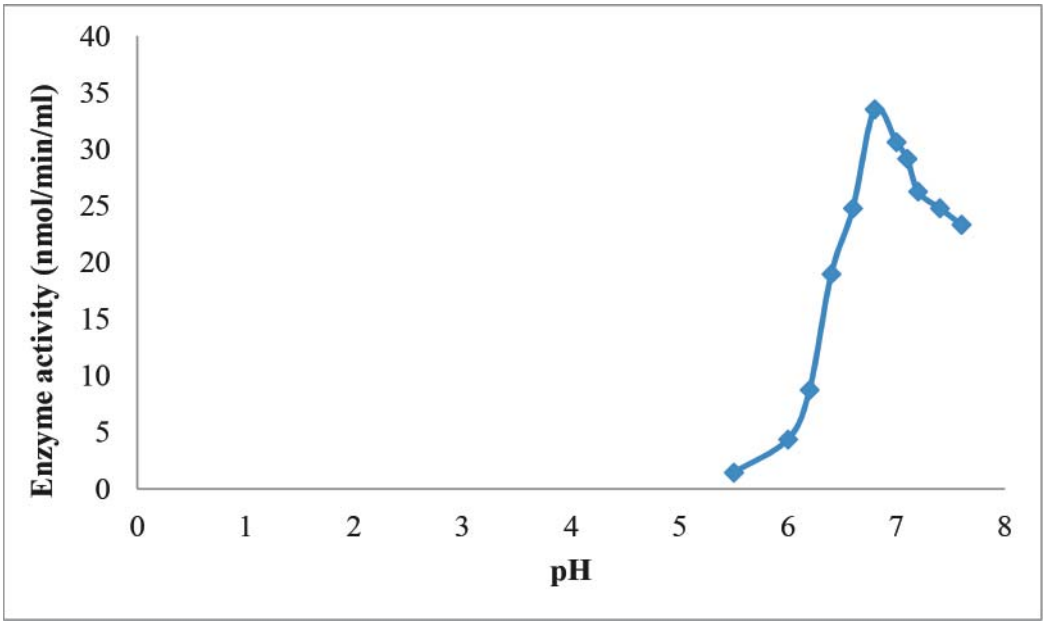


**Figure 2: Effect of Temperature on enzyme activity**

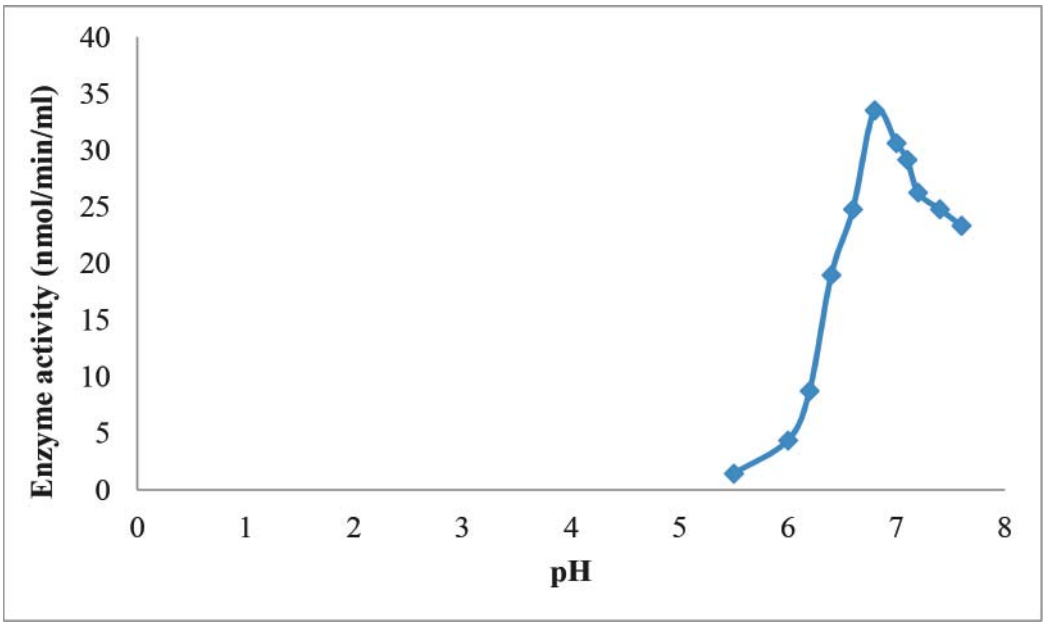


**Figure 3: Effect of pH on Enzyme activity**

**Effect of incubation time, temperature and pH on specific activity:** Like an enzyme activity, highest specific activity of enzyme was observed with 70 min incubation, at 37°C temperature and at 6.8 pH (Table 1).

**Table 1: Effect of incubation time, temperature and pH on specific activity of enzyme**

| Incubation time (min) | Specific activity (nmol/min/mg) | Temperature (°C) | Specific activity (nmol/min/mg) | pH | Specific activity (nmol/min/mg) |
|---|---|---|---|---|---|
| 0 | 0.00 | 0 | 15.61 | 5.5 | 3.10 |
| 15 | 9.53 | 14 | 37.63 | 6.0 | 9.40 |
| 30 | 27.33 | 22 | 53.25 | 6.2 | 18.82 |
| 45 | 47.63 | 27 | 62.67 | 6.4 | 40.73 |
| 60 | 62.75 | 37 | 72.06 | 6.6 | 53.24 |
| 70 | 72.06 | 40 | 37.63 | 6.8 | 72.06 |
| 90 | 55.94 | 45 | 12.52 | 7.0 | 65.84 |
| 120 | 42.01 | 50 | 9.40 | 7.1 | 62.67 |
| 150 | 33.61 | | | 7.2 | 56.43 |
| | | | | 7.4 | 53.25 |
| | | | | 7.6 | 50.13 |

**Effect of substrate concentration on enzyme activity:** The $V_{max}$ and $K_m$ values of enzymes were determined to 58.82nmol/min/ml and 2.23mM of ONPG respectively using Lineweaver-Burk plot while $V_{max}$ and $K_m$ values were found to be 54.43nmol/min/ml and 2.036 mM of ONPG respectively using Eadie-Hofstee plot (Table 2, Figure 4 and Figure 5).

**Table 2: Effect of substrate Concentration on enzyme activity**

| Concentration of substrate [S] (mM) | Enzyme activity (V) (nmol/min/ml) | 1/[S] | 1/V | V/ [S] |
|---|---|---|---|---|
| 0.285 | 6.6286 | 3.5088 | 0.1509 | 23.2584 |
| 0.571 | 11.00 | 1.7513 | 0.0909 | 19.2643 |
| 0.857 | 16.7143 | 1.1669 | 0.0598 | 19.5039 |
| 1.14 | 20.6286 | 0.8772 | 0.0485 | 18.0954 |
| 1.43 | 22.0571 | 0.6993 | 0.0453 | 15.4245 |
| 1.71 | 25.5714 | 0.5848 | 0.0391 | 14.9542 |
| 2.00 | 27.2857 | 0.5000 | 0.0366 | 13.6429 |
| 2.28 | 27.8857 | 0.4386 | 0.0359 | 12.2307 |

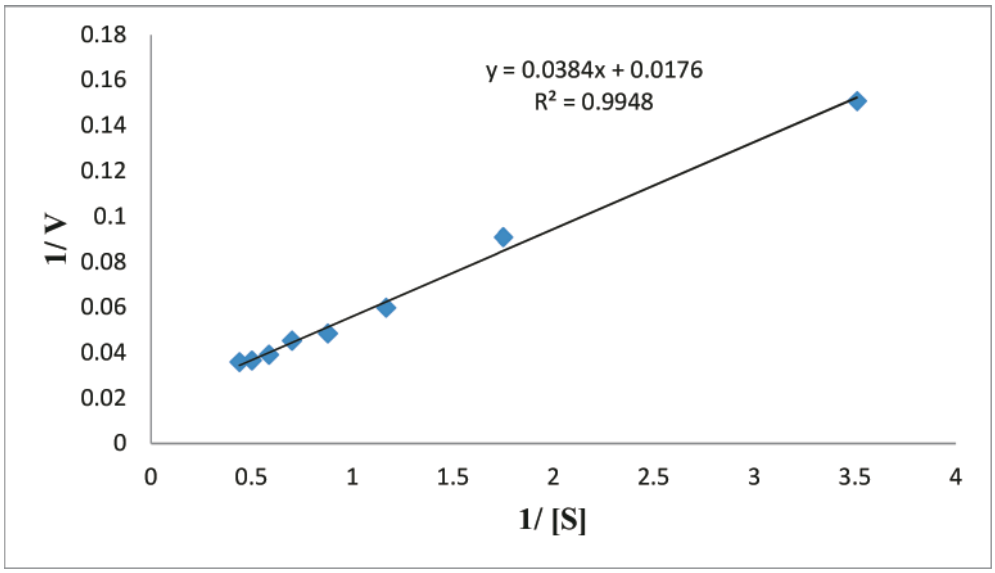


**Figure 4: Lineweaver-Burk/Double reciprocal plot**

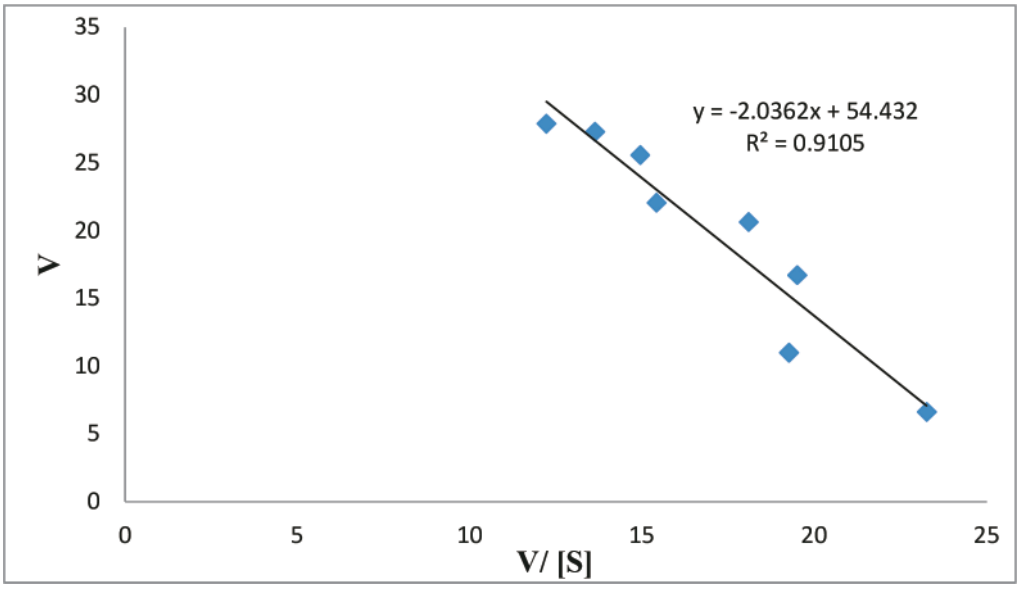


**Figure 5: Eadie-Hofstee plot (V/ [S] Vs. V]**

**Calculation of specific and total enzyme activity:** The specific and total enzyme activity were calculated to be 59.97nmol/min/mg and 3346.33nmol/min respectively (Table 3).

**Table 3: Summary of enzyme characteristics**

| Enzyme parameters | Values |
|---|---|
| Protein concentration of enzyme extract | 0.465 mg/ml |
| Initial volume of enzyme extract | 120 ml |
| Total protein of enzyme extract | 55.8 mg |
| Optimum time | 70 min. |
| Optimum pH | 6.8 |
| Optimum temperature | 37°C |
| $K_m$ | 2.23 mM of ONPG |
| $V_{max}$ | 58.82 nmol/min/ml |
| Enzyme activity | 27.88 nmol/min/ml |
| Specific enzyme activity | 59.97 nmol/min/mg |
| Total enzyme activity | 3346.33 nmol/min |

## DISCUSSION

The knowledge of enzyme stability can provide information on the structure of an enzyme which can aid in the economical industrial production of enzyme. Activity and stability of an enzyme depend upon the spatial conformation of the protein and is affected by various environmental factors such as pH, temperature, reaction medium, presence of ions. It is important to optimize these factors so, as to allow economical industrial production of enzyme. It is also important to determine the enzyme kinetic parameters such as Michaelis-Menten constant ($K_m$), maximum enzyme activity ($V_{max}$), optimum time duration which are necessary to characterize an enzyme and to optimize the enzymatic process.

The optimum time of reaction was found to be 70 mins. The activity gradually fell beyond 70 mins indicating the inactivation of the enzyme with time. The activity curve for effect of pH gave a bell-shaped curve with maximum activity in the near neutral pH of 6.8. The optimum pH of 6.8 suggests that the enzyme can be classified as slightly acidic. However, the enzyme showed activity over a wide range of pH from 5.5-7.6. In general, β-galactosidase from yeast and bacteria has optimum pH at near neutral range, whereas the enzyme from molds acts well at more acidic level (Gurr, 1987; Sani et al., 1999).

The activity curve of effect of temperature on enzyme activity gave a bell-shaped curve with maximum activity at 37°C. The enzyme had wide range of

working temperature from 0-50°C. However, greater than 50% hydrolyzing ability was maintained in the range of 14-40°C. The enzyme even showed slight activity at freezing temperature (0°C). This shows that the enzyme has potential application for managing the lactose pollution at the normal as well as low ambient temperatures. The enzyme showed least activity at temperatures 45°C or greater. This must be due to the denaturation of the enzyme at higher temperatures (Sani et al., 1999; Wolosowska and Synowiecki, 2004).

The activity curve for effect of substrate concentration on enzyme activity (Michaelis-Menten plot) showed that at low substrate concentration, almost linear increment in enzyme activity with increase in substrate concentration was seen. But at higher substrate concentration the enzyme activity increased by smaller and smaller amount in response to increases in substrate concentration and almost a plateau like region was seen. The activity/rate of an enzymatic reaction depends upon the concentration of the enzyme substrate [ES] complex. At low substrate concentration most of the enzyme is present in uncombined form [E], so the rate is proportional to substrate concentration. This explains for almost linear increment in enzyme activity with increase in substrate concentration, at low substrate concentration. At high substrate concentration most of the enzyme is present as ES complex i.e. virtually all the active sites in an enzyme are saturated with substrate, so that further increase in substrate concentration doesn't produce an appreciable increment in the enzyme activity. This explains the almost plateau like region observed in the enzyme activity at higher substrate concentration (Pivarnik et al., 1995; Prenosil et al., 1987).

From Lineweaver-Burk plot the $K_m$ value and $V_{max}$ was found to be 2.23mM of ONPG and 58.82nmol/min/ml respectively. From Eadie-Hofstee plot the $K_m$ value and $V_{max}$ was found to be 2.036 mM of ONPG and 54.43nmol/min/ml respectively. The low $K_m$ value of the enzyme indicated that the enzyme had high affinity for the substrate ONPG and the substrate is tightly bound to the enzyme (Fernandes et al., 2002).

The protein concentration of the crude enzyme extract was found to be 0.465 mg/ml. The low protein concentration and the high enzyme activity indicated that the enzyme β-galactosidase comprised most of the protein fraction of the crude enzyme extract. It might be due to the induction of β-galactosidase as a result of high lactose content of mass culture broth (YEP broth).

## CONCLUSION

The enzyme was slightly acidic (near neutral pH) and showed maximum activity at 37°C but showed activity over a wide range of temperature 0-50°C. $K_m$ value of the enzyme was 2.23 mM of ONPG, thus the enzyme has high affinity for ONPG. The overall properties of enzyme showed the potential use of the enzyme as industrial application.


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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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