VOL. 5, NO. 1

2018

ISSN: 2382-5499 (Print)
ISSN 2661-6076 (Online)

TRIBHUVAN UNIVERSITY JOURNAL OF MICROBIOLOGY

Tribhuvan University Journal of Microbiology

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

VOL. 5, NO. 1 2018 ISSN: 2382-5499 (Print)
ISSN 2661-6076 (Online)

Tribhuvan University

Journal of Microbiology

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

Tribhuvan University Journal of Microbiology

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.

CONTACT

Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu
Phone : 00977-1-4331869
E-mail : cdm1990@microbiotu.edu.np

THE EDITORIAL PROCESS

The manuscript will be reviewed with the understanding that it has not been submitted to other journal at a time or has not been published or accepted for publication elsewhere. Manuscript is reviewed for originality, scientific and technical ideas, and significant message. The poor articles with insufficient originality, serious scientific and technical mistakes and lack of significant message will be rejected. Manuscript is sent to expert reviewer without revealing the identity of the authors to the reviewers. Each manuscript is then reviewed by the TUJM editor based on the comments of the reviewers and make final decision for publication or rejection of the manuscript.

PUBLISHED BY **Central Department of Microbiology**
Tribhuvan University
Kirtipur, Kathmandu, Nepal
Tel.: 00977-1-4331869, E-mail : cdm1990@microbiotu.edu.np, URL: microbiotu.edu.np

Author's guidelines

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.

2. Format of papers

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.

3. Submissions

Authors are advised to submit their manuscripts through e-mails (cdm1990@microbiotu.edu.np, manita_aryal11@yahoo.com, upendrats@gmail.com, or adhikarinaba2004@yahoo.com) or electronic copy and three hard copies of the manuscript to the Research Management Cell, Central Department of Microbiology at Kirtipur. A signed cover letter mentioning that the article has not been submitted elsewhere for publication should be submitted with the manuscript.

3. 1 Full-length papers

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.

(b) INTRODUCTION: A balance should be maintained between the pure and applied aspects of the subject.

(c) MATERIALS AND METHODS: Ensure that the work can be repeated according to the details provided. By submission of a manuscript, the authors consent that biological material, including plasmids, viruses and microbial strains, unobtainable from national collections will be made available to members of the scientific community for non-commercial purposes subject to national and international regulations governing the supply of biological material. In the case of a new diagnostic PCR, you should consider the need for an internal amplification control. Ethical approval letter Reg no. form authorised institution should be given if applicable.

(d) RESULTS: Well-prepared tables and figures must be a feature of the 'Results' section because they convey the major observations to readers. Information provided in tables and figures should not be repeated in the text, but attention on the importance of the principal findings of the study should be focused.

(e) DISCUSSION: This must not recapitulate the results and should explain the meaning of results.

(f) CONCLUSION:

(g) ACKNOWLEDGEMENTS:

(h) REFERENCES: Citation of references having three or more names should be cited in the text as Jones et al. (1992) at the first and Green and Smith (1992) would have to be quoted in full. A series of references should be given in ascending date order (Green and Smith 1946; Jones et al. 1956). Different publications having the same author(s) and year will be distinguished by, for example, 1992a, 1992b. This also applies to the Bibliography. Papers or other publications having no obvious author(s) should usually be cited as 'Anonymous' with the year in the text and bibliography. Web sites should be quoted in the text with an access date.

Layout of references

The Harvard system should be used. Names with the prefixes de, do van, von, etc. will be placed in alphabetical order of the first letter of the prefix, e.g. von Braun would appear under 'V'. Where italics are

intended, words must either be typed in roman and underlined or printed in italics from a word processor. Abbreviate journal titles according to Index Medicus (). The following is an example of order and style tobe used in the manuscript:

Examples:

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.

Garner JS and Favero MS (1985) *Guidelines for Handwashing and Hospital Environment Control*. US Public Health Service, Centers for Disease Control HHS Washington DC: Government Printing Office No. 99-117.

Fricker CR (1995) Detection of *Cryptosporidium* and *Giardia* in water. In *Protozoan Parasites in Water* Eds

Personal communications should be cited in the text with initials and family name of all individuals.

Abbreviations and units

The Journal uses SI units: g/l; d, h, min, s (time units) but week and year in full; probability is p; centrifugation conditions relative to gravity (g or rpm). Please refer to the Biochemical Journal 'Instructions to Authors'.

Microbial nomenclature

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).

Common names will not have an initial capital letter nor will they be underlined in the manuscript, viz. pseudomonad, salmonellas. The specific name will be given in full in the captions to tables and figures. Major ranks are written in Roman with an initial capital (e.g. Enterobacteriaceae).

At the first citation of a serotype the genus name is given followed by the word 'serotype' and then the serotype name. Names of serotypes should be in Roman type with the first letter capitalized (for example *Salmonella* serotype Typhimurium). Subsequently the name should by written with the genus (abbreviated) followed directly by the serotype name (for example S. Typhimurium).

Nucleotide sequences

1. Nucleotide sequence data should be deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries and the accession number referenced in the manuscript.

2. Sequence data should only be included if they are new (unpublished), complete (no unidentified nucleotides included) and if the sequence information itself provides important new biological in sights of direct relevance to the question addressed in the manuscript. Generally, sequences should not be submitted if the same gene has been reported in another species unless a comparison with related sequences contributes important new information.

3. Presentation of nucleotide sequences should include clear indications of nucleotide numbers and points of interest, e.g. promoter sequences, ribosome binding sites, mutations, insertions, probe sequences, etc. In the case of comparisons, nucleotides which differ between the sequences should be readily visible to the reader, e.g. by the use of bold face, shading, boxing or by the use of a dash to represent identical nucleotides. The font size used in the manuscript should facilitate appropriate reduction of the figure.

Statistics

Tests must be presented clearly to allow a reader with access to the data to repeat them. It is not necessary to describe every statistical test fully, as long as it is clear from the context what was done. In particular, null hypotheses should be clearly stated. Authors are urged to give consideration to the assumptions underlying any statistical tests used and to assure the reader that the assumptions are at least plausible. Authors should be prepared to use nonparametric tests if the assumptions do not seem to hold.

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.

Figures

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.

Photographs

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.

Supporting data

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.

Footnotes

Not permitted other than on the first page of a manuscript where they are used to show the author's change of address and the address for correspondence.

Experimental hazards

Chemical or microbiological hazards that may be involved in the experiments must be explained. Authors should provide a description of the relevant safety precautions adopted or cite an accepted 'Code of Practice'.

Supporting information

Authors wishing to submit supporting information material (such as multimedia adjuncts, large data sets, extra colour illustrations, bibliographies or any other material for which there is in sufficient space in the print edition of the Journal) must do so at the time of first submission. This supporting information is an integral part of the article and will be reviewed accordingly. The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting information' and providing titles of figures and tables.

Letter of Conflict of Interest (If applicable)

3.2 Review Articles

Preparation of manuscript

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.

Manuscript presentation

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 not exceeding 35 letters and spaces; (d) the name,

postal and e-mail address of the author to whom all correspondence should be addressed and who will check the proofs. A short SUMMARY of 150-200 words must be included, as well as an INTRODUCTION, DISCUSSION, CONCLUSION (possibly referring to future prospects) sections. References must be chosen carefully as their number is limited by the size limitation of the review article.

3.3 Letters to the editor

The Chief Editor will consider letters which will provide further debate on a particular topic arising from the publication of a paper. Author(s) of the paper will be sent an edited copy of the letter and they will have the right of reply. Both letters will be published in the Journal.

3.4 Notes to the editor

The Chief Editor will consider notes which will provide further confirmatory information on a particular topic, or a novel aspect of a methodology (e.g. detection) or a microorganism (e.g. virulence factor) for which results are preliminary but the impact for Microbiology deemed to be important and requires rapid publishing. Notes should be concise (2000 words; including references), with no headings and present results in 1 table or 1 figure only. The abstract should be a brief summary of the work under the following four headings: Objectives; Methods and Results; Conclusion; Significance and Impact of the Study.

Disclaimer

Whilst every effort is made by the Publishers and Editorial Board to see that no inaccurate or misleading data, opinion or statement appears in this Journal, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the sole responsibility of the contributor or advertiser concerned. Accordingly, the Publishers and Editors and their respective employees, officers and agents accept no responsibility or liability what so ever for the consequences of any such inaccurate or misleading data, opinion or statement.

Tribhuvan University Journal of Microbiology

VOL. 5, NO. 1 2018 ISSN: 2382-5499 (Print), ISSN 2661-6076 (Online)

EDITORIAL

Microbiology Education in Nepal

Microbiology education in bachelor level was started in Nepal since 1978 from Tri-chandra campus. Master's in Microbiology was started since 1990 from Central Department of Microbiology, Tribhuvan University. Now, M.Sc. programme has been expanded to three constituents and eight affiliated campuses of Tribhuvan University. Since 2012, M.Sc. Microbiology programme has been changed into semester system from annual system.

The curriculum of microbiology is always designed to upgrade B.Sc. and M.Sc. Microbiology degree of Institute of Science and Technology, Tribhuvan University to international level in accordance to latest advances in microbiology. The curriculum emphasizes on professional as well as research based teaching learning practice. The curriculum has aimed to produce qualified microbiologists, molecular biologists, researchers and scientists as per national and international demand. Bachelor of Science in Microbiology emphasizes to produce students with fundamental knowledge and practical skills of microbiology, microbiology related subjects including biochemistry, microbial biotechnology, and applied subjects of microbiology including agriculture and food microbiology, public health and medical microbiology. Bachelor degree holders are also learned to conduct microbiology research and report writing independently or in group. The master's degree holders are able to use skills of modern molecular biology techniques in basic science research or in applied research areas of Microbiology. The graduates are able to work as a qualified scientist for investigating the potential uses of microorganisms to produce antibiotics, antibodies, steroids, vaccines, hormones and other products of microbial origin. The Master's degree holders are able to work as a professional microbiologist and research scientist in the laboratories for monitoring the diagnostic tests, identifying the causative agents of infectious diseases and helping to control those infectious diseases. They are able to work as qualified professionals and researchers in the institutions related to food production, food quality control, crop protection and soil fertility. The Master's degree holders are eligible to be the lecturers of Microbiology programme offered by Institute of Science and Technology and other institutions of Tribhuvan University or other universities for teaching, mentoring and supervising bachelors and masters level microbiology students. Microbiologists from Nepal are always in position to contribute to the science and service to the nation.

Dr. Megha Raj Banjara, Associate Professor
Chief Editor
Tribhuvan University Journal of Microbiology (TUJM)

Antimicrobial Activity of Ethanolic Extract of Medicinal Plants against Human Pathogenic Bacteria

Hira Sakha[1], Rejila Hora[1], Shilpa Shrestha[1], Shreeya Acharya [1], Dinesh Dhakal [1*], Srijana Thapaliya[1], Kamil Prajapati [1]

[1]Department of Microbiology, Sainik Awasiya Mahavidhyalaya, Sallaghari, Bhaktapur

***Corresponding author:** Dinesh Dhakal, Department of Microbiology, Sainik Awasiya Mahavidyalaya, Sallaghari, Bhaktapur; E-mail: dineshdhakal7@hotmail.com

ABSTRACT

Objectives: To evaluate the antimicrobial activity of medicinal plants against human pathogenic bacteria and perform Minimum Inhibitory Concentration (MIC) of plant extracts.

Methods: Rhizome of *Curcuma longa,* dried buds of *Synzygium aromaticum,* seeds of *Zanthoxylum armatum* and leaves of *Elaeocarpus ganitrus, Psidium guajava, Azadirachta indica,* and *Artemisia vulgaris* were collected from hilly regions of Nepal. The plant parts were air-dried at room temperature and grinded to powder form. The ethanolic extracts of medicinal plants were prepared by percolation process using separating funnel and tested against human pathogenic bacteria by disc diffusion method. Then, Minimum Inhibitory Concentration (MIC) of the plant extracts were determined.

Results: All plants extracts exhibited antibacterial properties against bacteria under study. However, extract from *S. aromaticum* (Clove), *P. guajava* (Guava) and *E. ganitrus* (Rudraksh) leaves showed most promising result against *Staphylococcus aureus* with zone of inhibition of 14mm, 16mm and 16 mm respectively. Likewise, *S. aromaticum* (Clove), *C. longa* (Turmeric) and *P. guajava* (Guava) showed good antibacterial activity against *Escherichia coli* with zone of inhibition of 11mm, 11mm and 10mm respectively. *A. vulgaris* (Titepati) and *A. indica* (Neem leaves) showed promising activity against *Pseudomonas aeruginosa* with zone of inhibition of 11mm. *Z. armatum* (Timur) showed good result against *E. coli* with zone of inhibition 10mm. MIC values of ethanolic extracts of *S. aromaticum* and *E. ganitrus* were found to be at the range of 12.5-25mg/ml.

Conclusion: This study has helped to understand the use of these plants as traditional medicine as an economic and safe alternative to treat infectious diseases.

Key words: Plant extracts, antimicrobial activity, zone of inhibition, Minimum Inhibitory Concentration

INTRODUCTION

Bacterial infections cause problem for human kind beyond historical age. Researches to find antimicrobial medicine have been launch for over 50 years (Rudrappa and Bais 2008). However, even many antibiotics have been discovered, we still are facing multidrug resistance bacterial infections (Dowzicky and Park 2008; Saonuam et al. 2008; Tillotson et al. 2008) and the side effects of antibiotic treatment for patients who are allergic to it.

Traditional plant refers to the health practices, approaches, knowledge and beliefs incorporating plant and mineral based medicine to treat, diagnose and prevent illness or maintain well-being (Selvamohan et al. 2012). Several ethnomedicinal plants of Nepal have been identified and their usage documented. These documented plants have been used as antibacterial, antifungal, antiviral and for other general treatments. But, a scientific and systematic investigation on

Date of Submission: August 3, 2018
Date of Acceptance: November 15, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22292

antibacterial properties of Nepalese medicinal plants is still lacking (Rudrappa and Bais 2008).

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics gets diminished. The use of plant extract for medicinal treatment has become popular when people realized that the effective life span of antibiotic is limited and over prescription and misuse of traditional antibiotics are causing microbial resistance (Alam et al. 2009).

The use of crude extracts of plants parts and phytochemicals, of known antimicrobial properties are of great significance in the therapeutic treatments. Extraction is the separation of medically active portions of the plant tissues using selective solvent known as menstruum through standard procedures. The products contain complex mixture of medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, and flavonoids, tannins and lignans. In order to be used as a modern drug an extract may be further processed through various techniques of fractionation to isolate individual chemical entities such as vincristine, vinblastine, hyoscyamine, hyoscine and codeine (Sukhdev et al. 2008).

In this study, an attempt has been made to evaluate the antimicrobial activity of extracts of selected medicinal plants against human pathogenic bacteria and perform minimum inhibitory concentration of plant extracts. This is with a view to contribute knowledge for utilization of locally available medicinal herbs in comparison to the antibiotics which can causes various side effects.

MATERIALS AND METHODS

Collection of plant samples and bacterial strains: The plant samples were collected from the various areas of Lalitpur, Bhaktapur and Kavrepalanchok district of Nepal and the study was carried out in the laboratory of the Sainik Awasiya Mahavidhayala, Bhaktapur. ATCC strains *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enterica* Typhi and *Citrobacter freundii* obtained from National Public Health Laboratory, Teku were used for antimicrobial activity of plant extracts.

Extraction of antimicrobial compounds: The ethanolic extracts of medicinal plants were prepared by dissolving 20gm of dried powder into 100ml of ethanol separately and then kept in the separating funnel for 7 days with continuous shaking in every 24 hours. After 7 days extract was filtered where the sediment settled down at the bottom and left ethanol was evaporated at evaporator. The left over filtrate called as extract containing the chemical constituents of each medicinal plants were dried in the hot air oven at 40°C and stored under refrigeration for further use. Whattman's No 3 filter paper was punched into 2mm disc and were sterilized. Each disc was soaked in the respective concentration of the extracts that was prepared by using DMSO and methanol in the ratio of 10:90 i.e. 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml.

Disc diffusion method: The 20ml of sterilized Muller Hinton Agar was poured into sterile petri plates, after solidification, 100µl of fresh culture of human pathogens were swabbed on the respective plates. The discs were kept over the agar plates using sterile forceps and incubated at 37°C for 24 hours. After incubation the diameter of inhibitory zones formed were measured.

Standardization of bacterial suspension: McFarland standard was used as a reference to adjust the turbidity of bacterial suspensions. The bacterial suspensions were standardized following the CLSI guidelines for aerobic bacteria. All of the tested bacteria were grown in Mueller Hinton broth for 18–24 h, followed by the matching of bacterial suspension to the turbidity equivalent to 0.5 McFarland solutions ($1\text{-}2\times10^8$ cfu/ml).

Determination of MIC: The minimum inhibitory concentration (MIC) of the aqueous extract was determined by micro broth dilution method (Andrews JM 2001). For MIC, two-fold serial dilutions of the extracts were prepared (200, 100, 50, 25 and 12.5µg/ml). Incubation of the tubes was carried out at 37° C for 18-24 hours for bacteria and were observed for any visible growth. The bacterial suspensions were used as positive control and extracts in broth were used as negative control. The MIC was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control tubes that contained only the extracts.

RESULTS

In this study, we tested the ethanolic extracts of seven medicinal plants for their antimicrobial activity against human pathogenic bacteria. ATCC strains of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,*

Salmonella enterica Typhi and *Citrobacter freundii* were used. All the plants extracts exhibited good antimicrobial properties against the microorganisms tested, as exhibited by disc diffusion method (Table 1). At higher concentration (200mg/ml), activity was remarkable in comparison to lower one (12.5mg/ml).

Extract of *C. longa* and *S. aromaticum* showed good antibacterial activity against *Escherichia coli* with similar zone of inhibition of 11mm, at concentration of 200mg/ml. Extract from *E. ganitrus*, *P. guajava* and *S. aromaticum* leaves showed most promising result against *Staphylococcus aureus* with zone of inhibition of 16, 16 and 14 mm respectively at 200mg/ml concentration. *Pseudomonas aeruginosa* was found highly sensitive to the action of *S. aromaticum* (14mm at 200mg/ml) followed by *E. ganitrus* and *A. indica* with zone of inhibition of 12 and 11mm respectively. At the concentration of 200mg/ml, extracts of *E. ganitrus, S. aromaticum* and *C. longa* were effective against *S. enterica* Typhi with significant zone of inhibition 13, 11, and 10mm respectively. *Citrobacter freundii* was found most sensitive against *S. aromaticum* with inhibition zone 15mm. On the contrary, *C. freundii* was resistant against *A. indica and A. vulgaris. S. aureus* and *P. aeruginosa* were resistant against *Z. armatum.*

Among the seven medicinal plants, the crude extracts of *S. aromaticum*, *E. ganitrus, C. longa* and *P. guajava* showed good antimicrobial activity against all tested microorganisms. *A. indica* and *A. vulgaris* showed good results against *Pseudomonas aeruginosa* with zone of inhibition 11mm. *Z. armatum* was effective against *E. coli* (10mm). MIC values of ethanolic extracts of *S. aromaticum* and *E. ganitrus* were found to be at the range of 12.5-25mg/ml.

Table 1: Antimicrobial activity of plant extracts against the human pathogenic bacteria by disc diffusion method.

Extract	Concentration	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)	*S. enterica* Typhi (mm)	*C. freundii* (mm)
S. aromaticum	200mg/ml	11	14	14	11	15
	100mg/ml	9	11	9	9	7
	50mg/ml	8	9	-	7	6
	25mg/ml	6	6	-	5	5
	12.5mg/ml	5	5	-	-	-
C. longa	200mg/ml	11	9	10	10	9
	100mg/ml	10	8	9	9	8
	50mg/ml	9	6	7	7	7
	25mg/ml	8	-	6	6	-
	12.5mg/ml	6	-	-	-	-
E. ganitrus	200mg/ml	9	16	12	13	9
	100mg/ml	7	14	8	10	8
	50mg/ml	-	13	-	6	7
	25mg/ml	-	11	-	5	-
	12.5mg/ml	-	10	-	-	-
P .guajava	200mg/ml	10	16	9	10	9
	100mg/ml	8	9	7	7	8
	50mg/ml	7	8	-	-	7
	25mg/ml	-	7	-	-	-
	12.5mg/ml	-	-	-	-	-
A. indica	200mg/ml	10	7	11	8	-
	100mg/ml	9	6	8	7	-

Extract	Concentration	*E. coli* (mm)	*S. aureus* (mm)	*P. aeruginosa* (mm)	*S. enterica* Typhi (mm)	*C. freundii* (mm)
	50mg/ml	8	6	7	6	-
	25mg/ml	-	-	-	-	-
	12.5mg/ml	-	-	-	-	-
A. vulgaris	200mg/ml	9	8	11	8	-
	100mg/ml	7	7	8	7	-
	50mg/ml	6	6	7	5	-
	25mg/ml	-	-	-	-	-
	12.5mg/ml	-	-	-	-	-
Z. armatum	200mg/ml	10	-	-	8	7
	100mg/ml	8	-	-	7	6
	50mg/ml	7	-	-	6	5
	25mg/ml	-	-	-	-	-
	12.5mg/ml	-	-	-	-	-

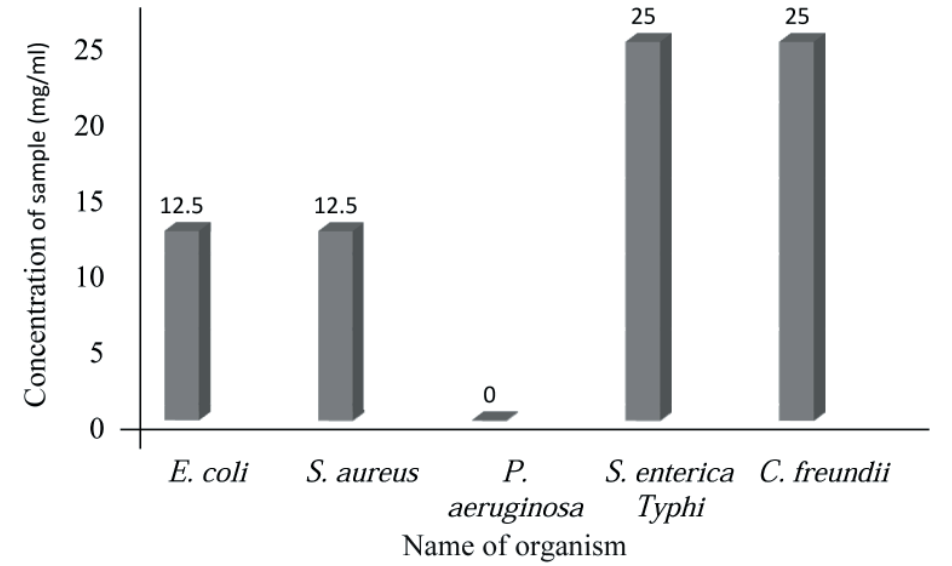


Figure 1: Minimum Inhibitory Concentration (MIC) of *S. aromaticum*

DISCUSSION

Medicinal plants are considered as a rich resource of ingredients which can be used in drug development and synthesis. The compounds found in plants are of many kinds, but most are in four major biochemical classes, alkaloids, glycosides, polyphenols, and terpenes. Some plants consider as important source of nutrition while others are recommended for their therapeutic values. These plants include ginger, green tea, walnuts and some others plants (Lichterman 2004). In this study plants *Syzygium aromaticum*, *Curcuma longa*, *Elaeocarpus ganitrus*, *Psidium guajava*, *Azadirachta indica*, *Artemisia vulgaris* and *Zanthoxylum armatum* were used. Maximum antimicrobial activity was shown by *E. ganitrus* against *S. aureus* at all concentrations.

The main component of *Syzygium aromaticum* is eugenol which is the major component for its antibacterial activity. In this study, it was observed that the ethanolic extracts of *S. aromaticum* has maximum activity against *C. freundii* (15mm) followed by *S. aureus* and *P. aeruginosa* i.e.14mm whereas the least activity was seen in the case of *E. coli* and *S. enterica* Typhi i.e.11mm. However, in the study conducted by Maharjan et al.

(2011) and Wankhede (2015) using *E. coli*, *S. aureus*, *S. enterica* Typhi and *P. aeruginosa*, maximum activity was seen in the case of *Salmonella enterica* Typhi while least activity was seen in case of *S. aureus*.

Curcuma longa contains curcuminoids, which include mainly curcumin (Chainani 2003), which are believed to be the most important fraction responsible for the biological activities of *C. longa*. In this study, the maximum activity was seen in case of *E. coli* (11mm) followed by *P. aeruginosa* and *S. enterica* Typhi (10mm) and minimum activity against *S. aureus* and *C. freundii* each with 9mm. Similarly, in the study conducted by Maharjan et al. (2011), among *E. coli*, *S. aureus*, *S. enterica* Typhi, *P. aeruginosa* used in this study, the maximum antibacterial activity was seen against *E. coli* (15mm) and lowest antibacterial activity against *S. aureus* (14mm) at 100mg/ml concentration whereas, no activity was seen against other organisms.

The *Elaeocarpus ganitrus* fruit have reported to contain many phytoconstituents such as alkaloids, flavonoids, tannins, steroids and triterpenoids. In this study, ethanolic extracts of *E. ganitrus* was most active against *S. aureus* (16mm) whereas lowest antimicrobial activity was seen against *E. coli* and *C. freundii* (9mm) in 200mg/ml concentration of extracts. According to Kumar et al. (1998), antimicrobial activity of the aqueous extract of leaves of *E. ganitrus* was tested against clinical isolates of bacteria and fungi where maximum activity was seen in case of *E .coli* (12.3mm) and minimum activity was seen in case of *S. aureus* (11mm).

The major chemical constituent of *Pisidium guajava* is said to be tannins. In this study, ethanolic extract of leaves of *P. guajava* showed maximum activity against *S. aureus* (16mm) and lowest activity was observed in case of *P. aeruginosa* and *C. freundii* (9mm) at 200 mg/ml concentration. Similarly, Neha et al. (2017) screened the antibacterial effects of ethanolic extracts of guava against UTI causing pathogens i.e. *S. aureus* and *E. coli*. *P. guajava* leaves showed maximum activity against *E. coli* (10mm) while the growth of *S. aureus* was inhibited to a minimum extent (7mm).

Terpenes are considered to be the major chemical constituent of *Azdirachta indica*. In our study, the maximum activity was seen against *P. aeruginosa* (11mm) whereas lowest antimicrobial activity was seen against *S. aureus* (6mm) at highest concentration of the extracts i.e.200mg/ml. Similarly in the study conducted by Brindha et al. (2012) leaf extract showed highest antibacterial activity was detected against *P. aeruginosa* and its lowest antibacterial activity was detected against *S. aureus.*

The active components of *Artemisia vulgaris* are known to be flavonoids, inulin and traces of alkaloids, and volatile oil. In our study, the highest antimicrobial activity was seen against *P. aeruginosa* (11mm) followed by *E. coli* (9mm), *S. aureus* (8mm) and *S. enterica* Typhi (8mm) respectively. Similarly, in the study conducted by Kolume et al. (2011) maximum activity was seen in case of *E. coli* (20mm) whereas least activity was shown by *S. aureus* (10mm).

In our study, maximum antimicrobial activity of ethanolic extracts of leaves of *Zanthoxylum armatum* was seen in case of *E. coli* (10mm) and lowest antimicrobial activity in case of *C. freundii* (7mm). According to Sadia et al. 2014, maximum antimicrobial activity of *Z. armatum* was observed against *P. aeruginosa* whereas lowest activity was seen against *S. aureus*.

The contradiction in the zone of inhibition may be due to concentration variation, various test environment and methods. On the basis of this finding, the ethanolic extract of plant parts possess a good candidate in the search for a natural antimicrobial agent against infections or diseases caused by the test organisms. The extracts of these plants should be further analyzed to isolate the specific antibacterial properties in them. Clinical trials should be carried out to explore the potential of these plant extracts in the treatment of the infectious diseases.

CONCLUSION

This study has helped in demonstrating the potential bioactive compound of natural plant extracts that are economical. Among seven extracts examined *Snyzygium aromaticum, Pisidium guajava and Elaeocarpus ganitrus s*howed the best antibacterial activity against *S. aureus*. Based on the results, we may conclude that secondary components of these plants showed antibacterial activity that can be used for the treatment of diseases caused by the organism. This study has helped us to understand the importance of traditional medicine in the treatment of different bacterial disease than antibiotics as it shortens the length of treatment, increase patient compliance as well as reduce overdose which may lead to toxicity or other side effects.

ACKNOWLEDGEMENTS

We would like to express our sincere gratitude to Sainik Awasiya Mahavidhyalaya for providing us with laboratory facilities that are required for smooth and efficient passage of work required for the completion of the project. We are also thankful to the supervisors for the proper guidance, support, and encouragement throughout the project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alam MT, Karim MM, and Khan SN (2009). Antibacterial activity of different organic extracts of *Achyranthes aspera* and *Cassia alata*. *Journal of Scientific Research* **1**: 393-398.

Brindha S, Maragathavalli S, Kaviyarasi NS, Annadurai BB and Gangwar SK (2012). Antimicrobial activity in leaf extract of neem (*Azadirachta indica* linn). *International Journal of Science and Nature* **3**(1): 110-113.

Chainani WN (2003). Safety and anti-inflammatory activity of curcumin: a component of turmeric (*Curcuma longa*). *J Altern Complement Med* **9**: 161-168.

Dowzicky MJ and Park CH (2008). Update on antimicrobial susceptibility rates among gram-negative and gram-positive organisms in the United States: Results from the tigecycline evaluation and surveillance trial (TEST) 2005 to 2007. *Clin Ther* **30**: 2040-2050.

Kolume DD, Hiremath SK and Muddapur UM (2011). Antimicrobial activity of *Artemisia vulgaris* Linn (Damanaka). *International Journal of Research in Ayurveda and Pharmacy* **2**(6): 1674-1675.

Kumar G, Karthik L, Rao B and Venkata K (1998). Antimicrobial activity of *Elaeocarpus ganitrus* Roxb (Elaeocarpaceae): An In vitro study. *Elixir International Journal* **40**: 5384-5387.

Lichterman BL (2004). Aspirin: The Story of a Wonder Drug. *Curr Med Chem* **11**: 1451–1460.

Maharjan D, Singh A, Lekhak B, Basnyat S and Gautam LS (2011). Study on Antibacterial Activity of Common Spices. *Nepal Journal of Science and Technology* **12**: 312-317.

Neha S, Jana AM, Pathak N, Singh C and Singh P (2017). A preliminary study on the antibacterial effects of ethanolic extract of *Psidium guajava* leaves on Bacteria isolated from urinary tract infection with special reference to *Escherichia coli* and *Staphylococcus aureus*. *International Journal of Biotechnology and Biochemistry*: **13**(2): 83-89

Rudrappa T and Bais HP (2008). Curcumin, a known phenolic from *Curcuma longa*, attenuates the virulence of *Pseudomonas aeruginosa* PA01 in whole plant and animal pathogenicity models. *J Agric Food Chem* **56**: 1955-1962.

Sadia A, Abdul M , Shahzad H, Rehman AU, Khan T, Jadoon MA and Rehman MU (2014). Comparative In vitro activity of ethanol and hot water extracts of *Zanthoxylum armatum* to some selective human pathogenic bacterial strains. *International Journal of Biosciences* **4**(1): 285-291.

Saonuam P, Hiransuthikul NC, Suankratay KM and Danchaivijitr S (2008). Risk factors for nosocomial infections caused by extended-spectrum a-lactamase producing *Escherichia coli* or *Klebsiella pneumoniae* in Thailand. *Asian Biomed* **2**: 485-491.

Selvamohan T, Ramadas V and Kishor SSS (2012). Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. *Advances in applied science Research* **3**(5): 3374-3381.

Sukhdev HS, Sumanpreet KS, Longo Gennaro and Dev RD (2008). Extraction Technologies for Medicinal and Aromatic Plants. International Centre for Science and High Technology, Trieste, Italy 6-266.

Tillotson GS, Draghi DC, Sahm DF, Tomfohrde KM, Fabro TD and Critchley IA (2008). Susceptibility of *Staphylococcus aureus* isolated from skin and wound infections in the United States 2005-07: Laboratory-based surveillance study. *J Antimicrobial Chemother* **62**: 109-115.

Quality Analysis of Milk in Kathmandu Valley

Astha Parajuli[1], Prasiddhi Rimal[1], Rujisha Maharjan[1], Richa Chaudhary[1], Shashi Bhushan Chaturwedi[1*]

[1]Department of Microbiology, DAV College of Science and Humanities, Lalitpur, Nepal
***Corresponding author:** Shashi Bhushan Chaturwedi, Department of Microbiology, DAV College of Science and Humanities, Lalitpur, Nepal; E-mail: sbhushan2016@gmail.com

ABSTRACT

Objectives: This study was carried out to evaluate physiochemical, adulteration and microbial quality of milk sold in Kathmandu valley.

Methods: The total of 20 milk samples randomly collected from different places of the valley including 10 pasteurized milk sample and 10 were raw milk sample, were processed for physiochemical and microbiological parameters.

Results: The laboratory analysis revealed that the pasteurized samples has less mesophilic count as well as coliform count than raw milk samples. About 55% milk samples showed neutralizer test positive and 10% of milk samples were found to be positive for sugar test. However, none of the samples were found to contain starch as an adulterant. The average fat content of milk samples was 3%. Fat percent was significantly different among different sources of sampling points. The highest milk fat content value was recorded at Pulchowk (3.7%). The average SNF was 7% in which the pasteurized sample had the highest average SNF (7.3%) and the raw milk had lowest average SNF (6.8%).

Conclusion: The significant variation in the physiochemical properties and microbial properties of the milk samples showed that people should be conscious about the consumption of market milk.

Key words: Fat, SNF, acidity, coliform count, adulteration

INTRODUCTION

Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, five days after and 15 days before parturition, which contains not less than 8.5 percent milk solids-not-fat and not less than 3.5 percent milk fat (U.S. Public Health Services, 1965).

When milk is drawn from the udder of a healthy animal, milk contains organisms from the teat canal. They are mechanically flushed out during milking. Milking under hygienic conditions with strict attention to sanitary practices will result in a product with low bacterial content and good keeping quality. But if maintained under conditions that permit bacterial growth, then the raw milk will develop a clean, sour flavor. This is due to fermentation of lactose to lactic acid (Pelczar et al. 2013)

Raw milk is milk that has not been pasteurized, a process of heating liquid foods to decontaminate them for safe drinking. Pasteurizing milk involves exposing milk to high temperatures for a short period of time to destroy all harmful bacteria that might be lurking in the milk.

Due to the fact that milk-borne diseases, chemical and physicalquality of milk are of public health importance, there is a need to screen the milk in informal market for the sake of consumer health protection (Mansouri & Sharifi, 2013).

The main purpose of this study is to assess quality of milk in Kathmandu valley.

Date of Submission: September 11, 2018
Date of Acceptance: December 7, 2018
Published Online: January 2019
DOI: http://doi.org/10.3126/tujm.v5i0.22295

MATERIALS AND METHODS

The milk samples were collected from Kathmandu valley, 10 pasteurized samples and 10 raw milk samples. A cross-sectional study was carried out in the department of Microbiology, D.A.V. College of Science and Humanities from January to May, 2018.All of the collected milk samples were placed for physiochemical analysis (fat percentage, total solid and solid not fat), adulteration test (starch, neutralizer, table sugar) and microbial analysis(bacterial count, coliform count) by following the standard laboratory manual as suggested by Marth (1978).

RESULTS

As shown in table 1, physiochemical analysis of the milk samples revealed that half of the pasteurized milk samples contained less percentage of fat whereas most of the raw milk samples contained good percentage of fat (Table 1).

Table 1: Physiochemical analysis of raw and pasteurized milk

Samples(milk)	Fat		TS		SNF		Total
	≥3%	< 3%	≥ 12.5%	< 12.5%	≥ 8%	< 8%	
Pasteurized	5(50%)	5(50%)	-	10(100%)	-	10(100%)	10
Raw	8(80%)	2(20%)	-	10(100%)	1(10%)	9(90%)	10

The milk samples were tested for adultarants such as starch, neutralizer and table sugar where neutralizer was found commonly used adultrant in pasteurized milk than raw milk. (Table 2).

Table 2: Adulteration test of milk for starch, neutralizer and table sugar

Adulterant	Pasteurized milk		Raw milk	
	Positive	Negative	Positive	Negative
Starch	-	10 (100%)	-	10 (100%)
Neutralizer	7 (35%)	3 (30%)	4(40%)	6(60%)
Table sugar	2(10%)	8(80%)	-	10(100%)
Total	10		10	

Among the tested milk, pasteurized milk showed 50% mesophilic count (≤10^5)whereasonly 25% in case of raw milk. Presence of coliforms inraw milk was 40% while only 20% in pasteurised milk (Table 3).

Table 3: Microbial analysis of milk samples

Samples(milk)	Samples with total mesophilic count		Coliform count		Total
	≤10^5 (cfu/ml)	>10^5 (cfu/ml)	Presence	Absence	
Pasteurized	10(100%)	-	4(40%)	6(60%)	10
Raw	5(50%)	5(50%)	8(80%)	2(20%)	10

Among the coliform, *E. coli* was found to be most predominant organism followed by *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp. in both sample.

Table 4: Distribution of coliform among samples

Sample	Pasteurized milk samples		Raw milk samples	
	No.	%	No.	%
E.coli	2	20	5	50
Klebsiella spp.	-	-	1	10
Enterobacter spp.	1	20	-	-
Citrobacter spp.	-	-	1	10
Total sample	**10**		**10**	

DISCUSSION

National Dairy Development Corporation, Nepal recommended a minimum of 3% fat and our study showed 3% as an average fat content of milk samples of Kathmandu Valley unlike the study showed by Teklemickeal (2012) and Janstora et al. (2010) who reported 3.86% and 3.79% of fat content, respectively. The fat content was significantly affected by the factor such as feed, parity, and stage of lactation. The average SNF of milk samples tested was found to be 7%. The SNF content of milk in this study is less than the finding of Debebe (2010) who reported a minimum (8.3 ± 0.30) and maximum (8.7 ± 0.36). According to NDDB, the SNF of milk should be 8%. The low SNF of the samples could have been attributed to a variety of factors including the feed, genetics, season of the year, stage of lactation and disease. The average total solid (TS) content of milk was found to be 10%. This value is less than the finding of Tekelemichael (2012) who reported TS of 12.58%. According to European Union, a recognized quality standard for total solids content of cow milk should not to be less than 12.5%. The variation could be due to difference in breed, feeding and management practices which have important effect on milk composition quality.

In this study, 55% of the tested milk samples were found to be adulterated with soda whereas among 10% of the milk sample with table sugar. The added percentage of soda as an adulterant was found to be more than that reported by Bastola, 2016. Soda and table sugar is commonly used as an adulterant to increase the SNF content of milk. Starch was not found to be used as an adulterant in this study as well as in the study by Bastola, 2016. It may be because as starch is expensive, difficult to be homogenized and can be detected and discovered by the consumer.

The study showed the average total mesophilic count of milk samples of Kathmandu valley was in the range of 10^5 bacterial colony forming unit per ml of milk. From our study, 60% of the total sample showed coliform which is more than the findings of Nahas et al. (2015) who found 55% coliform. The higher coliform count observed in the current study might be attributed to the initial contamination of the milk through the milkers, milk containers and milking environment, improper handling, storage and transport facilities.

In previous study by Ali (2006) on pasteurized milk, 2.6% *E. coli* and 1.3% *Enterobacter* spp. were detected. Similarly, this study showed 30% *E. coli* and 20% *Enterobacter* spp. which is higher than the previous study. In case of *Klebsiella* spp.and *Citrobacter* spp. the finding is similar with our study. In this study, 50% of the raw milk samples were found to be contaminated with *E. coli* which was less than that reported by Nahas et al. (2015) who found 55% milk samples contaminated with *E. coli*. In a study by Kaloianov and Gogov (1977) most encountered coliforms were *Citrobacter* (35%), *Enterobacter* (29.8%), *Klebsiella* (23.9%) and *E. coli* (11.3%) which is much higher than our study. The higher coliform count observed in this study may be due to poor hygiene of farm, the water used while milking and lack of knowledge of hygiene in farmers. Since it is not practical to produce milk that is always free of coliforms, even at high level of hygienic condition; their presence in raw milk to a certain extent may be tolerated. The presence of coliforms in pasteurized milk sample may be due to defective pasteurization, adulteration of pasteurized milk with raw milk and unsanitary handling.

CONCLUSION

The physiochemical properties of both milk samples should be maintained within the standard limits. To control the microbial contamination in raw as well as pasteurized milk the hygienic condition should be maintained. It is concluded that routine analysis of milk should be done regularly which helps to enhance their quality.

ACKNOWLEDGEMENTS

We would like to express our heartfelt gratitude to the entire team of Microbiology Department, DAV College, who made available every materials and place required for our study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Asamineu T and Eyassu S (2010). Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. *Agricultural and Biological Journal of North America* 2(1): 29-33.

Debebe W (2010). Physicochemical properties and

safety of street-vended milk in and aroundAddis Ababa city (Kotebe, Bishoftu and Chancho), M.Sc. (Agriculture) Thesis. *Haramaya University*, Ethiopia.

Manandhar S and Sharma S (2013). Practical Approach to Microbiology. National Book Center Pvt. Ltd. pp:245-250.

Marth EH (1978). Standard Methods for the Examinations of Dairy Products. American Public Health Association, Washington, DC. pp:416.

Mansouri-Najandi L and Sharifi HR (2013). Quality of raw milk in Kerman province.

O'Connor CB (1994). Rural Dairy Technology. ILRI Training Manual No. 1. International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia. pp:133.

Pelczar MJ, Chan E and Krieg N (2013). Microbiology. McGraw Hill Education(India) Private limited, pp:620

Teklemichael T (2012). Quality and Safety of Raw and Pasteurized Cow Milk Produced and Marketed in Dire Dawa Town, M.Sc.Thesis.Haramaya University, Ethiopia.

Walstra P, Geurts TJ, Noomen A, Jellema A and Van Boekel MAJS (1999). Dairy Technology: Principles of Milk Properties and Processes. 1st edition. New York: Marcel Dekker pp:149-170.

Diversity of Insecticidal Crystal Proteins (ICPs) of Indigenous *Bacillus thuringiensis* Strains

Ganga GC[1*], Charu Arjyal[1], Yamuna Khadka[1] and Sabina Dhamala[1]

[1]Tri-Chandra Multiple Campus, Ghanta Ghar, Kathmandu, Nepal

***Corresponding author:** Ganga G.C, Tri-Chandra Multiple Campus, Ghanta Ghar, Kathmandu, Nepal; E-mail: gangagc2@gmail.com

ABSTRACT

Objectives: The purpose of this study was to characterize the indigenous *Bacillus thuringiensis* (Bt) isolated from the soil samples of Terai.

Methods: A total of 50 soil samples were collected from cultivated and barren fields of Terai region. Isolation was carried out using the acetate selection protocol Nutrient broth (NB) was acetated by using 0.25M sodium acetate which is a selective enrichment method for isolation of Bt. Characterization of the isolate was done by phenotyping methods (microscopy and biochemical).

Results: No distinct variation was observed between the isolates of cultivable and uncultivable lands. Bt were categorized into 7 different types based on colony morphology. The dominant colony was fried egg type identical with the reference strain, followed by flat white type of colony. The result showed that even though the colony morphology was same but the ICPs (Insecticidal crystal proteins) shapes produced by them varied, rod shapes (53.57%), spherical (10.71%), ovoid (8.3%), amorphous (17.85%), capheaded (9.5%). ICPs morphology revealed the *cry1, cry2, cry3, cry4, cry8, cry 9, cry10* and *cry11* types of gene may be present in the native isolates.

Conclusion: This study represents the first report of several indigenous *Bacillus thuringiensis* strains with significantly different ICPs producing strains from hot tropical climate.

Key words: ICPs, amorphous, indigenous, microbial pesticides

INTRODUCTION

Biopesticides falls in major three categories Microbial pesticides, Plant pesticides, Biochemical pesticides (Çetinkaya, 2002; Kachhawa, 2017). They appear to be ecologically safer then commercial pesticides. Bt is a bacterium known for producing protein crystals with pesticidal properties. *Bacillus thuringiensis* biopesticide is commonly known as Bt (Jisha et al. 2013). Bt has been used commercially in the biological control of insect pests for the last 4 decades (Glare and Callaghan 1998). In Nepal farmers use chemical pesticides to control the pest of the crops. This is due to the unavailability of other agents to control the pest. These chemical pesticides are not specific and hazardous to the people and environment. The use of biopesticides in crop protection leads to decrease level of chemical pesticides in food chain and environment (Mishra et al. 2012). This study aims at isolating Bt from soil samples. There are a large number of toxins showing toxicity to one of many diverse pests produce by Bt. For these reasons there is current great interest in isolating novel strains of Bt with either unique host specificity or elevated toxicity so that it can be used in future as a biopesticide to control the pest of the crops. The genetic diversity and toxic potential of Bt strains differ from region to region (Hernández-soto et al. 2014). Bt strains have been collected and characterized in many parts of the country. But in case of Nepal the types of strain present in different region is not yet known. So the study aimed in collection and characterization of Bt found from the native soil of Nepal.

Date of Submission: November 13, 2018
Date of Acceptance: December 8, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22296

MATERIALS AND METHODS

Sample collection: About 10gm of soil was collected from Terai region (Province No: 2 and 3) of Nepal. The sample was collected from the soil where but was not used in the past. Fifty soil samples were collected. Samples were collected by scraping of the surface soil material, and from 5cm depth (Barathi et al. 2012). All samples were aseptically placed in sterile plastic bags. Soil samples were collected from cultivable land and uncultivable land. Collected samples were stored at 4°C before processing.

Isolation was carried out using the acetate selection protocol as described by Russell and Al 1987 with a slight modification. The nutrient broth was acetated by using 0.25M sodium acetate which is a selective enrichment method for isolation of *Bacillus thuringiensis* var *Kurstaki*, serotype 3a, 3b, 3c. Strain DOR Bt-1 was included in this study as a reference strain. To the sterile 9ml enriched media 1g of soil sample was added and incubated overnight at 35°C. After incubation the broth was heated at 100°C for 5 minute. Following heat treatment, the suspension was plated on nutrient agar plate (NA) by spread plate technique. The colony was enumerated, isolated and preserved in 60% glycerol containing NA as described by Ammouneh et al. 2011; Çetinkaya, 2002; Ralte et al. 2016 and stored at 4°C for further study.

Phenotypic characterization: The isolated organisms were identified by standard microbiological techniques including morphological, physiological and biochemical characteristics. The colony morphology was recorded by studying the shape, size, colony margin, opacity of the isolated colonies. Morphology of the vegetative cell, size, spore and crystal was studied by various staining technique like Gram staining, spore staining, negative staining, and Coomassie brilliant blue staining after incubation of culture for 72- 90 hours in a Nutrient broth. Physio-chemical characterization was done by biochemical test like, Indole test, MR test, VP test, citrate test, starch test, gelatin test, beta haemolysis test, sucrose, fructose, mannitol, lactose fermentation test, motility test and lecithinase test after incubation of the culture in the respective biochemical test media.

RESULTS

From the 50 soil samples collected from cultivable land and uncultivable land 84 isolates of Bt were obtained (Table 1).

Colony characterization: Bt showed different colony morphology in NA. On the basis of colony morphology the isolates were categorized into 7 different types. The dominant colony was fried egg type which was isolated from 50 soil samples followed by flat white type of colony, the colony code A resemble with the reference strain used in this study. On enumeration 10^6cfu/gm (colony forming unit) of soil Bt was obtained. The existing Bt showed biodiversity in morphology. On analysis of 25 samples from cultivable and 25 from uncultivable land Bt distribution in both types of samples was equal. There was no significant difference in the isolates obtained from both the soils (Table 2).

Microscopic characterization: The microscopic morphology reveals that they were Gram positive, spore producing and their vegetative size varies. For instance size of the vegetative cell of SN1 (1) by negative staining is 0.5x0.1µm and the size of SN1 (3) 5x2µm.

Table 1: Distribution of Bt isolates in soil samples collected from different localities

Province	District	No of samples	Sample coding	No of isolates
Province No: 3	Sindhuli	5	SN1(1), SN1(2), SN1(3), SN2(1), SN2(2), SN2(3), SN2(4), SN2(5), SN2(6), SN3(1), SN3(2), SN3(3), SN4(1), SN4(2), SN5(1), SN5(2)	16
Province No: 2	Chitwan	4	CW1(1),CW1(2),CW2,(1), CW2(2), CW3(1), CW4(1)	6
	Makwanpur	7	MP1(1), MP1(2), MP2(1), MP3(1), MP3(3), MP4(1), MP4(2), MP5(1), MP6(1), MP7(1)	10
	Parsa	6	P1(1), P1(2), P2(1),P2(2), P3(1), P4(1), P5(1), P6(1),	8
	Bara	6	B1(1),B1(2),B1(3), B2(1), B3(1), B4(1), B5(1), B6(1)	8
	Rautahat	4	CHA1(1), CHA1(2), CHA2(1), CHA2(2), CHA3(1), CHA3(2), CHA4(1), CHA4(2)	8
	Sarlahi	6	S1(1), S2(1), S2(2), S3(1), S3(3),S4(1), S4(2), S5(1), S6(1)	9
	Mahottari	6	M1(1), M1(2), M1(3) M1(4), M2(1), M2(2), M3(1), M4(1), M5(1), M6(1)	10
	Dhanusa	6	D1(1), D1(2), D2(1), D2(2), D3(1), D3(2), D4(1), D5(1), D6(1)	9
Total		50 soil samples		84 Bt isolates

Coomassie brilliant blue staining (CBBS): The isolates were further analyzed by Coomassie brilliant blue staining (CBBS) technique for observing the presence of crystal protein and the shape of the crystal shows that even though the colony morphology was same but the ICPs shapes produced by them varied (Table 2). On calculating the types of ICPs produced by the 84 isolates in terms of percentage the highest prevalent ICPs was rod shaped crystal protein (Figure 1). In this study the rod shaped ICPs was found to be dominant. The rod shaped ICPs produced by the isolates were different (Table 2)

Table 2: Colony morphology of the native isolates

S.No.	Colony code	Shape of the crystal	Morphology	No. of isolates	cfu/gm of soil
1	A	Amorphous, rod shaped, ovoid, spherical	White, raised wavy (fried egg type)	50	$5x10^6$
2	B	Long rod, short rod, spherical	White, flat, irregular	23	$5x10^6$
3	C	Short rod shaped	Yellow, raised, smooth	1	$1x10^2$
4	D	Short rod shaped	White, raised, round, smooth, mucoid	1	$1x10^1$
5	E	Long rod	Shiny(watery type),raised, round	1	$1x10^2$
6	F	Rod shaped	White rhizoid type of colony	1	$1x10^2$
7	G	Cap headed, spherical	white membrane slightly raised center	7	$3x10^4$

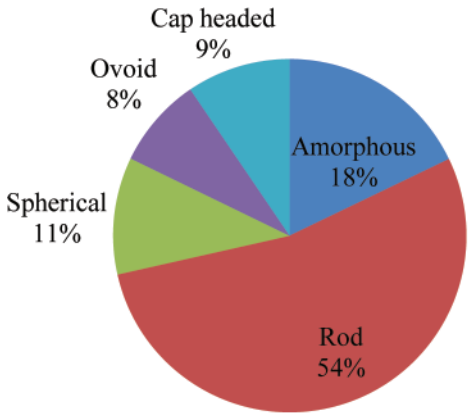


Figure 1: Distribution of ICPs% in the native isolates

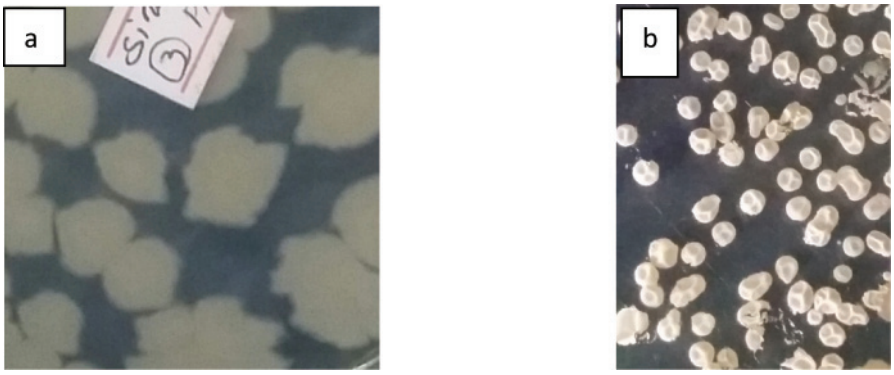
Based on ICPs morphology, the suspected cry gene present in the native isolates might be *cry1, cry2, cry3, cry4, cry8 cry 9 cry10* and *cry11*(Table3).

Table 3: ICPs types, their percentage and suspected *cry* gene in 84 isolates

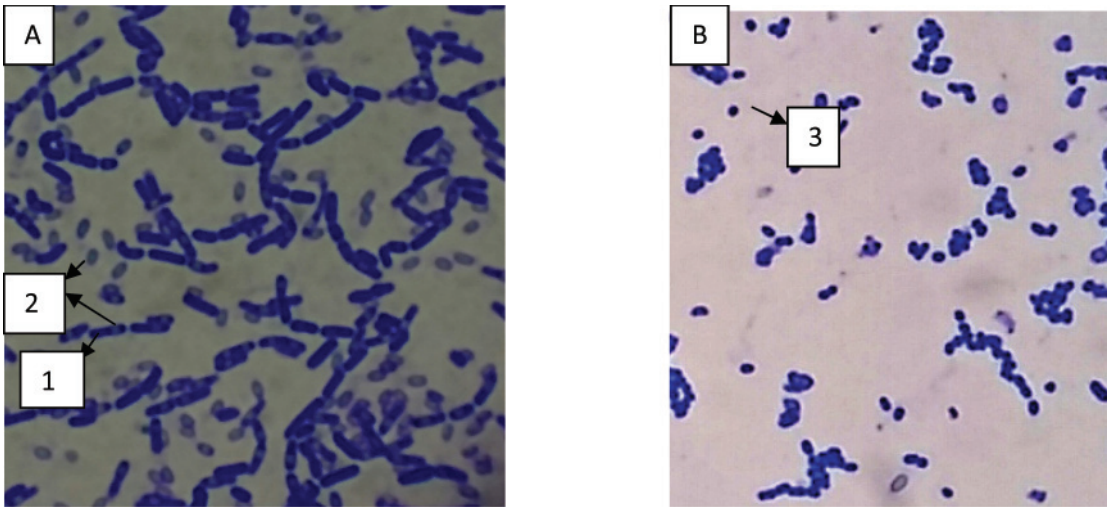
S.No.	ICPs shapes	ICPs%	Suspected *cry* gene
1	Amorphous	17.85%	*cry 4 cyt*
2	Rod	53.57%	*cry 1*
3	Spherical	10.71%	*cry1, cry3, cry9, cry8*
4	Ovoid	8.3%	*cry1, cry3, cry9, cry8*
5	Cap headed	9.5%	*cry9*

Source for *cry* gene: Ibrahim et al. 2010; Lenina et al. 2014

The dominant colony was fried egg type photo1 b and the flat white irregular type photo1a.



Photograph 1: Colony morphology of the native isolates a: SN1 (2) flat white irregular, b:ML5(1) fried egg type



Photograph 2: Insecticidal crystal morphology. A) Spore attached and separated rod shaped crystal shapes.(1: rod attached to the colourless spore rod is dark blue in colour. 2: colour less spores). B) 3: spherical spores

Biochemical profiling: Based on the biochemical characteristics all the isolates were positive for catalase, oxidase, starch, and gelatin hydrolysis, beta hemolytic, sucrose, fructose mannitol, lactose fermentation and lecithinase test but showed a variable reaction in Indole, MR, VP, Motility and citrate test.

DISCUSSION

Bacillus thuringiensis entomopathogenic pesticide has become as safe and successful microbial pesticide worldwide. In Nepal, in comparison with chemical pesticide the biopesticides are less commonly used due to the unavailability or unawareness. Screening the environment for a potent Bt strain for insect management has become necessary. In this study isolation of indigenous Bt was performed by collecting 50 soil sample from tropical region of Terai of province 2 and 3 (Table 1). Soil sample was collected from uncultivable and cultivable land; collected 10g of soil was stored at 4˚C before isolation. From the 50 soil samples 84 isolates of Bt strains were obtained. Similar type of result was also obtained by Ralte et al. 2016. There is no distinct variation in the isolates obtained from the cultivable and uncultivable land as well as the cfu/gm of soil in both types of soil. This may be due to the sporulating capacity of Bt strain the spore can remain in a dormant stage until the favorable condition

prevail. There was no significant difference in the isolates number as well as the colony type obtained from both type of soil. On enumeration the distribution of Bt in indigenous soil showed an average of 10^6cfu/g of soil. The colony morphology of Bt shows biodiversity in strain present in native soil.

The isolation technique used during the study period was effective for the isolation of Bt from the native samples. The enrichment was done in 0.25M sodium acetate broth for overnight at 35°C during this period the spores of Bt remain in dominant stage as the sodium acetate inhibit the germination of Bt spore, where as other spore forming *Bacillus* species germinate to produce vegetative cell. After incubation the broth was heat treated at 100°C for 5m to kill the vegetative cell of other *Bacillus* species were as the spore of the Bt withstand the heating and on spreading on NA the spore of Bt germinated to produce a visible colony. As the selection process for isolation of Bt differed from the conventional methods used for isolation of spore forming bacteria, the new finding in this research work is heating the overnight incubated enrichment broth at 100°C for 5m enhance the isolation of axenic culture of Bt strain. Heat treated at 80°C performed by others shows that the mix culture of *Bacillus* species isolation. So the heat shocked 100°C used for isolation is efficient to obtain the pure culture of Bt from soil samples, the reason behind the isolation may be due to the accumulation or synthesis of spore peptidoglycan which is different from the other *Bacillus* endospore producing bacteria. According to Peng et al. 2016 Bt endospore are encircled by an additional loose-fitting layer called the exosporium, which is not present on other species such as *Bacillus subtilis*, for which the coat constitutes the outermost layer of the mature spore. The exosporium is a balloon-like layer that acts as the outer permeability barrier of the spore and contributes to spore survival and virulence as it contains approximately 20 different proteins.

On observing the colony morphology of the 84 isolates, based on colony morphology the isolates are categorized into 7 different types (Table 2). The dominant colony type was fried egg type (A) Photograph 1(b). Which was present in 50 soil samples followed by flat white type of colony (B) Photograph 1(a).The phenotypic characterization based on morphology reveled that Bt strain of native soil shows a wide range of colony types. Some of the colony morphology (Table2) like (Yellow, raised, smooth,) (White, raised, round, smooth, mucoid) (Shiny (watery type) raised, round) (White, rhizoid type of colony) (white membrane slightly raised center) this types of colony morphology was not found in all the soil samples. As well as they were not numerous as the other dominant types of colony code A and B. The colony code A was identical with the reference strain.

Characterization of Bt can be done in various ways such as biotyping, flagella serotyping, protein profiling, plasmid profiling, PCR amplification, molecular finger print etc. In this study isolated Bt strains were characterized by phenotyping methods like morphological and biochemical. The 84 isolates on Gram staining the microscopic morphology revels that they are rod shaped, Gram positive, the vegetative cell size differ among the isolates. The size variation determines that the native Bt strains differ in the capacity to produce different types of ICPS. On spore staining the native Bt isolates produced a central ellipsoidal spore on incubation in NB at 35°C for 72 hours. The 84 isolates were confirmed as Bt by detecting the crystal protein which was observed by coomassie brilliant blue staining (CBBS) technique. coomassie brilliant blue R-250 (CBB) is a popular and widely used dye for detection of proteins by gel electrophoresis. As the parasporal body or crystal protein of ICPS (insecticidal crystal protein) is made of protein compound which can be stained by CBB as dark blue color and the spore remain unstained and the vegetative cell takes up the light blue stain (Rampersad et al. 2002). The presence of insecticidal crystal protein (ICPS) in an 84 isolates proves that the native soil of Nepal posses diverse Bt subspecies with different ICPs (*cry* gene), thus the assessment of Cry proteins is a good basis to study insecticidal activity of Bt as well as assess the habitat containing a novel Bt strains. The authors Kebdani et al. 2016 could not discriminate between the two species until after the observation by scanning electron microscope, which allowed the visualization of the parasporal crystal which is present only in *B. thuringiensis* and responsible for entomopathogenic activity of this bacterium against several devastating

species. Morphology of ICPS provides valuable information about the type of *cry* gene harboring Bt strain. In this study varied morphology of the ICPS was observed, the ICPs were characterized into 5 major groups' viz., amorphous, spherical, rod shaped (long rod, short rod), ovoid and cap headed, the bipyramid type of ICPs was not seen in this 84 isolates. This result is consistent with the result of Ralte et al. 2016 isolated two types of ICPs spherical and oval from 55 soil samples. According to Rana et al. 2002 only 8 Bt strains from 350 soil samples collected from the five development region of Nepal produced bipyramidal ICPs and were effective against the cabbage butterfly, *Pieris brassicae nepalensis* and the cotton bollworm, *Helicoverpa armigera Hubner,* indicate the presence of few bipyramid ICPs in native soil. Based on the position of ICPs while observing in a microscope two different types of ICPs were observed viz., free ICPs and spore attached ICPs photograph2(A,B). Analysis of this ICPs in this 84 native isolates showed most of the ICPs were attached with the spore, the amorphous, rod shapes and cap headed. The SDS-PAGE analysis of spore coat profile carried by King et al. 2012 showed that spore coat of HD-1 and SN5 isolates spore coat contain Cry1 and Cry2 proteins. So the ICPs attached to the spore isolated from native soil sample may code for the Cry1 or Cry2 protein. *B. thuringiensis* strains can carry one or more *cry* genes, (Crickmore et al. 2014; Ibrahim et al. 2010) and therefore, they may synthesize one or more crystal protein. Diversity, distribution and abundance of *cry* gene type 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 (Çetinkaya, 2002). According to Crickmore et al. 1998 133 crystal proteins were categorized .

Based on the morphology of ICPs the indigenous Bt stains can be related to the type of *cry* gene present in it. The amorphous type of ICPs producing isolates may possess *cry4* gene (Çetinkaya, 2002; Ralte et al. 2016) similarly spherical and ovoid related to *cry1or cry3 or cry8 or cry9,* (Ralte et al. 2016; Shishir et al. 2015). Rod shaped ICPs may be related to rectangular type according to other research articles that relates to *cry1* gene (Çetinkaya, 2002). During this study the dominant type of ICPs was rod shaped in different size was observed. Cap headed related to *cry9* gene. According Noguera and Ibarra 2010 *B. thuringiensis* shows great variability, as has been demonstrated by the huge number of strains isolated around the world by the number of serotypes known to date a total of 84 and by the great number of different *cry* gene sequences accumulated so far a total of 492 as well as by the number of molecular characterization tools that have been developed, such as sequencing of the flagellin gene and of the *gyrB* genes, the band patterns from repetitive extragenic palindromic-PCR analyses, and the plasmid patterns, among others all indicating the great variability within this species. While many Cry proteins have useful pesticidal properties and may be exploited for the control of insect pest in agriculture (Palma et al. 2014). 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). The parasporal crystal of Bt subsp. *kurstaki* HD-73 contains Cry1Ac protein only, whereas the parasporal crystal of HD1 strain, which belongs to the same subspecies, is comprised of five different Cry toxins Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab (Ibrahim et al. 2010). The spherical ICPs producing native 11% isolates may also posses Cry1 protein according to (Shishir et al. 2015). So the isolates with the spherical crystal protein may be *B. thuringiensis* subsps *Kurstaki.* For the identification of subspecies genotyping characterization has to be done, mainly the PCR methods for the detection of *cry or cyt* gene along with the SDS-PAGE may help in identifying the subspecies as well as the molecular weight of the crystal protein.

Biochemical profiling: Biochemical test was performed but it doesn't provide any evidence for the identification of Bt subspecies.on the basis of ICPS production by coomassie brilliant blue staining(CBBS) technique (Rampersad et al. 2002). According to Chen and Tsen 2002 when a large number of *Bacillus* strains was tested, results showed that discrimination between *B. cereus* and Bt is difficult to distinguish, a single feature, such as the presence of a ICPs or *cry* gene, may be reliable. One of the confidential methods to distinguish BT from other *Bacillus* spp is by observing the ICPs production

by microscopy. Identification of *cry* gene content by PCR is the most effective techniques in screening large Bt.

CONCLUSION

This study revealed that several indigenous Bt strains with significantly different morphological and ICPs producing stains exists in hot tropical climate of Nepal. Some native Bt strain shows identical crystal protein with the reference strain. This shows the possibility of using natural bioinsecticides, based on the local strains of Bt.

ACKNOWLEDGEMENTS

The authors express sincere gratitude and heartiest honor to University Grants Commission, Sanothimi Bhaktapur for providing grant for this research. Also like to thank the Department of Microbiology, Tri-Chandra Multiple Campus, Ghanta Ghar, Kathmandu, Nepal, for providing the laboratory facilities to carry the research successfully.

REFERENCES

Ammouneh H, Harba M, Idris E and Makee H (2011) Isolation and characterization of native bacillus thuringiensis isolates from Syrian soil and testing of their insecticidal activities against some insect pests. *Turkish Journal of Agriculture and Forestry 35*(4): 421–431.

Barathi S, Sangeetha P, Karthick C, Govindaraju S and PI A (2012) Diversity in Protein Profile of Bacillus thuringiensis Strains Isolated From Varied Soil Environments. *Journal of Pharmacy Research 5*(9): 4645–4647.

Çetinkaya F (2002) Isolation of Bacillus thuringiensis and Investigation of Its Crystal Protein Genes. A Dissertation Submitted to the Graduate School in Partial Fulfillment of the MASTER OF SCIENCE Department Biotechnology and Bioengineering Major B. *Biotechnology and Bioengineering* pp 62.

Chen ML, and Tsen HY (2002) Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and gyrB gene based PCR primers and sequencing of their annealing sites. *Journal of Applied Microbiology* 92: 912–919.

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, and Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews : MMBR*, *62*(3): 807–813.

Crickmore N, Zeigler DR, Schnepf E, Vanrie J, Lereclus D, Baum J, Bravo A, Dean DH (2018) *Bacillus thuringiensis* toxin nomenclature. *http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/*

Glare TR, and Callaghan MO (1998) Environmental and health impacts of *Bacillus thuringiensis israelensis*. Report for the Ministry of Health. Biocontrol and Biodiversity,Grassland Division,Ag Research Lincoln pp 5-10

Hernández-soto A, José RS, Rica C, Agronomía EDe, Agroalimentarias FDC, Rica UD and Rica C (2014) Diversity of *Bacillus thuringiensis* strains isolated from coffee plantations infested with the coffee berry borer *Hypothenemus hampei*. *Revista Biologia Tropical* 52(3): 757-764.

Ibrahim MA, Griko N, Junker M and Bulla LA (2010) A genomics and proteomics perspective. *Bioengineered Bugs*.

Jisha VN, Smitha RB, Benjamin S and Al ET (2013) An Overview on the Crystal Toxins from *Bacillus thuringiensis*. *Advances in Microbiology 3:* 462–472.

Kachhawa D (2017) Microorganisms as a biopesticides. *Journal of Entomology and Zoology Studies 5*(3): 468–473.

Kebdani M, Mahdjoubi M, Cherif A, Gaouar BN and Rebiahi SA (2016) Molecular characterization of local strains of *Bacillus thuringiensis* in the North of Algeria. *African Journal of Microbiology Research 10*(45):1880–1887.

King PJH, Ong KH, Sipen P and Mahadi NM (2012) Toxicity of local Malaysian *Bacillus thuringiensis* subspecies *kurstaki* against *Plutella xylostella*. *African Journal of Biotechnology* 11(56):11925-11930

Lenina NK, Naveen Ak, Sozhavendan AE, Balakrishnan N, Balasubramani V. Udayasuriyan V (2014) Characterization of parasporin gene harboring Indian isolates of *Bacillus thuringiensis*. *Biotech*

3(4): 545–551.

Mishra J, Tewari S and Singh S (2012) Biopesticides: Where We Stand? *Plant Microbes Symbiosis: Applied Facets* 322-2068-8

Noguera PA, and Ibarra JE (2010) Detection of new cry genes of *Bacillus thuringiensis* by Use of a Novel PCR primer system. *Applied and Environmental Microbiology 76*(18): 6150–6155.

Palma L, Muñoz D, Berry C, Murillo J, Caballero P and Caballero P (2014) *Bacillus thuringiensis* toxins: An overview of their biocidal activity. *Toxins 6*(12): 3296–3325. https://doi.org/10.3390/toxins6123296

Peng Q, Kao G, Qu N, Zhang J, Li J and Song F (2016) The Regulation of Exosporium-Related Genes in *Bacillus thuringiensis. ScientificReports 6*: 1–12.

Ralte Z, Nachimuthu S and Guruswami G (2016) Diversity and toxicity of *Bacillus thuringiensis* from shifting cultivation (jhum) habitat. *Biocontrol Science 21*(2): 99–112.

Rampersad J, Khan A and Ammons D (2002) Usefulness of staining parasporal bodies when screening for *Bacillus thuringiensis*. *Journal of Invertebrate Pathology 79*(3): 203–204.

Rana P, Sijapati J, Rana N and Regmi C (2002) Distribution of *Bacillus thuringiensis* in the Soils of Nepal and Their Bioassay. *Nepal Journal of Science and Technology 4*: 27–31.

Russell S, and Al T E T (1987) Selective Process for Efficient Isolation of Soil *Bacillus. Applied and Enivironmental/ Microbiology 53*(6): 1263–1266.

Shishir A, Akter A, Khan SN and Hoq MM (2015) Novel Toxicity of *Bacillus thuringiensis* Strains against the Melon Fruit Fly *Bactrocera cucurbitae* (Diptera : Tephritidae). *Biocontrol Science 20*(2): 115-123.

Methicillin Resistant *Staphylococcus aureus* Isolated from Wound Infections

Jyoti Shrestha[1], Krishna Govinda Prajapati[2], Om Prakash Panta[1], Pramod Poudel[1] and Santosh Khanal[1*]

[1]Department of Microbiology, National College, Kathmandu, Nepal

[2]Department of Microbiology, B and B Hospital, Lalitpur, Nepal

***Corresponding author:** Santosh Khanal, Department of Microbiology, National College, Kathmandu, Nepal
E-mail: santoshkhanal007@gmail.com

ABSTRACT

Objectives: The aim of this study was to determine the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and assess antibiotic resistance pattern of the isolates from wound infections.

Methods: A total of 706 wound specimens including pus and wound swab were processed in the laboratory of B and B Hospital, Lalitpur from May to October 2014. The specimens were cultured on blood agar and mannitol salt agar plates and incubated at 37°C for 24 hours. Antibiotic susceptibility test was performed by modified Kirby-Bauer disc diffusion method. Strains resistant to cefoxitin (30mcg) with inhibition zone ≤ 21mm were identified as MRSA.

Results: Out of 366 bacterial isolates, 90 (24.6%) were *S. aureus* and among them 16.7% were MRSA and 54.4% multi-drug resistant (MDR). All isolates were sensitive to vancomycin and most of the isolates were sensitive to cefoxitin (83.3%). High rate of resistance was observed towards penicillin (98.9%) and ampicillin (86.7%). All MRSA isolates and 52.9% of methicillin sensitive *S. aureus* (MSSA) were MDR.

Conclusion: MRSA incidence is increasing in the population, and therapeutic measures are few and accompanied by diverse side effects. It is noteworthy to state that vancomycin is still the first line drug although vancomycin-resistant strains have been reported.

Key words: Wound infection, antimicrobial resistance, MRSA, MDR

INTRODUCTION

Wound is a breach in the skin, which can lead to infections with the presence of replicating microorganisms with the discharge of pus (Dulon et al. 2011). *Staphylococcus aureus* has been recognized as an important cause of disease around the world ranging from relatively mild infections of the skin and soft tissue to life-threatening sepsis. The emergence of strains resistant to methicillin and other antimicrobial agents has become a major concern, especially in the hospital environment (Spagnolo et al. 2014).

Methicillin resistance is mediated by PBP-2a, a penicillin binding protein encoded by the *mecA* gene that is located on a mobile genetic element called a Staphylococcal cassette chromosome (Mahasenan et al. 2017). The relative ease of transfer of this genetic element explains the growing resistance to β-lactam antibiotics such as penicillin and its chemical derivatives as well as cephalosporins. MRSA is now endemic in both community and hospital environments (Sit et al. 2017).

MRSA strains have spread among hospitals and disseminated worldwide. The development of resistance to multiple antibiotics and control of disease transmissions by MRSA isolates in hospitals have been recognized as a major challenge (Chen and Huang 2014). The recent studies conducted in different parts of Nepal reported the rate of MRSA to be 21.1% (Khanal et al. 2018), 35.5% (Adhikari et al. 2017) and 43.6% (Raut et al. 2017). The knowledge on prevalence of MRSA and their current antimicrobial profile has become

Date of Submission: August 7, 2018
Date of Acceptance: December 10, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22297

necessary in the selection of appropriate empirical treatment of these infections. This study aimed to determine the prevalence of MRSA in wound infections among the patients attending B and B hospital and their susceptibility pattern towards various antimicrobial agents.

MATERIALS AND METHODS

Study site and population

The research work was conducted at laboratory of Department of Microbiology, B and B Hospital, Gwarko, Lalitpur from May to October 2014. During this period, a total of 706 aspirated pus and wound swabs from male and female patients of age group 1-87 years was collected, properly labelled and transferred to laboratory for further processing.

Isolation and identification of *S. aureus*

The specimens were directly inoculated on blood agar and mannitol salt agar plates and incubated at 37°C for 24 hours. The isolates were identified by morphological appearance of the colonies, microscopic findings; biochemical properties like catalase production test and coagulase production test by slide and tube methods. The colonies with golden yellow pigmentation on mannitol salt adgar and cream colored hemolytic or non-hemolytic on blood agar; Gram-positive cocci in grape-like cluster in Gram staining and catalase and coagulase tests positive were identified as *S. aureus* (Forbes et al. 2007).

Antibiotic susceptibility testing

Antibiotic susceptibility tests of all *S. aureus* isolates towards various antibiotics were performed by modified Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI 2018). In this study the antibiotics used were amoxicillin (AMX/10mcg), cefoxitin (CX/30mcg), ciprofloxacin (CIP/5mcg), cotrimoxazole (COT/25mcg), erythromycin (E/15mcg), gentamicin (GEN/10mcg), ofloxacin (OF/5mcg), penicillin (P/10mcg) and vancomycin (VA/30 mcg). Screening for methicillin resistance was performed by cefoxitin disc diffusion method and interpreted according to CLSI (2018). Isolates with diameter of zone of inhibition (ZOI) ≥ 22mm were identified as MSSA and isolates with ZOI ≤ 21mm identified as MRSA. Isolates resistant to three or more classes of antibiotics were considered MDR (Nair et al. 2013).

RESULTS

A total of 706 wound specimens included 39 aspirated pus and 667 wound swabs in which bacterial growth was observed in 366 wound specimens (Table 1).

Table 1: Bacterial growth pattern in wound samples

Specimens	Bacterial growth N (%)	No growth N (%)	Total sample
Wound swab	352 (52.8)	315 (47.2)	667 (94.5)
Aspirated pus	14 (35.9)	25 (64.1)	39 (5.5)
Total	**366 (51.8)**	**340 (48.2)**	**706 (100)**

Among 366 isolated different organisms, 90 (24.6%) were identified as *S. aureus*. *Klebsiella* species was found to be most predominent Gram negative bacteria constituting 89 (24.3%). Other most frequently isolated organisms were *E. coli* (22.1%) and *Enterococcus* spp (11%) (Table 2).

Table 2: Different bacterial isolates from wound infection

Organisms	Number of isolates	%
Staphylococcus aureus	90	24.6
Klebsiella spp	89	24.3
E. coli	81	22.1
Enterococcus spp	40	11
Proteus spp	35	9.6
Pseudomonas spp	21	5.7
Citrobacter spp	6	1.6
Acinetobacter spp	4	1.1
Total	**366**	**100**

Out of 706 patients under study, 470 (66.6%) were inpatients and 236 (33.4%) were outpatients. The culture positive cases in inpatients were 216 (59%) and 150 (41%) in outpatients (Table 3).

Table 3: Case wise distribution of patients

Patient type	Specimen N (%)	Culture positive N (%)
Inpatients	470 (66.6)	216 (59)
Outpatients	236 (33.4)	150 (41)
Total	**706**	**366**

The higher numbers of isolates of *S. aureus* were recovered from the age group 31-40 years (20%) followed by age group 21-30 years (17.8%). Least isolates were from age group 71-80 years (10%) and none were isolated from 80 years and above (Table 4).

Table 4: *S. aureus* isolates from different age groups of patients

Age group (years)	Total	*S. aureus*	
		N	%
<10	41	6	6.7
11 - 20	72	11	12.2
21 - 30	85	16	17.8
31 - 40	196	18	20
41 - 50	174	13	14.4
51 - 60	35	12	13.3
61 - 70	60	5	5.6
71 - 80	33	9	10
80 above	10	0	0
Total	**706**	**90**	**100**

Out of 366 culture positive cases, a total of 90 *S. aureus* were isolated. Among them, 61 isolates (67.8%) were from male patients and 29 (32.2%) were from female patients. The distribution of *S. aureus* was higher in males than in females and the result was statistically significant ($p<0.05$) (Table 5).

Table 5: Distribution of *S. aureus* according to the gender of patients

Gender	*S. aureus*		*P*-value
	N	%	
Male	61	67.8	
Female	29	32.2	**0.005**
Total	**90**	100	

S. aureus isolated were tested with different antibiotics by using modified Kirby-Bauer disc diffusion method. Antibiotic susceptibility pattern of *S. aureus* isolates showed that the high proportion of isolates were resistant to penicillin (n=89, 98.9%) and amoxicillin (n=78, 86.7%). All the isolates of *S. aureus* were susceptible to vancomycin and most of the isolates were susceptible to cefoxitin (n=75, 83.3%) and gentamicin (n=48, 53.3%). The prevalence of MRSA was found to be 16.7% as shown by resistance with cefoxitin (Table 6).

Table 6: Antibiotic susceptibility pattern of *S. aureus* (N = 90)

Antibiotics	Sensitive N (%)	Intermediate N (%)	Resistant N (%)
Amoxicillin	10 (11.1)	2 (2.2)	78 (86.7)
Penicillin	1 (1.1)	0	89 (98.9)
Gentamicin	48 (53.3)	8 (8.9)	34 (37.8)
Cotrimoxazole	41 (45.6)	27 (30)	22 (24.4)
Cefoxitin	75 (83.3)	0	15 (16.7)
Erythromycin	12 (13.3)	29 (32.2)	49 (54.4)
Vancomycin	90 (100)	0	0
Ofloxacin	30 (33.3)	9 (10)	51 (56.7)
Ciprofloxacin	30 (33.3)	7 (7.8)	53 (58.9)

Among 90 *S. aureus* isolates, 49 (54.4%) were found to be MDR. All 15 MRSA isolates and 34 (45.3%) MSSA were MDR (Table 7).

Table 7: MDR pattern of *S. aureus*

Drug resistance	MRSA N (%)	MSSA N (%)	Total *S. aureus* N (%)
MDR	15 (30.6)	34 (69.4)	49 (54.4%)
Non-MDR	0	41 (100)	41 (45.6%)
Total	**15 (16.7)**	**75 (83.3)**	**90 (100%)**

DISCUSSION

MRSA has emerged as a serious public health problem globally. Because of the ability of Staphylococci to acquire antimicrobial resistance over time, MRSA has been and will continue to be a problem in the future. Today, most of the MRSA are multi-drug resistant thus causing a clinical problem as antibiotic treatment becomes useless. As such, this study was undertaken to determine the prevalence of *S. aureus* and MRSA, along with their antibiotic susceptibility patterns.

Out of 706 specimens, 366 were culture positive cases and *S. aureus* (24.6%) was found to be predominant bacteria causing wound infection. Pandey et al. (2012) and Hussain et al. (2005) reported similar results with bacterial growth of 26.1% and 20% respectively. This suggested that *S. aureus* is the constantly isolated pathogen in hospital settings and regular intervention is required for the control of infection caused by this organism.

The present study showed that male (67.8%) had a higher infection rate of wounds than females, which was statistically significant ($p<0.05$) and similar result was found in a study carried out by Mama et al. (2014). Some other studies showed statistically insignificant results in the distribution of *S. aureus* between males and females (Adhikari et al. 2017; Khanal et al. 2018). The number of wound specimens was highest in 31-40 years of age group with higher incidence of *S. aureus* infection (20%). This might be explained by the fact that this group of population is mainly involved in occupations such as farming, construction works, transportation and industry works where the likely exposure to trauma is common.

Among 470 samples from inpatients, 216 culture positive results were observed and 150 positive cases were observed from 236 samples of outpatients. The prevalence of *S. aureus* was higher in outpatients (33.3%) as compared to inpatients (18.5%). The result was not in the agreement with the study done by Bhatta et al. (2014) who have reported higher prevalence of *S. aureus* in hospital setting accounting 54% as compared to outpatients (46%).

S. aureus isolated in this study in overall showed higher rate of sensitivity towards cefoxitin (83.3%) followed by gentamicin (53.3%) and cotrimoxazole (45.6%) whereas higher rate of resistance was observed towards penicillin (98.9%) and amoxicillin (86.7%) followed by ciprofloxacin (58.9%) and erythromycin (54.4%). In this study, most of *S. aureus* isolates were resistant towards β-lactam antibiotics making them the least effective drugs. The high resistance to penicillin and total susceptibility to vancomycin is commonly noted for *S. aureus* isolated at different hospitals worldwide (Adhikari et al. 2017). Bacterial

resistance to β-lactam antibiotics is primarily due to the production of β-lactamase that opens its β-lactam ring rendering them to deactivate and also its penicillin binding protein (PBP2a) (Richmond 2000). None of the isolates were resistant to vancomycin as this antibiotic has unique mode of action to bacteria. It acts by inhibiting the second stage of cell wall synthesis of various susceptible bacteria altering bacterial cell wall membrane permeability and RNA synthesis (Rijal et al. 2008). Knowledge about MRSA and carrier status needs to be raised among the health staffs of the hospital and control measures need to be implemented consistently in order to reduce the burden of MRSA infection in the hospital environment (Holmes et al. 2005).

Among 90 isolates of *S. aureus* 49 isolates (54.4%) were found to be MDR. Similar studies by Banjara (2002), Rajbhandari et al. (2003) and Surucuoqlu et al. (2005) reported 40%, 54.9% and 31% MDR *S. aureus* respectively from wound sample. The overuse of antibiotics clearly drives the evolution of resistance. In bacteria, antibiotic resistance occurs due to horizontal gene transfer among different species of bacteria and spontaneously through mutation. Antibiotics remove drug-sensitive competitors, leaving resistant bacteria behind to reproduce as a result of natural selection. Despite warnings regarding overuse, antibiotics are overprescribed worldwide (Read and Woods 2014).

The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives (Golkar et al. 2014). The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (Spellberg and Gilbert 2014).

CONCLUSION

S. aureus was the most common bacteria causing wound infection and the prevalence of MRSA and MDR *S. aureus* was 16.7% and 54.4% respectively. Most of the isolates were sensitive towards and gentamicin and resistant towards penicillin and amoxicillin. Continuous surveillance on antimicrobial susceptibility of *S. aureus* is essential for the detection of emerging trends and the development of appropriate therapeutic strategies.

ACKNOWLEDGEMENTS

We are most grateful to the managing director of B and B Hospital for giving us the clinical facilities and the necessary support. We would like to thank all the staff of the National College for their support and suggestions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adhikari R, Pant ND, Neupane S, Neupane M, Bhattarai R, Bhatta S, Chaudhary R and Lekhak B (2017). Detection of Methicillin Resistant *Staphylococcus aureus* and Determination of Minimum Inhibitory Concentration of Vancomycin for *Staphylococcus aureus* Isolated from Pus/Wound Swab Samples of the Patients Attending a Tertiary Care Hospital in Kathmandu, Nepal. *Can J Infect Dis Med Microbiol* Volume 2017, Article ID 2191532.

Banjara MR (2002). Study of air, water and wound Infection in different wards of TUTH. A dissertation submitted to the central department of Microbiology, TU, Kathmandu, Nepal. pp 42-47.

Bhatt CP, Karki BMS, Baral B, Gautam S, Shah A and Chaudhary A (2014). Antibiotic susceptibility pattern of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in a tertiary care hospital. *Journal of Pathology of Nepal* **4**: 548 – 551.

Chen CJ and Huang YC (2014). New epidemiology of *Staphylococcus aureus* infection in Asia. *Clin Microbiol Infect* **20(7)**: 605-623.

CLSI, Wayne, PA, USA (2018). Performance standards for anitmicrobial susceptibility testing, 28th informational supplement M100-S28.

Dulon M, Haamann F, Peters C, Schablon A and Nienhaus A (2011). MRSA prevalence in european healthcare settings : a review. *BMC Infect Dis* **11:** 138.

Forbes BA, Sahm DF and Weissfeld AS (2007). *Staphylococcus*, *Micrococcus* and Similar Organisms. In Baily and Scott's Diagnostic Microbiology. 12th ed. Mosby Inc: St. Louis, pp. 254-280.

Golkar Z, Bagazra O and Pace DG (2014). Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Count* **8(2)**: 129–136.

Holmes A, Ganner M, McGuane S, Pitt TL, Cookson BD and Kearns AM (2005). *Staphylococcus aureus*

isolates carrying frequency, characterization and association with clinical disease. *J Clin Microbiol* **43(5)**: 2384-2390.

Hussain S, Shams R, Ahmad K, Perveen R and Riaz B (2005). Prevalence of methicillin Resistant *Staphylococcus aureus* (MRSA) in Surgical Site Infections in a Tertiary Care Hospital. *Int J Path* **3(2)**: 81-85.

Khanal LK, Adhikari RP and GuragainA (2018). Prevalence of Methicillin Resistant *Staphylococcus aureus* and Antibiotic Susceptibility Pattern in a Tertiary Hospital in Nepal. *J Nepal Health Res Counc* **16(39)**: 172-174.

Mahasenan KV, Molina R, Bouley R, Batuecas MT, Fisher JF, Hermoso JA, Chang M and Mobashery S (2017). Conformational Dynamics in Penicillin-Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus*, Allosteric Communication Network and Enablement of Catalysis. *J Am Chem Soc* **139**: 2102-2110.

Mama M, Abdissa A and Sewunet T (2014). Antimicrobial susceptibility pattern of isolates from wound infection and their sensitivity to alternative topical agents at Jimma University Specialized Hospital, South West, Ethiopia. *Ann Clin Microbiol Antimicrob* **14**: 13:14.

Nair R, Hanson BM, Tundev O and Smith TC (2013). Antimicrobial resistantance and molecular epidemeology of *Staphylococcus aureus* from Ulanbattar, Mongolia. *Peer J* **1**: e176**.**

Pandey S, Raza MS and Bhatta CP (2012). Prevalence and Antibiotic Sensitivity Pattern of Methicillin-Resistant *Staphylococcus aureus* involved in a hospital outbreak. *J Clin Microbiol* **37**: 2858-2862.

Rajbhandari R, Manandhar SP and Shrestha J (2003). Comparative study of MRSA and its antibiotic susceptibility pattern in indoor and outdoor patients in Bir Hospital, Nepal. *Nepalese J Microbiol* **1**: 62-65.

Raut S, Bajracharya K, Adhikari J, Pant SS and Adhikari B (2017). Prevalence of methicillin resistant *Staphylococcus aureus* in Lumbini Medical College and Teaching Hospital, Palpa, Western Nepal. *BMC Res Notes* **10**:187.

Read AF and Woods RJ (2014). Antibiotic resistance management. *Evol Med Public Health* **1**: 147.

Richmond VA (2000). Role of Penicillin Binding Protein Four (PBP4) in Vancomycin Susceptibility Among Clinical Vancomycin Intermediate Susceptible Staphylococcs *aureus* (VISA) Isolates. *Antimicrob Agent Chemther* **20**: 40-75.

Rijal KR, Shrestha N, Pahari N, Shrestha B, Paudel B, Nepal A, Ghimire P and Rijal B (2008). Methicillin Resistant *Staphylococcus aureus* in patients visiting Western Regional Hospital, Pokhara. *Journal of Institute of Medicine* **30***:* 21-25.

Sit PS, Teh CSJ, Idris N, Sam IC, Omar SFS, Sulaiman H, Thong KL, Kamarulzaman A and Ponnampalavanar S (2017). Prevalence of methicillin-resistant Staphylococcus aureus (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC Infect Dis* **17**: 274.

Spagnolo MA, Orlando P, Panatto D, Amicizia D, Perdelli F and Cristina LM (2014). *Staphylococcus aureus* with reduced susceptibility to vancomycin in healthcare settings. *J Prev Med Hyg* **55 (4)**: 137-144.

Spellberg B and Gilbert DN (2014). The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clin Infect Dis* **59(S2)**: S71–S75.

Surucuglu S, Gazi H, Kurutepe S, Ozkutak and Ozbakkaloglu B (2005). Bacteriology of surgical wound infection in Tertiary care Hospital in Turkey. *Afr Med J* **82(7)**: 3-6.

In vitro Antibacterial Effect of Medicinal Plants against Multidrug Resistant Gram Negative Bacteria

Bishnu Thapa[1], Anjana Singh[1], Reshma Tuladhar[1*]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal

[*]**Corresponding author:** Reshma Tuladhar, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal; E-mail: reshma.tuladhar@microbiotu.edu.np

ABSTRACT

Objectives: The aim of this work was to determine the antibacterial activity of methanol extract of herbal plants against the multidrug resistant (MDR) Gram negative bacteria isolated from clinical samples.

Methods: Gram negative bacteria isolated from various clinical samples were processed for antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method and MDR bacteria were selected. Methanol extracts of six different medicinal plants *Acorus calamus* (bojho), *Ocimum sanctum* (tulsi), *Azadirachta indica* (neem), *Cinnamomum tamala* (tejpatta), *Aloe vera* and *Zanthoxylum alatum* (timur), were tested for antibacterial activity against the selected MDR bacteria by agar well diffusion method.

Results: From clinical samples, 8 different MDR Gram negative bacteria isolated were *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter* spp., *Proteus mirabilis, Proteus vulgaris*, *Acinetobacter* spp. and *Pseudomonas* spp. with *E. coli* dominated the number. Out of six medicinal plants extracts, *Z. alatum*, *C. tamala* and *Ocimum sanctum* were found to be effective with zones of inhibition ranging from 9-13 mm. The medicinal plants with antibacterial activity can be an alternative source of medicine against MDR Gram negative bacteria.

Conclusion: Several herbal plants extracts exhibit antibacterial activity against MDR Gram negative bacteria. Antibacterial activity of plant extracts can vary with type of plant and extraction methods. Thus, for optimal benefit of plant extract, an appropriate extraction method and use of purified product is essential.

Key words: Multidrug resistant, medicinal plants, phytocompounds, antibacterial activity

INTRODUCTION

The efficacy of existing antibiotics is being threatened by the emergence of Multidrug resistant (MDR) bacteria (Dahiya and Purkayastha 2013). Treatment of infections due to MDR strains have clinically become intractable (Davies and Davies 2010) leading to prolonged hospitalizations, increased cost, and greater risk of morbidity and mortality (Dhital 2000). Resistance is a vexing problem especially for people with impaired immune systems, such as Acquired Immune Deficiency Syndrome, cancer patients and recipients of organ transplants (Russell 2002).

Historically, pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Random screening of new biologically active molecules has been most productive in the area of antibiotics discovery (Ahmad et al. 2002). According to World Health Organization more than 80% of the world's populations rely on traditional medicine for their primary healthcare needs (WHO 1993). Use of herbal medicines in Asia represents a long history of human interactions with the environment and represents a rich source of antimicrobial agents and powerful drugs (Ahmad et al. 2002).

Date of Submission: November 2, 2018
Date of Acceptance: December 9, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22298

Researchers are increasingly turning their attention towards the medicinal plants and it is estimated that, plant materials are present in, or have provided the models for 25-50% western drugs (Crag et al. 2013). Many commercially available drugs used in modern medicine were initially used in crude forms in traditional or folk healing practices, or for other purposes that suggested potential biological activity (Anthony and Livermore 2015). Plant derived medicines are relatively safer than synthetic alternatives and offer affordable therapeutic benefits (Hammer et al. 1990).

Plants produce enormous variety of small molecules classified as phytoalexins which have been assumed to play important role in the defenses mechanisms of the plants (Rathanyaka and Obreshkova 2013). Phytoalexins are low molecular weight, anti-microbial compounds that are synthesized and accumulated in plants after exposure to microorganisms or abiotic agents (Khan et al. 2010). Phytoalexins structural spaces contain terpenoids, glycosteroids, flavonoids and polyphenols and these biologically active compounds are supposed to exhibit antimicrobial properties (Azwaninda 2016). Medicinal and aromatic plants have potential for contributing to the local economy, subsistence health needs, and improved natural resource management.

Thus, it was deemed important to consider for investigation of antimicrobial activity of herbal plants against MDR Gram negative isolates from the clinical samples. In this study the medicinal plants were selected based on their common uses in Nepali traditional systems. In view of vast potential of plants as sources for antimicrobial drugs with reference to antibacterial agents, six common floras, *Acorus calamus* (bojho), *Ocimum sanctum* (tulsi), *Azadirachta indica* (neem), *Cinnamomum tamala* (tejpatta), *Aloe vera* and *Zanthoxylum alatum* (timur) were selected to evaluate the antibacterial activity.

MATERIALS AND METHODS

Gram negative bacteria isolated from different clinical samples (urine, pus and sputum) were identified by standard microbiological techniques which included colonial morphology, Gram staining and biochemical tests**.** Antibiotic susceptibility tests of the identified bacteria were performed by modified Kirby-Bauer disk diffusion method in Mueller Hinton agar (MHA)

according to CLSI guideline (2014). MDR bacteria were categorized as per the definition given by Magiorakos et al. (2012).

The medicinal plants *Acorus calamus* (bojho), *Ocimum sanctum* (tulsi), *Azadirachta indica* (neem), *Cinnamomum tamala* (tejpatta), *Aloe vera* and *Zanthoxylum alatum* (timur) selected for this research were collected from Jhapa and Kathmandu. Identification of these plants and processing for extraction was done in Department of plant resources, Thapathali, Kathmandu.

Root of *A. calamus*, leaves of *O. sanctum*, *A. indica*, *C. tamala* and *Aloe vera*, and seeds of *Z. alatum* were washed thoroughly. Roots and leaves were cut into tiny pieces (3-5 cm) with the help of sterile knife. They were spread and left to air dry for 10-12 days with periodical turning. Fine powders of dried samples were obtained by grinding in an electrical grinder. Extraction from approximately 70-90 gm of powdered form was carried out by Soxhlet extraction method using methanol as solvent. Rotatory evaporator was used under negative pressure at water bath below 55°C to remove the solvent. The crude extract obtained in round bottom flask was further assayed for antibacterial activity.

Agar well diffusion method was used for in-vitro antibacterial activity of the extracts against the MDR Gram negative bacterial isolates. The fresh inoculums of MDR bacterial isolates compared with McFarland standard were spread uniformly on the surface of sterile MHA plates. Six wells were prepared in each plate with the help of 8 mm diameter cork borer. Then, 50 µl of methanol extract (100µg/ml) from the selected plants were loaded in each well with the help of micropipette (Redfern et al. 2014). The plates were left for diffusion of extract for 15 minutes and was incubated overnight at 37°C and observed for the zone of inhibition.

RESULTS

Out of different clinical samples processed, 33.5 % showed positive for bacterial growth among which 40.9% were Gram negative isolates. Eight different MDR Gram negative bacteria isolated were *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter* spp., *Proteus mirabilis, Proteus vulgaris*, *Acinetobacter* spp. and *Pseudomonas* spp. with the highest number of *E. coli* (Figure 1). Of the total *E. coli* isolated 32.1% were MDR. Similarly, 17.9% *K. oxytoca*, 14.1% of *K. pneumoniae,* 3.8% of each of *P. vulgaris* and *P. mirabilis*, 2.6% *Acinetobacter* spp., and 1.3% each of *Citrobacter* spp. and *Pseudomonas* spp. were isolated.

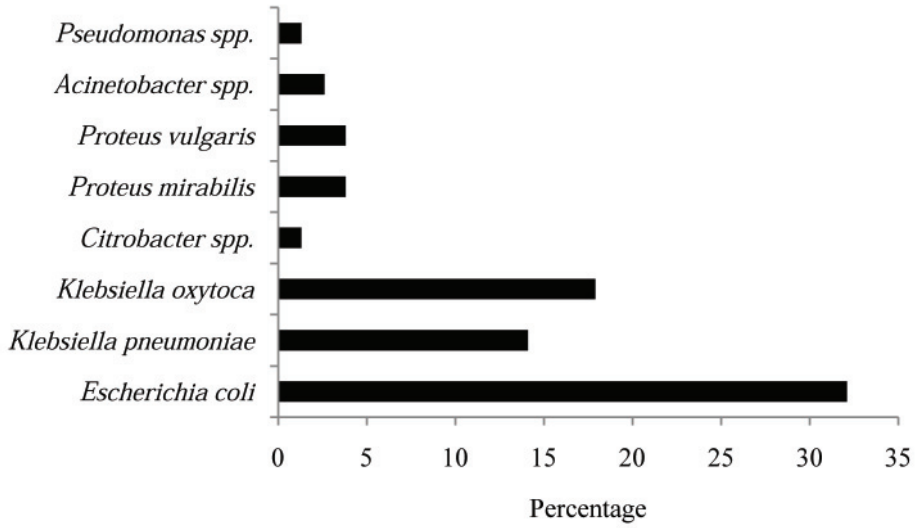


Figure 1: Gram negative MDR bacteria prevalent in clinical specimens

Antimicrobial activity of crude extracts of medicinal plants, *A. calamus*, *O. sanctum*, *A. indica*, *C. tamala, Aloe vera*, and *Z. alatum* when tested against the isolated Gram negative MDR bacteria, some of them showed activity against few bacteria. Table 1 shows the number of *E. coli* isolates inhibited by the plant extracts. Out of 24 isolates tested for antibacterial activity, 5 were inhibited by *Z. alatum* and 4 by *O. sanctum* but other plant extracts did not show any activity.

Table 1: Number ofAntibacterial effect of methanol herbal extracts against MDR *E. coli* (n=24)

S.N.	Plant extract	Active	Inactive
1	*A. calamus*	0	24
2	*A. indica*	0	24
3	*A. vera*	0	24
4	*C. tamala*	0	24
5	*Z. alatum*	5	19
6	*O. sanctum*	4	20

Among 12 *K. pneumoniae* tested, only 2 were inhibited by *C. tamala* and none were inhibited by remaining plant extracts (Table 2). Similarly, each of 2 isolates of *K. oxytoca* were inhibited by *Z. alatum* and *O. sanctum*, and one isolate was inhibited by *A. vera* and *C. tamala* each (Table 3).

None of the isolate of *P. vulgaris*, *P. mirabilis*, *Acinetobacter* spp., *Citrobacter* spp. and *Pseudomonas* spp. was inhibited by the plant extracts.

Table 2: Antibacterial effect of methanol herbal extracts against MDR *K. pneumoniae* (n=12)

S.N.	Plant extract	Active	Inactive
1	*A. calamus*	0	12
2	*A. indica*	0	12
3	*A. vera*	0	12
4	*C. tamala*	2	10
5	*Z. alatum*	0	12
6	*O. sanctum*	0	12

Table 3: Antibacterial effect against MDR *K. oxytoca* (n=14)

S.N.	Plant extract	Active	Inactive
1	*A. calamus*	0	14
2	*A. indica*	0	14
3	*A. vera*	1	13
4	*C. tamala*	1	13
5	*Z. alatum*	2	12
6	*O. sanctum*	2	12

Among the extracts, *Z. alatum* was found to be effective with 11.7% (n=7) showing active effect against MDR Gram negative bacteria followed by *O. sanctum* and *C. tamala* with 10% (n=6) and 5.1% (n=5). *A. calamus* and *A. indica* were found to be most ineffective medicinal plants (Figure 2).

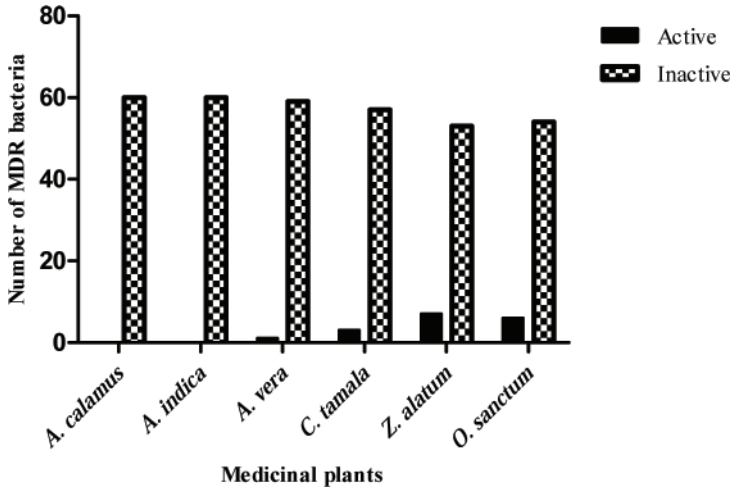


Figure 2: Comparison of antibacterial effect of medicinal plants against the MDR Gram negative bacteria

DISCUSSION

Diverse plants possess a variety of bioactive compounds at different concentration. Bioactive compounds derived from plants extracts can differ in their chemical composition and polarities. Thus, it is imperative to select solvent wisely to dissolve most of the compounds from the plants extract (Alekshun and Vijayalaxmi 2013). Methanol was used for the extraction of bioactive compounds from the selected plants in this research since it has polarity index of 5.1, which makes it an efficient solvent. Besides it is relatively cheap, free of regulation and gets evaporated easily (Crag et al. 2013). We selected the six medicinal plants in view of the fact that they are easily available and most commonly used by people in Nepal.

From the antibacterial assay, the selected plants exhibited zone of inhibition within the range of 10-12 mm. In a similar study by Ahmad et al. (2002), zone of inhibition from 11-14 mm against MDR Gram negative isolates was observed. While Rastogi et al. (2015) concluded that the inhibition zone was found within the range of 12-26 mm from different medicinal plants (*Cassia fistula, Holarrhena antidysenterica, Terminalia alata, T. arjuna* and *Paederia foetida*). Variation in zone of inhibition by the extracts against the MDR Gram negative isolates might be due to the differences in the type of solvents used for the extraction which is responsible for dissolving bioactive compounds and also based on the selection of plants for bioactive compounds.

Medicinal plants contain complex phenol compounds and the mechanism of action of each phenolic compound against various bacteria is also very complicated. Therefore, it is necessary to investigate further with purified extract instead of crude extract to understand the relationship between the antibacterial activity and

chemical structure of each phenolic compound in the extracts (Burt 2005).

A. calamus was found to be inactive against MDR Gram negative strains in the present study. However the rhizome of *A. calamus* (Chhatopadhyay et al. 2010) exhibited 16 mm zone of inhibition, which did not support the present finding. The possible reason for this might be different extraction methods adopted by other researcher for the extract preparation resulting in antibacterial effect.

Similarly, *A. indica* was found to be inactive against MDR Gram negative strains in the present study. However, a study from India revealed that *A indica* successfully exhibited zone of inhibition at the range of 12-19 mm against MDR Gram negative pathogens (Monali et al. 2015). Neem has been known to possess antibacterial activity. But the reason for not observing any activity by this compound might be due to possible damage of the compound during extraction or insufficient amount of active compound in crude extract.

The methanol extracts of *Z. alatum* was the most effective compound to exhibit antibacterial effect against MDR Gram negative isolates with 11.7 % active effect which was similar to a study conducted by Hanberger et al. (2003). The methanolic stem extract of *Z. alatum* indicated moderate inhibition of *E. coli*, *K. pneumoniae* (11.0±1.0 mm; 11.0±1.0 mm) while leaf extracts showed the greatest effect on *E. coli*, *K. pneumoniae*. (14.0±1.0 mm) at 100 mg/ml (Guleria et al. 2014). The bioactive constituents of the *Z. alatum* corresponding to Za1 and Za2 were identified as -fenchol and linalool, which might be the cause of antibacterial property (Guleria et al. 2014). Several workers have reported that the phenolic compounds present in *Z. alatum* acts as reducing agent and owe to the antibacterial activity against MDR pathogens (Jain 2016) while others revealed that bioactive compounds like 2-decanone, 4-terpineol and linalol can be possible factor for antibacterial activity in *Z. alatum* (Ebrahimabadi et al. 2010).

C. tamala, on the other hand, exhibited antibacterial effect with 5% activity against the MDR Gram negative isolates. Studies suggested that the antibacterial activity of cinnamon was probably due to their major component, cinnamaldehyde, a natural antioxidant (Monali et al. 2015). Cinnamaldehyde was found to completely inhibit both sensitive and resistant strain of *Helicobacter pylori* (Joshi and Edington 1990). Leaves of *O. sanctum* exhibited antibacterial effect with 10% activity which is in consistent with result from a study conducted by Jain et al. (2012), in which the leaves inhibited growth of *E. coli* and *S. aureus*. However the result obtained was different with Ahmad et al. (2002) where the leaves of *O sanctum* did not show any zone of inhibition against MDR *E. coli*.

All the extracts were found to be inactive against the MDR *P. vulgaris*, *P.mirabilis, Acinetobacter* spp., *Citrobacter* spp. and *Pseudomonas* spp. Similarly, a study from India by Jacoby et al. (2012), revealed that among the Gram negative tested for the antibacterial activity, only two strains of *P. aeruginosa* (ATCC 2642) were the least sensitive to the oil extracts (MIC value > 100 mg/ml).

The differences in the activities of extracts of various plants may be due to varying degrees of solubility of the active constituents in methanol. It has been documented that different solvents have different solubility capacities for different phytoconstituents (Hammer et al. 1990). Besides, differences were observed in antibacterial activities of the different extracts. These differences could be due to the dissimilar secondary metabolites of plants (Djeussi et al. 2013).

Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Vieira and Simon 1999).

CONCLUSION

Three of the selected medicinal plants (*O. sanctum, Z. alatum* and *C. tamala*) were successful in exhibiting antibacterial effect against common MDR Gram negative isolates. In response to the propagation of bacterial resistant to many antibiotics, also called MDR bacteria, the discovery of new and more efficient antibacterial agents is essential.

ACKNOWLEDGEMENTS

We are grateful to all the staff of Sahid Memorial Hospital, Kalanki, Kathmandu, Nepal for their assistance. We are also thankful to Department of Plant Resources, Thapathali, Kathmandu for helping in plant extraction process.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahmad I, Mehmood Z and Mohammad F (2002).

Screening of some Indian medicinal plants for their antimicrobial properties. *J Ethno Pharmacol* **62**: 183-193.

Alekshun K and Vijayalakshmi G (2013). Improved shelf life of protein-rich tofu using *Ocimum sanctum* (tulsi) extracts to benefit Indian rural population. *J Food Sci* **72**: 300-305.

Anthony CM and Livermore DM (2015). Resistance to antimicrobial drugs: a worldwide calamity. *Ann Intern Med* **18**: 557-561.

Azwaninda NN (2016). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med Aromat Plants* **4**: 196.

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods – a

review. *Int J Food Microbiol* **94**: 223-253.

Chhatopadhyay M, Ali SR and Aboud S (2010). Antimicrobial activities of aqueous and methanolic extracts from *Acorus calamus* used in the treatment of wound infection isolates. *Int J Infect* **2**: 18-25.

Clinical and Laboratory Standards Institute (2014). Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement, M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA pp 121-148.

Crag GM, Newman DJ and Sander KM (2013). Natural products in drug discovery and development. *J Nat Prod* **60**: 52-60.

Dahiya P and Purkayastha S (2013). Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. *Indian J Pharm Sci* **74**: 443-450.

Davies J and Davies D (2010). Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**: 417-433.

Dhital S (2000). Determination of antibiotic resistant Gram negative urinary pathogen in pediatric patients at Kanti Children's Hospital. A dissertation submitted to Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal pp 27-32.

Djeussi DE, Noumedem JA, Seukep JA, Fankam AG, Voukeng IK and Tankeo SB (2013). Antibacterial activities of selected edible plants extract against multidrug-resistant Gram negative and Gram positive bacteria. *BMC Complement Altern Med* **13**: 164.

Ebrahimabadi AH, Ebrahimabadi EH, Bidgoli Z, Kashi FJ, Mazoochi A and Batooli H (2010). Composition and antioxidant and antimicrobial activity of the essential oil and extracts of *Dioscorea hispida* from Iran. *J Rural Trop Health* **19**: 452-458.

Guleria S, Tiku AK, Koul A, Gupta S, Singh G and Razdan VK (2014). Antioxidant and antimicrobial properties of the essential oil and extracts of *Zanthoxylum alatum* grown in North-Western Himalaya. *J Med Plants Res* **6**: 971-976.

Hammer KA, Carson CF and Riley TV (1990). Antimicrobial activity of essential oils and other plant extracts. *J App Microbiol* **86**: 985-990.

Hanberger HL, Didier M and Antoine T (2003). Antimicrobial resistance among uropathogens that cause community acquired UTI in Bangui, Central African Republic. *J Antimicrob Chemother* **51**:192-194.

Jacoby B, Ebadi A, Aghdam BM and Hassanzade Z **(**2012). Antibacterial activities of lemon grass methanol extract and essence in pathogenic bacteria. *American-Eurasian J Agri Env Sci* **12:** 1042-1046.

Jain R, Katare N and Kumar V (2012). In vitro antibacterial potential of different extracts of *Ocimum* spp. *J Nat Sci Res* **2**: 84-90.

Jain N, Srivastava SK, Aggarwal KK, Ramesh S, and Kumar S (2016). Essential oil composition of *Zanthoxylum alatum* seeds from northern India. *J Med Plants Res* **16**: 408-410.

Joshi AR and Edington JM (1990). The use of medicinal plants by two village communities in the central development region of Nepal. *Economic Botany* **44**: 71-83.

Khan R, Islam B, Akram M, Shakil S, Ahmad A and Ali SM (2010). Antimicrobial activity of five herbal extracts against multidrug resistant strains of bacteria and fungus of clinical origin. *Pak J Bot* **14**: 586-597.

Magiorakos M, Fazilah A, Norzials MH and Karim AA

(2012). Antimicrobial activity and mechanical properties of partially hydrolyzed sago starch-alginate edible film containing lemon grass oil. *J Food Sci* **72**: 324-330.

Monali P, Mishra R, Shasank S, Goutam G, Debajyoti Das and Rabindra N (2015). In vitro antibacterial activity of crude extracts of nine selected medicinal plants against UTI causing MDR bacteria. *J King S U* **4**: 36-44.

Rastogi RP and Mehrotra BN (2015). Glossary of Indian medicinal plants. *Indian J Med Microbiol* **9**: 102-118.

Rathnayaka G and Obreshkova D (2013). Comparitive studies on the activity of basil-an essential oil from *Ocimum basilicum* L.-against multidrug resistant clinical isolates of the genera *Staphylococcus*, *Enterococcus* and *Pseudomonas* by using different test methods. *J Microbiol Methods* **54**: 105-110.

Redfern J, Kinninmonth M, Burdass D and Verran J (2014). Using soxhlet ethanol extraction to produce and test plant material (essential oils) for their antimicrobial properties. *J App Microbiol* **1**: 45-46.

Russell AD (2002). Antibiotic and biocide resistance in bacteria: Introduction. *J App Microbiol* **92**: 15-35.

Sasidharan S, Chen Y, Saravanan D, Sundram KM and Latha L (2010). Extraction, isolation and characterization of bioactive compounds from plant extracts. *Indian J Pharma Sci* **3**:1-10.

Vieira RF and Simon JE (1999). Chemical characterization of basil (*Ocimum* spp.) found in the markets and used in traditional medicine in Brazil. *J Sociedade Botanica de Sao Paulo* **54**: 207-216.

WHO (1993). Summary of WHO guidelines for the assessment of herbal medicines. *Herbal Gram* **28**: 13-14.

Microbial Profile of Various Catheter Tips among Hospitalized Patients

Pushpa Man Shrestha[1], Nisha Thapa[2], Navraj Dahal[3], Nabaraj Adhikari[4], Upendra Thapa Shrestha[4]*

[1]Assistant Professor, Ayurved Campus, Institute of Medicine, TU, Kirtipur

[2]Kantipur College of Medical Science, Kathmandu

[3]Senior Medical Microbiologist, B&B Hospital, Gwarko, Lalitpur, Nepal

[4] Assistant Professor, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu

***Corresponding author:** Upendra Thapa Shrestha, Assistant Professor, Central Department of Microbiology, TU, Kirtipur, Kathmandu; E-mail: upendrats@microbiotu.edu.np

ABSTRACT

Objectives: This study aimed to identify the microbiological profile of various catheter tips, and multidrug resistance pattern of extended spectrum β-lactamase (ESBL) producing *E. coli and Klebsiella* spp. isolates.

Methods: A descriptive analysis of 263 catheter tip specimens processed for culture and antimicrobial susceptibility testing was carried out in B&B Hospital, Lalitpur. Five different types of catheter tips were analyzed for microbiological growth and antimicrobial susceptibility testing.

Results: Among catheter tips, the highest percentage of microbial growth was observed in tracheostomy tip. Monomicrobial growth was recorded in 82.9% catheter tips and polymicrobial growth was observed in 17.1% tip samples. Of 180 isolates, gram negative rods (76.6%) followed by yeast (19.4%) and gram-positive cocci (3.9%) were isolated. Gram negative *Acinetobacter* spp. (25%) *and Pseudomonas* spp. (23.3%) and gram-positive *Enterococcus* spp. (2.2%) were the most frequently isolated bacteria. However, carbapenam was the most effective antibiotic for both groups.

Conclusion: Of the total isolates tested, 61.4% were found to be multidrug resistant (MDR). Among gram negative rods, 22.2% *E. coli* and 27.3% *Klebsiella* spp. were confirmed as ESBL producer. It is recommended to apply standard protocol during insertion and removal of catheter which may help in managing nosocomial infection associated with catheters.

Key words: Indwelling devices, catheters, nosocomial infection, MDR, ESBL

INTRODUCTION

Catheter is a tube that can be inserted into a body cavity, duct, or vessel thereby allowing drainage, injection of fluids, or access to surgical instruments. The number of intravascular catheters, urinary catheters, endotracheal tubes and other temporary devices inserted each year probably ranges into the millions. The variety of available devices and the frequency with which they are implanted will undoubtedly continue to increase in the coming years (Dickinson and Bisno 1989).

Due to the frequent and sometimes unnecessary use of indwelling catheters during hospitalization in 21 to 50% of patients, many patients are placed at risk for complications associated with the use of these devices (Jain et al. 1995). A study of 1,540 nursing home residents determined that the risk of hospitalization, length of hospitalization, and length of antibiotic therapy were three times higher in catheterized residents than in non-catheterized residents (Kunin et al. 1992). They are also the main source of bacteremia and septicemia in hospitalized patients (Elliott et al. 1997). Primary blood stream infections (BSIs) comprise the majority (64%) of nosocomial infections reported by the Centers for Disease Control and Prevention (CDC)'s National Nosocomial Infection Surveillance (NNIS) system, and

Date of Submission: August 10, 2018
Date of Acceptance: November 21, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22299

most are due to infected intravascular, mostly central venous catheters. More than 250,000 vascular catheter related bacteremia and fungemia occur annually in the USA with an attributable mortality ranging from 12% to 25% in critically ill patients (O'Grady et al. 2002). The prolonged use of intravascular catheters and its improper management is a major risk factor for development of nosocomial BSIs. The worldwide increase in the incidence of nosocomial BSIs is mainly attributed to the increased use of invasive devices and aggressive drug therapy along with increased frequency of invasive procedures (Mermel 2000). Approximately 25% of central venous catheters (CVCs) inserted have been reported to become colonized with rates of catheter related blood stream infection (CRBSI) varying between 0% and 11% (Maki et al. 1997).

It is estimated that 10-12% of hospital patients and 4% of patients in the community have urinary catheters in situ at any given time (Stamm and Coutinho 1999). Nosocomial urinary tract infections (UTIs) develop in 5% of catheterized patients per day in the US, with associated bacteremia in 4% and as many as 80% are a consequence of urinary catheters. Fever, pyelonephritis, urinary tract stones and chronic renal inflammation are some of the other complications of this procedure (Sedor and Mulholland 1999).

Bacteria have the capacity to adhere to and multiply on the surfaces of catheters. It may contaminate the system at the insertion site with spread along the external surface of the catheter, in the fluids being infused, or at junctions in the external line. The latter is frequently the result of manipulation of the line by medical personnel. Organisms colonizing CVCs include Coagulase negative Staphylococci (CoNS), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Candida albicans* (Elliott et al. 1997; Raad et al. 2007)

This study is an attempt to summarize the pattern of microorganisms and their resistance pattern that are isolated from the various catheter tips from hospitalized patient in B & B hospital.

MATERIALS AND METHODS

This was a cross sectional study carried out at B & B Hospital (Pvt.) Ltd., Lalitpur, Nepal. During this study, catheter tips were enrolled from both sexes and all age group but catheter tips arrived in saline or transport media, and without proper labeling were excluded.

During this study period, a total of 263 catheter tips samples were collected from hospitalized patients and processed in microbiology laboratory of the hospital. Catheter tips were kept in body for not more than 13 days.

Various tips were removed by nursing staff. The skin was cleaned with 70% alcohol prior to catheter removal. Observing aseptic technique, the exposed end of catheter was hold and it was removed from the patients with a sterile instrument, taking care to avoid contact with exposed skin. Holding the distal end over a sterile tube, the tip was cut with a sterile scissors, dropping the last 2 to 3 inches into the tube[11]. The tube with tip was labeled with patient name, sample collection date and hospital number. The tube was sealed to avoid the drying and was submitted to Microbiology laboratory as soon as possible.

All the tips were laid on the Blood Agar Plate (BA) and MacConkey Agar Plate (MA). The tip was rolled back and forth exerting a slight downward pressure across the entire surface of a BA and MA using sterile forceps. The plates of BAP were incubated at 35^0C in CO_2. The plates were read at 24, 48, 72, and 96 hours. Each type of colony isolated was counted comparing growth on each medium. Plates were observed for fungal growth.

The identification of various gram-negative isolates was done by using standard microbiological techniques as described in Bergey's Manual of systemic bacteriology and Clinical Microbiology Procedure Handbook (Isenberg 2004).

Antimicrobial susceptibility test towards isolated organisms were performed by Kirby-Bauer disk diffusion method and interpreted according to Clinical Laboratory Standard Institute (CLSI). The initial screen test for the production of ESBL was performed by using both ceftazidime (CAZ) (30µg) and ceftriaxone (CTR) (30µg) disks. Isolates those were suspected as ESBL- producer by screen test were tested further by Combination disk method (CD).

RESULTS

In this study, a total of 263 catheter tip samples were taken from hospitalized patients at B & B Hospital, Lalitpur. Out of 263 samples, 152 (57.8%) were found to be culture positive. Eighty percent of tip culture positive result was observed in a patient re-catheterized for 10 times and 45.7% tip showed positive culture result from 70 patients with single time of catheterization.

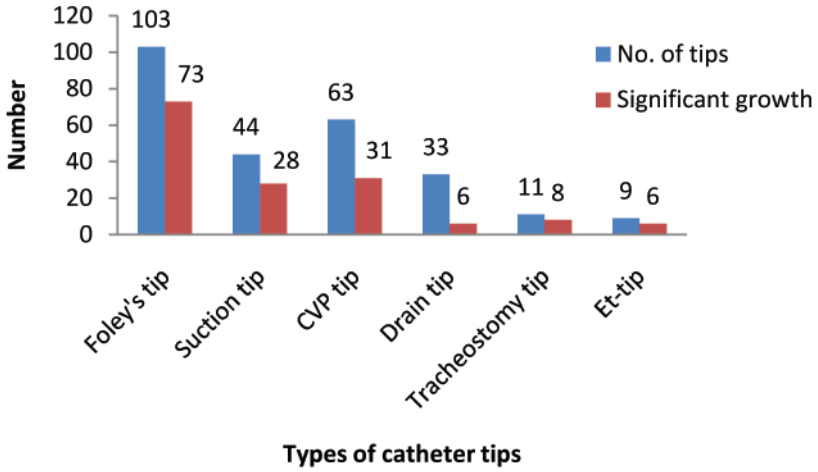


Figure 1: Growth pattern according to sample distribution

In total 152 culture positive sample, 180 organisms were isolated. Out of 180 organisms, 138(76.6%) were Gram negative rods followed by 35 (19.4%) yeast and 7(3.9%) gram positive cocci. The most predominant organism was *Acinetobacter* spp. accounting for 25 % of the total isolates.

Table 1: Distribution of various organisms in growth positive tips

Organisms	Number	Percentage
Bacteria	145	80.5
Gram positive cocci	7	3.9
Enterococcus spp.	4	2.2
MR-CONS	1	0.5
MS-CONS	1	0.5
NHS	1	0.5
Gram negative rods	138	76.6
Acinetobacter spp.	45	25
Pseudomonas spp.	42	23.3
Klebsiella spp.	22	12.2
E. coli.	21	11.7
Proteus spp.	4	2.2
Enterobacter spp.	3	1.7
Citrobacter spp.	1	0.6
Yeast	35	19.4
Candida albicans	6	3.3
Non-albican Candida	29	16.1

Ten different antibiotics were used for all isolates for antibiotic susceptibility test but additional antibiotic colistin sulphate was used for *Pseudomonas* spp. and *Acinetobacter* spp. The antibiotic colistin sulphate was found to be 100% sensitive towards *Pseudomonas* spp. and 97.8 % *Acinetobacter* spp. and both of them were resistant to ciprofloxacin, ofloxacin and ceftriazone. Imipenam was found drug of choice for *E. coli, Proteus* spp., *Enterobacter* spp. *and Citrobacter* spp. with sensitivity of 95.4 %, 100%, 100%, 100%, 100% of isolates respectively.

Table 2: Antibiotic susceptibility pattern of Gram-negative rods

Antibiotics used	Percentage resistant to antibiotics in						
	Acinetobacter spp.	*Pseudomonas* spp.	*Klebsiella* spp.	*E. coli*	*Proteus* spp.	*Enterobacter* spp.	*Citrobacter* spp.
Cip	97.8	50	81.8	76.1	75	33.3	0
Of	97.8	50	77.2	76.1	75	33.3	0
C	95.5	47.6	50	80.9	0	33.3	100
Gen	95.5	54.7	68.1	38	25	100	100
Ak	95.5	40.4	27.2	38	25	100	100
Ctr	97.8	80.9	90.9	80.9	50	100	100
Imp	20	14.2	4.5	0	0	0	0
Mrp	22.2	7.1	13.6	4.7	0	0	0
Ptz	93.3	50	40.9	23.8	50	66.6	0
Cs	73.3	73.3	13.6	14.3	0	33.3	100
Co	_	0	_	_	_	_	_

(Note: Cip- Ciprofloxacin, Of- Ofloxacin, C- Chloramphenicol, Ctr- Ceftriazone, Ptz- Piperacillin/ Tzobactum, Cs- Cefoperazone/ Sulbactum, Imp- Imepenam, Mrp- Meropenam, , Gen- Gentamycin, Ak- Amikacin, Co- Colistin sulphate)

Out of 7 Gram positive cocci, *Enterococcus* spp. was found 100% resistance to six antibiotics. 50 % of CoNS was found resistant to methicillin. Non-haemolytic streptococci (NHS) was found 100% sensitive towards Chloramphenicol, Amoxycillin and Vancomycin.

Out of 138 Gram negative rods, *Citrobacter* spp. was found 100 % resistant to ≥ 3 common antibiotics. While 27.3 % *Klebsiella* spp. were found to be MDR. Out of 7 Gram positive bacteria, *Enterococcus* spp. and NHS were found 100 % MDR and 50 % CoNS were MDR.

Table 3: MDR among Gram negative rods

Organisms	Total number	MDR (n)	Percentage
Acinetobacter spp.	45	43	95.5
Pseudomonas spp.	43	20	47.6
Klebsiella spp.	22	6	27.3
E. coli	21	9	42.8
Proteus spp.	4	2	50
Enterobacter spp.	4	3	75
Citrobacter spp.	1	1	100

Table 4: MDR among Gram positive bacteria

Organisms	Total	MDR (n)	Percentage
Enterococcus spp.	4	3	100
CoNS	2	1	50
NHS	1	1	100

Table 5: ESBL producing *E. coli* and *Klebsiella* spp. among Gram negative rods

Organisms	ESBL(n)	Percentage
E. coli	2	22.2
Klebsiella spp.	6	27.3

Out of 21 *E. coli* and 22 *Klebsiella* spp., 22.2% of *E. coli* was confirmed to be ESBL producer while 27.3% *Klebsiella* spp. was detected as ESBL producer.

DISCUSSION

This study showed that among 263 catheter tip samples, 152 (57.7%) samples were found to be culture positive. In previous study on growth positive tip culture was found 37.8% (Atela et al. 1997) and other investigators elsewhere in the world have reported higher growth positive which is in agreement with the findings of the present study (Kalsoom and Abdul 2006; Mahto et al. 2013). Possible reasons for these differences in rates could be the use of central catheters only for very sick patients, absence of dedicated IV catheter insertion teams and lack of standardized protocol for replacement/ change of catheters. The slightly higher growth in our setting may be due to the lack of aseptic technique during insertion and removal of catheter tips, long duration of catheterization and irregular sterilization of patient's room. The study showed that the prevalence of tip colonization increases with the increase in times of catheterization. In this study lowest of 45.7% growth was observed in tips with single time of catheterization and maximum growth was observed in a patient recatheterized for 10 times with 80% growth positive results. The catheter tips were kept inside body for maximum 13 days.

In this setting, highest growth was recorded in tip samples received from patients in ICU showing ICU to be more prone to infection. Intensive care unit (ICU) is one of the potential sources of nosocomial infections even in countries where extensive infection control measures are routinely implemented. The international study of infections in ICU, which was conducted in 2007, demonstrated that the patients who had longer ICU stays had higher rates of infection, especially infections due to resistant *Staphylococci, Acinetobacter* spp., *Pseudomonas* spp., *Candida* spp. (Radji et al. 2011).

This study showed a highest growth in tracheostomy-tip followed by Urinary catheter, Et- tips, Suction tip, CVP and the least growth was in Drain tips. 70.9% urinary catheters were found to have significant growth in our setting which was comparable with the result of a study done in India in which 69.6% catheters were found to have microbiological growth (Deep et al. 2004). In our study 66.7% endotracheal tips were found to be tip culture positive.

Furthermore, in the present study 26 (17.1%) tips to have polymicrobial growth. In the previous studies found that catheter tips with polymicribial growth were 41.2% and 43.5% respectively (Storti et al. 2006; Xu et al. 2012). Risk factors for polymicrobial infections in children and adults include the presence of a central venous catheter, administration of parenteral nutrition, gastrointestinal pathology, especially short gut syndrome, use of broad-spectrum antibiotics and immunosuppression (Downes et al. 2008).

In this study, out of 180 isolated organisms, gram negative bacteria accounted for higher percentage with 76.6% followed by yeast 19.4% and gram-positive cocci 3.9%. The high prevalence of gram-negative bacteria may be due to immune compromised state of patients, contaminated infuscate and misuse of antibiotics (Maki et al. 1997; Mermel 2000). The hands of health-care workers often introduce Gram-negative organisms during the manipulation of catheters or intravenous tubing (Gaynes 2009). An intravascular catheter tip colonized with Enterobacteriaceae and *Pseudomonas* spp. was predictive of subsequent Gram-negative bacteremia in 20 and 14% of the cases, respectively (Peacock et al. 1998).

Acinetobacter spp. was the most predominant organism accounting for 25% of total isolates and *Pseudomonas* spp. was second most prevalent organism. In previous study, *Acinetobacter* spp. (26.67%) was most frequently isolated organism followed by *Pseudomonas* spp. (Tullu et al. 1998). In contrast to our result, it was found that *Pseudomonas* spp. as most frequent organism followed by *Acinetobacter* spp. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the most prevalent nonfermentative bacterial species isolated from clinical specimens of hospitalized patients (Karlowsky et al. 2004). In contrast, CoNS was the most predominant organism in many studies (Riboli et al. 2014).

In the study, yeast was isolated in 19.4% among all isolates. This result was comparable with the previous study in which prevalence of *Candida* spp. was 20% (Rao et al. 2005).

Furthermore, Imipenam was drug of choice for *Acinetobacter* spp. with efficiency of 80% and ciprofloxacin, ofloxacin, ceftriazone were found ineffective for the organism. This result was concurrent with previous study showing imipenam as most sensitive drug (75% sensitive) (Amin, Shrestha and

Bhat 2013). Amikacin and cefoperazone – sulbactum were effective drug (35.7% each) for the organism in previous study (Khanna et al. 2013). The most effective antibiotic for *Pseudomonas* spp. was colistin sulphate with 100% sensitive result followed by meropenam (92.9% sensitive) and cefoperazone –sulbactum (73.3% resistant) was ineffective drug in the study.

In addition, overall 61.4% organisms were found to be MDR which were resistance to three or more than three different group of antibiotics. In previous studies showed that rate of MDR organism was 31.48% and 30.2% respectively (Khanna et al. 2013; Parameswaran et al. 2011). The study showed highest number of MDR in *Enterococcus* spp., *Citrobacter* spp. and NHS (100% each) followed by *Acinetobacter* spp. (95.5%), *Enterobacter* spp. (75%), *Proteus* spp. and CoNS (50% each), *Pseudomonas* spp. (47.6%), *E. coli* (42.8%) and *Klebsiella* spp. (27.3%). Isolating MDR *A. baumannii* from hospitalized patients depends on external ecological variables and risk factors related to the patients themselves (Bonten et al. 1998). Several previous reports have discussed the risk factors associated with the development of MDR *A. baumannii* infections in hospitalized patients (Allen and Hartman 2004).

In this study 18.6 % of enteric bacilli (*E. coli and Klebsiella* spp.) were ESBL producer which is in congruous with previous study which recorded 19% of these organisms to be ESBL producer. Our study confirmed 22.2% *E. coli* as ESBL producer. This finding differed from previous studies which confirmed 27.2% *E. coli* as ESBL producer (Khanna et al. 2013). The prevalence of ESBL producing Enterobacteriaceae varies greatly from country to country and among the hospitals within the country. Less than 1% to greater than 70% ESBLs is reported worldwide. The prevalence rates of ESBL in Nepal are reported frequently increasing from year to year.

CONCLUSION

The infection rates of indwelling devices used for various reasons are found to be very high. These infections have increased the morbidity and mortality of the hospitalized patients and also increased the duration of hospital stay.

ACKNOWLEDGEMENTS

We are grateful to KCMS family and all the staff members of Pathology Department of B & B Hospital for their help and kind cooperation during laboratory work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Allen DM and Hartman BJ (2004). Acinetobacter species. In Bennett's principles and practice of infectious diseases. 6th ed. *Churchill Livingstone, Philadelphia*, pp. 2631-2635.

Amin NS, Shrestha ND and Bhat S (2013). Evaluation of semi quantitative culture method in the diagnosis of central venous intravascular catheter related infections. *J Pharm Biomed Sci* **26**(26): 724-728.

Atela I, Coll P, Rello J, Quintana E, Barrio J, March F, Sanchez F, Barraquer P, Ballus J, Cotura A and Prats G (1997). Serial surveillance cultures of skin and catheter hub specimens from critically ill patients with central venous catheters: molecular epidemiology of infection and implications for clinical management and research. *J Clin Microbiol* **35**(7): 1784-1790.

Bonten MJ, Slaughter S, Ambergen AW, Hayden MK, van Voorhis J, Nathan C and Weinstein RA (1998). The role of "colonization pressure" in the spread of vancomycin-resistant enterococci: an important infection control variable. *Arch Intern Med* **158**: 1127–1132.

Deep A, Ghildiyal R, Kandian S and Shinkre N (2004). Clinical and Microbiological Profile of Nosocomial Infections in the Pediatric Intensive Care Unit (PICU). *Indian Pediatrics* **41**: 1238-1246.

Dickinson GM and Bisno AL (1989). Infections associated with indwelling devices: infections related to extravascular devices. *Antimicrob Agents Chemother* **33**: 602-607.

Downes KJ, Metlay JP, Bell LM, McGowan KL, Elliott MR and Shah SS (2008). Polymicrobial Bloodstream Infections among Children and Adolescents with Central Venous Catheters Evaluated in Ambulatory Care. *Clinical Infectious Diseases* **46**(3): 387-94.

Elliott TS, Moss HA, Tebbs SE, Wilson IC, Bonser RS, Graham TR, Burke LP and Faroqui MH (1997). Novel approach to investigate a source of microbial contamination of central venous catheters. *Eur J Clin Microbiol Infect Dis* **16:** 210–213.

Gaynes R (2009). Definitions and epidemiology of nosocomial intravascular catheter-related (primary) bloodstream infections. Available: http://www.uptodate.com.

Isenberg HD (2004). Clinical Microbiology Procedure Handbook. 2nd Edition, ASM Press, Washington DC.

Jain P, Parada JP and David A (1995). Overuse of the indwelling urinary tract catheter in hospitalized medical patients. *Arch Intern Med* **155**(13): 1425-1429.

Kalsoom F and Abdul H (2006). Resistance pattern of clinical isolates of *Staphylococcus aureus* against five groups of antibiotics, Multan. *Pakistan J res* Sci **17**: 19-26.

Karlowsky JA, Jones ME, Draghi DC, Thornsberry C, Sahm DF and Volturo GA (2004). Prevalence and antimicrobial susceptibilities of bacteria isolated from blood cultures of hospitalized patients in the United States in 2002. *Ann Clin Microbiol Antimicrob* **3**:1-8.

Khanna V,Mukhopadhayay C, K. E. V, Verma M and Dabke P (2013). Evaluation of central venous catheter associated blood stream infection: a microbiological observational study. *Journal of pathogen* 2-6.

Kunin CM, Douthitt S, Dancing J, Anderson J and Moeschberger M (1992). The association between the use of urinary catheters and morbidity and mortality among elderly patients in nursing homes. *Am J Epidemiol* **135:** 291 – 301.

Mahto R, Amatya J, Amatya R and Adhikari D (2013). staphylococcal colonization in intravenous cannula and antimicrobial patterns of isolated *Staphylococcus* species. *Nepal Journal of science and Technology* **14**(2): 131-136.

Maki DG, Stolz SM, Wheeler S and Mermel LA (1997). Prevention of central venous catheter-related bloodstream infection by use of an antiseptic-impregnated catheter: a randomized, controlled trial, *Ann Inter Med* **127**: 257-266.

Mermel LA (2000). Prevention of intravascular catheter-related infections. *Ann Intern Med* 7; **132**(5): 391-402.

O'Grady NP, Alexander M, Dellinger EP, Gerberding JL, Heard SO, Maki DG, Masur H, McCormick RD, Mermel LA, Pearson ML, Raad II, Randolph A and Weinstein RA (2002). Guidelines for the prevention of intravascular catheter-related infections, Centers for Disease Control and Prevention. *MMWR Recomm Rep*. 9; **51**(RR-10):1-29.

Parameswaran R, Sherchan JB, Varma D M, Mukhopadhyay C and Vidyasagar S (2011). Intravascular catheter-related infections in an Indian tertiary care hospital. *J Infect Dev Ctries* **5**(6): 452-458.

Peacock SJ, Eddleston M, Emptage A, King A and Crook DW (1998). Positive intravenous line tip cultures as predictors of bacteraemia. *J Hosp Infect* **40**(1): 35–38.

Raad I, Hanna H and Maki D (2007). Intravascular catheter-related infections: advances in diagnosis, pre vention, and management. *Lancet Infect Dis* **7**: 645–657.

Radji M, Fauziah S and Aribinuko N (2011). Antibiotic sensitivity pattern of bacterial pathogens in the intensive care unit of Fatmawati Hospital, Indonesia. *Asian Pac J Trop Biomed* **1**(1): 39-42.

Rao SD, Joseph MP, Lavi R and Macaden R (2005). Infections related to vascular catheters in a pediatric intensive care unit. *Indian Pediatrics* **42**(7): 667–672.

Riboli DFM, Lyra JC,Silva EP,Valadão LL, Bentlin MR,Corrente JE,de Souza Rugolo LMS andde Souza da Cunha MDLR (2014). Diagnostic accuracy of semi quantitative and quantitative culture techniques for the diagnosis of catheter-related infections in newborns and molecular typing of isolated microorganisms. *BMC Infectious Diseases* **14**: 283.

Sedor J and Mulholland SG (1999). Hospital-acquired urinary tract infections associated with the indwelling catheter. *Urol Clin North Am* **26**(4): 821-828.

Stamm AM and Coutinho MS (1999). Urinary tract infection associated with indwelling bladder catheter: incidence and risk factors. *Rev Assoc Med Bras* **45**(1): 27-33.

Storti A, Manzato AJ, Pizzolitto AC and Pizzolitto E (2006). Assessment of central venous catheterassociated infections using semi-quantitative or quantitative culture methods. *Journal of Basic and Applied Pharmaceutical Sciences* **27**(3): 213-220.

Tullu MS, Deshmukh CT and Baveja, SM (1998). Bacterial profile and antimicrobial susceptibility pattern in catheter related nosocomial infections. *J Postgrad Med* **44**(1):7-13.

Xu Y, Moser C, Al-Soud WA, Sørensen S, Høiby N, Nielsen PH andThomsen TR (2012). Culture-Dependent and -Independent Investigations of Microbial Diversity on Urinary Catheters. *J Clin Microbiol* **50**(12): 3901.

Bacteraemia and Antibiotic Susceptibility Pattern of Isolates from Patients Visiting a Private Hospital of Kathmandu

Nandalal Jaishi[1], Pramila Pathak[2], Pradeep Kumar Shah[2], Puspa Raj Dahal[1]*

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

* **Corresponding author**: Puspa Raj Dahal; Department of Microbiology, Tri-Chandra Multiple Campus, Ghantaghar, Kathmandu; E-mail: pusparajdahal@gmail.com

ABSTRACT

Objectives: This study was conducted to identify the causative agents of bacteraemia and to assess antibiogram of the isolates among the patients suspected of blood stream infection visiting Everest hospital, New Baneshwor Kathmandu.

Methods: Altogether 400 blood cultures were processed during March to August, 2015. Standard Operating Procedures (SOPs) was followed during the processing of the specimens. Antibiotic susceptibility testing of bacterial isolates was done by Kirby Bauer disc diffusion method with Muller-Hinton agar using the guidelines and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI 2013).

Results: The positivity of blood culture was found to be 48 (12%). Gram negative bacteria were found to be more predominant 27(56.2%) than Gram positive bacteria 21(43.7%) in causing bacteraemia. The most prevalent isolate was *Staphylococcus aureus* 15 (31.2%) followed by *Salmonella* Paratyphi A 10 (20.8%) and *Salmonella* Typhi 8 (16.6%), *Escherichia coli* & CoNS 4 (8.3%), *Pseudomonas aeruginosa* 3 (6.2%) and *Klebsiella pneumoniae and Streptococcus pneumoniae 2 (4.1%)* respectively. All Gram-positive isolates were found to be sensitive to Cefoxitin, Ceftriaxone and Vancomycin followed by Ampicillin (90.42%), Erythromycin (85.71%), Ciprofloxacin (83.33%), Doxycycline (75%) and Cephalexin (70.58%) whereas Gram negative isolates were sensitive to Ceftriaxone followed by Chloramphenicol (92%), Gentamicin (88.8%), Cefixime (85.71%), Ofloxacin (83.3%) and Amoxycillin and Ciprofloxacin (71.3%).

Conclusion: The isolation of etiological agents of blood stream infection should be assessed by proper microbiological analysis and it would be helpful for controlling of the outbreaks of resistance strains through effective empirical therapy.

Key words: Non-fermentative gram-negative bacteria (NFGNB), bloodstream infections, BHI

INTRODUCTION

Continuous or transient presence of bacteria with in the blood stream is bacteraemia which may indicate an intravascular infection, pneumonia or liver abscess (Forbes, et al., 2007). It is the most common cause of significant morbidity that leads to mortality especially in the developing countries (Karlowsky, et al., 2004), (Pittet, et al., 1994) despite the availability of potent antimicrobial therapy and advances in supportive care. Both gram positive and gram negative bacteria causes bacteraemia and septicemia. Gram negative septicemia, also known as endotoxic shock, is more severe than gram positive septicemia. *Staphylococcus aureus, Escherichia coli*, coagulase-negative staphylococci (CoNS), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Streptococcus* spp., *Candida albicans*, and *Enterobacter cloacae* are the most frequent etiological agents of bacteraemia and fungaemia in Europe and the United States (Cleven , et al., 2006). Infections caused by non-fermentative gram-negative bacteria (NFGNB) is a significant problem in both hospitalized and community-dwelling patients due to their ubiquitous

Date of Submission: August 20, 2018
Date of Acceptance: November 18, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22300

distributions in the environment and increasing incidence of multidrug resistance (Enoch, et al., 2007), (Suárez et al. 2005). Gram-negative bacillary sepsis with shock has a mortality rate of 12 to 38 percent; mortality varies depending, in part, on whether the patient receives timely and appropriate antibiotic therapy (Kang et al. 2005), (Kumar et al. 2006). Mortality appears to be higher with methicillin-resistant (MRSA) compared with methicillin-sensitive *S. aureus* (MSSA) bacteremia (Mylotte et al. 1987). Effective treatment of gram-positive bloodstream infections including those caused by *Staphylococcus*, *Streptococcus* and *Enterococcus* species, represents major clinical challenges (Christoph et al. 2009). This is necessary intervention program to monitor the spread of resistant bacteria and their antibiogram with the aim to identify the common bacterial agents involved in BSIs in Nepal. This would help health professionals technicians to know about the current situation of bacteraemia.

MATERIALS AND METHODS

The study was conducted on 400 blood samples collected during March, 2015 to August, 2015 from male and female patients of all age groups, who were suspected of bacteraemia by the clinicians attending Everest Hospital, New Baneshwor, Kathmandu, Nepal.

All blood samples, for culture 3ml from children and 5ml from adult were collected from peripheral vein aseptically before starting antibiotic therapy and inoculated into 45 ml of sterile brain heart infusion broth supplemented with Sodium Polyanethol Sulphonate (SPS). Inoculated medium was thoroughly mixed to avoid clotting of the blood and immediately transported to the microbiology laboratory for further procedures. BHI broth was incubated at 37°C upto7 days unless the visible growth was obtained. The culture bottles were examined daily for visual evidence of microbial growth such as haemolysis, turbidity, gas production, pellicle formation and "puffballs" and clotting which indicates the positive microbial growth. Subcultures were performed from positive culture bottles on blood agar and MacConkey agar. Plates were incubated at 37°C for 24 hours (Forbes, et al., 2007). The identification of bacteria from isolated colonies was done by standard microbiological procedures as described in Bergey's Manual (second edition 2004), which involve colony morphology, Gram stain and biochemical reaction. Antibiotic susceptibility testing of bacterial isolates was done by Kirby Bauer disc diffusion method with Muller-Hinton agar using the guidelines and interpretive criteria of the Clinical and Laboratory Standards Institute (CLSI 2013). Control strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter. Statistical analysis was done by using SPSS version 16. Frequency and percentages were calculated and Chi-square test was done whenever applicable with $P<0.05$ regarded as significant.

RESULTS

Out of total 400 blood samples processed for culture, 48 (12%) showed bacterial growth whereas 352 (88%) showed no growth. Among 48 culture positive isolates, 81.2% were from outpatients and remaining 18.7% were from inpatients. Out of total bacterial isolates (n=48), Gram negative bacteria were found to be 27 (56.2%) with *Salmonella* Typhi, Paratyphi A, *E. coli*, *pseudomonas aeruginosa* and *Klebsiella pneumoniae* being the common microbes. Out of 21(43.7%) gram positive bacteria, *Staphylococcus aureus* (31.2%) was found to be most predominance followed by CoNS and *Streptococcus pneumoniae*.

Table 1: List of bacterial isolates

Types of bacteria	Number	Percentage (N=48)
Gram Negative Bacteria	27	56.2
Salmonella Typhi	8	16.6
Salmonella Paratyphi A	10	20.8
Escherichia coli	4	8.3
Pseudomonas aeruginosa	3	6.2
Klebsiella pneumoniae	2	4.1
Gram positive bacteria	21	43.7
Staphylococcus aureus	15	31.2
CoNS	4	8.3
Streptococcus pneumoniae	2	4.1

Table 2: Distribution pattern of the bacterial isolates in blood cultures of different age group

Types of organism isolated	Age group (years)								Total
	0-10	11-20	21-30	31-40	41-50	51-60	61-70	>71	
Salmonella Typhi	4	0	1	0	2	0	1	0	**8**
Salmonella Paratyphi A	2	1	2	3	0	1	0	1	**10**
Escherichia coli	0	1	2	0	0	1	0	0	**4**
Pseudomonas aeruginosa	0	0	1	2	0	0	0	0	**3**
Klebsiella pneumoniae	2	0	0	0	0	0	0	0	**2**
Staphylococcus aureus	1	2	1	4	2	5	0	0	**15**
CoNS	0	0	2	1	0	1	0	0	**4**
Streptococcus pneumoniae	2	0	0	0	0	0	0	0	**2**
Total (No.)	**11**	**4**	**9**	**10**	**4**	**8**	**1**	**1**	**48**

Among the total patients, 262 (65.5%) were male showed 31/262 (11.8%) growth and remaining 138 (34.5%) female showed 17/138 (12.3%) growth. The age group between 21 to 30 years had the maximum requests of 110 (27.5%) for blood culture followed by age group between 11 to 20 years 89 (22.2%) whereas, age group above 71 years found to be least with 15 (3.7%) requests.

All gram-positive isolates were found to be sensitive to Cefoxitin, Ceftriaxone and Vancomycin followed by Ampicillin (90.4%), Erythromycin (85.7%), Ciprofloxacin (83.3%), Doxycycline (75%) and Cephalexin (70.5%) whereas Gram negative isolates were sensitive to Ceftriaxone followed by Chloramphenicol (92%), Gentamicin (88.8%), Cefixime (85.7%), Ofloxacin (83.3%) and Amoxycillin and Ciprofloxacin (71.4%) (Table 3 and Table 4).

Table 3: Antibiotic susceptibility pattern of Gram negative bacteria

Antibiotics	Total no. of isolates	Susceptibility pattern	
		Resistant (%)	Susceptible (%)
Amoxycillin	27	6 (28.5)	21 (71.4)
Cotrimoxazole	17	6 (35.2)	11 (64.7)
Cefixime	14	2 (14.2)	12 (85.7)
Ofloxacin	24	4 (16.6)	20 (83.3)
Ciprofloxacin	27	6 (28.5)	21 (71.4)
Ceftriaxone	23	0 (0)	23 (100)
Chloramphenicol	25	2 (8)	23 (92)
Nalidixic acid	18	12 (66.6)	6 (33.3)
Gentamicin	9	1 (11.1)	8 (88.8)
Cefotaxime	15	5 (33.3)	10 (66.6)

Table 4: Antibiotic susceptibility pattern of Gram positive bacteria

Antibiotics	Total no. of isolates	Susceptibility pattern	
		Resistant (%)	Susceptible (%)
Ampicillin	21	2 (9.5)	19 (90.4)
Cotrimoxazole	21	12 (57.1)	9 (42.8)
Chloramphenicol	4	0 (0)	4 (100)
Ciprofloxacin	6	1(16.6)	5 (83.3)
Cefoxitin	19	0 (0)	19 (100)
Ceftriaxone	17	0 (0)	17 (100)
Doxycycline	4	1 (25)	3 (75)
Vancomycin	19	0 (0)	19 (100)
Erythromycin	21	3 (14.2)	18 (85.7)
Cephalexin	17	5 (29.4)	12 (70.5)
Clindamycin	15	0 (0)	15 (15)

DISCUSSION

The present study was conducted to isolate the bacteria causing blood stream infection and their antimicrobial susceptibility pattern among the patient visiting Everest hospital, New Baneshwor Kathmandu. The isolation rate of blood culture positive cases was 12% which was similar to the study conducted by (Pandey et al. 2013) reporting a 12.6% blood culture positivity rate and a study from western Nepal in 2007 with isolation rate 10.2% (Easow et al. 2010), while a similar study which was done in Iran also showed a lower positivity rate of 5.6% (Mehdinejad et al. 2009). A very recent study done in south India also showed 8.3% culture positive samples (Vanitha et al. 2012) whereas (Meremikwu et al. 2005) reported 45.9% of positive blood culture. The variation in the blood culture positivity may be attributed to the factors like number and amount of blood culture taken for screen (Lee et al. 2007).

Gram negative bacteria (56.25%) were found to be predominant in our study which was similar as reported by (Osinupebi & Olajubu 2003) and (Sharma et al. 2006). The predominance of Gram-negative organisms reported as etiological agents of bacteraemia and septicaemia was also seen in the study done by (Mehta et al. 2005) where Gram-negative bacteria accounted for 80.96%. This is in contrast to the study by (Babay et al. 2005) revealed the predominance of gram positive bacteria (78.6%). And also, from finding by (Chaudhry et al. 2000); (Khanal et al. 2007), (Lee et al. 2007), Gram positive bacteria as the predominant. Variation of such etiology are due to changes in geographical location and antibiotic policy advocated in hospital which reflects the better isolation of patients in hospital and hand washing practices in ICU or high-risk unit in hospital (Mahmood 2001). *S. aureus* 31.2% (15/48) was isolated as the most common causative agent followed by *S.* Paratyphi A 20.8% (10/48), *Salmonella* Typhi 16.6% (8/48), *Klebsiella pneumoniae* 6.2% (3/48), *P. aeruginosa* 6.2% (3/48), *E. coli* 8.3% (4/48), *Streptococcus pneumoniae* 4.1% (2/48) and CoNS 6.2% (3/48). Similar finding reported by Swain and Otto, 2012, where they reported *Staphylococcus* was common organism (73%).

In this study, analysis of blood samples covers almost all aged patients in which the highest frequency of infection was in the age group (0-10) years that was (22.9%). Among the positive blood culture male was 11.8% (31/262) and the female was 12.3% (17/138) Similar finding was reported by (Manandhar et al. 2009), in their study conducted at Sahid Gangalal National Heart Centre, Bansbari, Nepal and studies from both Nepal and other parts of the world (Kandel et al. 2007); (Mylonakis & Calderwood, 2001). Physical stress, smoking habits and types of invasive procedures could contribute for impairments of cardiac conditions leading to bacteremia. Among the total population, the highest culture positive was from outpatients 81.2% which might be because the patients were in the initial phase of infection without undergone any antibiotic therapy. The highest peak of culture positivity was seen in the month of March 2015 followed by August, May, July, June and April. This may be possibly due to sewage mediated contamination of drinking water (Rai et al. 2005). Another factor influencing this occurrence might be scanty and contaminated community water supply in summer and rainy season (Bhatta et al. 2007).

In our study, gram-positive isolates showed 100% sensitive towards Cefoxitin, Ceftriaxone and Vancomycin followed by Ampicillin (90.4%), Erythromycin (85.7%), Ciprofloxacin (83.3%), Doxycycline (75%) and Cephalexin (70.5%) but showed resistance towards Cotrimoxazole similar pattern was reported by (Lee et al. 2007) whose found shows that gram positive isolates was sensitive to most of the antibiotics including Ampicillin, penicillin and vancomycin. Similarly, gram negative isolates showed 100% sensitive to Ceftriaxone followed by Chloramphenicol (92%), Gentamicin (88.8%), Cefixime (85.7%), Ofloxacin (83.3%) and Amoxycillin and Ciprofloxacin (71.4%). The antibiotic Nalidixic acid (66.6%) was found least effective towards Gram negative isolates resembling with the study conducted by (Tibrewal, 1999) found that ciprofloxacin was the most effective antibiotic (93.2%) whereas ampicillin was found to be 20.2% effective against all the 89 gram-negative isolates. It has been suggested that resistance to nalidixic acid may be an indicator of low-level resistance to ciprofloxacin (Vasallo et al. 1998).

CONCLUSION

Bacteraemia is emerging as an important disease in our community. Proper identification of isolates using microbiological tools should be undertaken.

ACKNOWLEDGEMENTS

We would like to thank all the patients whose samples were used in the study, all the staff of Everest Hospital,

New Baneshwor, Kathmandu, all the faculties and staff of Department of Microbiology, Tri-Chandra Multiple Campus, and all my friends and family members.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Karlowsky JA et al. (2004). Prevalence and antimicrobial susceptibilities of bacteria isolated from blood cultures of hospitalized patients in the United States in 2002. *Ann Clin Microbiol Antimicrob*, **3**: 7.

Babay HA, Twum-Danso K, Kambal AM & Al-Otaibi FE (2005). Bloodstream infections in pediatric patients. *Saudi Med J* **26**: 1555-1561.

Bhatta DR et al. (2007). Serotyping, PCR, phage-typing and antibiotic sensitivity testing of Salmonella serovars isolated from urban drinking water supply systems of Nepal. *App Microbial Newsletter* **44**: 588-94.

Chaudhry I et al. (2000). Etiologcal pattern of septicemia at three Hospitals in Lahore. *JCPSP* **10**: 375-379.

Christoph KN et al. (2009). Clinical Consensus Conference: Survey on Gram-Positive Bloodstream Infections with a Focus on *Staphylococcus aureus*. *Clin Infect Dis* **48**(Suppl 4): S260-S270.

Cleven BE et al. (2006). Identification and characterization of bacterial pathogens causing bloodstream infections by DNA microarray. *J Clin Microbiol* **44**(7): 2389-97.

Easow MJ et al. (2010). Blood Stream Infections among febrile patients attending a teaching hospital in Westren Region of Nepal. *AJM,* **3**(10): 633–37.

Enoch DA, Birkett CI and Ludlam HA (2007). Non-fermentative Gram-negative bacteria. *Int J Antimicrob Agents,***29**(Suppl 3): S33-S41.

Forbes BA, Sahm DF & Weissfeld AS (2007). *Bailey & Scott's Diagnostic Microbiology.*12th ed. s.l.:Mosby.

Kandel NP et al. (2007). Laboratory tests for infective endocarditis among patients visiting Sahid Gangalal National Heart Centre, Bansbari, Kathmandu, Nepal. *J Nepal Health Res Counc* **1**: 5.

Kang CI et al. (2005). Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. *Antimicrob Agents Chemother* **49**(2): 760-6.

Khanal B et al. (2007). Antimicrobial susceptibility patterns of Salmonella enterica serotype Typhi in Eastern Nepal. *J H Pop Nutr* **25:** 28-7.

Kumar A et al. (2006). Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit Care Med* **36**(6): 1589-96.

Lee A, Mirrett S, Reller LB and Weinstein MP (2007). Detection of bloodstream infections in adults: how many blood cultures are needed?. *J Clin. Microbial* **45**(11): 3546-8.

Mahmood A (2001). Blood stream infections in a medical intensive care unit: spectrum and antibiotic susceptibility pattern. *J Pak Med Assoc* **51**: 213–15.

Manandhar S et al. (2009). Bacteriological profile of heart valves resected from infective endocarditis patients. *J Nepal Health Res Counc* **7**: 108-11.

Mehdinejad A, Khosravi D and Morvaridi A (2009). Study of prevalence and antimicrobial susceptibility pattern of bacteria isolated from blood cultures. *J Bio Sci* 249–53.

Mehta M, Dutta P & Gupta V (2005). Antimicrobial susceptibility pattern of blood isolates from a teaching hospital in north India. *Jpn J Infect Dis* **58**: 174–76.

Meremikwu MM et al. (2005). Bacterial isolates from blood cultures of children with suspected septicaemia in Calabar, Nigeria. *BMC Infect Dis* **5**:110.

Mylonakis E and Calderwood SB (2001). Infective endocarditis in adults. *N Engl J Med* 1318-30.

Mylotte JM, McDermott C and Spooner JA (1987). Prospective study of 114 consecutive episodes of *Staphylococcus aureus* bacteremia. *Rev Infect Dis* **9**(5): 891-907.

Osinupebi OA and Olajubu FA (2003). *Afr J Med Med Sci* Volume **32**: 311-14.

Pandey S, Raza S and Bhatta CP (2013). The Aetiology of the Bloodstream Infections in the Patients Who Presented to a Tertiary Care Teaching Hospital in

Kathmandu, Nepal. *J Clin Diagn Res* **7**(4): 638-41.

Pittet D, Tarara D and Wenzel RP (1994). Nosocomial bloodstream infection in critically ill patients. Excess length of stay, extra costs, and attributable mortality. *JAMA* **271**(20): 1598-601.

Rai DR, Kshetry NT, Tamang MD and Pokharel BM (2005). Study of enteric fever and malaria incidence in southern part of Nepal. *J Inst Med* **27**(3): 46-51.

Sharma NP et al. (2006). A hospital-based study of bloodstream infections in febrile patients in Dhulikhel Hospital Kathmandu University Teaching Hospital, Nepal. *Southeast Asian J Trop Med Public Health* **37**: 315-56.

Suárez CJ, Lolans K, Villegas MV and Quinn JP (2005). Mechanisms of resistance to β-lactams in some common Gram-negative bacteria causing nosocomial infections. *Expert Rev Anti Infect Ther* **3**(6): 915-922

Tibrewal A (1999). *A* Prospective Study on Etiological Agents Causing Infective Endocarditis and Related Bacteraemic and Septicaemic Cases among Patients Visiting Bir Hospital. M.Sc. Dissertation, Submitted to Central Department of Microbiology, Tribhuvan University, Kathmandu.

Vanitha RN et al. (2012). A retrospective study on blood stream infections and antibiotic susceptibility patterns in a tertiary care teaching hospital. *Int J Pharm Pharm Sci* **4**: 543–48.

Vasallo FJ et al. (1998). Failure of ciprofloxacin therapy for invasive nontyphoidal salmonellosis. *Clin Infect Dis,* **26**: 335-36.

Wayne P (2003). Performances, Standards for Antimicrobial Disk Susceptibility. PA 19087 USA.

Extended Spectrum Beta Lactamase and Metallo Beta Lactamase Producing *Pseudomonas aeruginosa* at Tertiary Care Hospital of Nepal

Pallavi Shrestha[1], Saroj Sharma[2], Roshani Maharjan[3*]

[1]Department of Microbiology, St. Xavier's College, Maitighar, Kathmandu, Nepal

[2]Kanti Children's Hospital, Maharjgunj, Kathmandu, Nepal

[3]Department of Microbiology, Tri-Chandra Multiple College, Ghantaghar, Kathmandu, Nepal

***Corresponding author:** Roshani Maharjan, Department of Microbiology, Tri-Chandra Multiple College, Ghantaghar, Kathmandu, Nepal. E-mail: roshani_mh@hotmail.com

ABSTRACT

Objective: To assess the prevalence of extended spectrum beta lactamase (ESBL) and metallo beta lactamase (MBL) producing *Pseudomonas aeruginosa* from pus samples.

Methods: A cross-sectional study was conducted at Kanti Children's Hospital, Kathmandu, Nepal during which 316 pus samples were collected and tested using standard microbiological procedures. Antibiotic Susceptibility Test (AST) was done by Kirby-Bauer disk diffusion method and the detection of ESBL and MBL production were done using Ceftazidime/clavulanic acid combined disk test and Imipenem-Ethylenenediaminetetraacetic acid combined disk test respectively as per CLSI guideline 2014.

Results: The prevalence rate of *P. aeruginosa* was found to be 7.9% in pus samples. Out of 25 *P. aeruginosa* isolates 9(36%) were ESBL producers and 2(8%) were MBL producers. ESBL producers were predominant in the age group 2-3 years (33.3%) and in male patient (55.6%).Out of 2 MBL producing *P. aeruginosa,* 1(50%)was isolated from the age group below 2 years and male patient and 1(50%) from the age group 8-9 years and female patient. 96% of isolates showed sensitive to Polymyxin B.

Conclusion: The study showed increasing trend of ESBL and MBL production in *P. aeruginosa* so constant survey of prevalence of ESBL and MBL producing isolates is essential to control and manage spread of these isolates in different units of health institutions.

Key words: Pus, antibiotic susceptibility test, ESBL, MBL, *Pseudomonas aeruginosa.*

INTRODUCTION

Multidrug-resistant *Pseudomonas aeruginosa,* a major pathogen in pyogenic infections (Soumya and Nagmoti 2017) are serious problems to the successful treatment of the wounds leading to complications sometime even fatal sepsis.

P. aeruginosa is intrinsically resistant to most of the drugs making the therapeutic choices limited for its treatment (Murray et al. 2015). The acquisition of ESBL and MBL producing genes by *P. aeruginosa* has made the bacteria resistant to the antibiotics among the limited choice. Thus, making the treatment of infections caused by *P. aeruginosa* difficult or impossible to treat and increasing global threat in community and hospital settings (Ansari et al. 2016; Hirsh and Tam 2010; Solomon et al. 2017).

ESBLs are plasmid-mediated beta-lactamase that confer resistance to the penicillins, first-, second-, and third-generation cephalosporins, and Aztreonam and are inhibited by β-lactamase inhibitors such as clavulanic acid (Poudyal et al. 2011). The exposure of bacterial strains to a multitude of beta-lactams has induced mutation of beta-lactamase in many bacteria, expanding their activity even against carbapenems by the production of MBL carbapenamases which require zinc divalent cation, as cofactor for enzyme activity and are able to hydrolyze all β- lactams except monobactam and known to be inhibited by chelating divalent cations like Ethylenediaminetetraacetic acid (EDTA).

Date of Submission: August 3, 2018
Date of Acceptance: November 15, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22301

The ESBL and MBL producing *P. aeruginosa* infections have high mortality rates and have emerged worldwide rapidly. A very few study on ESBL and MBL producing *P. aeruginosa* has been conducted in Nepal thus necessitating the extensive study on its prevalence. This study was conducted with an objective toassess the prevalence of ESBL and MBL producing *P. aeruginosa* from pus samples.. This study would help to plan a proper hospital infection control strategy to prevent the spread of these isolates.

MATERIALS AND METHODS

A hospital based descriptive cross sectional study was carried out at Kanti Children's Hospital, Maharjgunj, Kathmandu Nepal from August 2015 to January 2016. This study included patients of age group below 14 years of both sex. A total of 316 samples sent for routine investigation were processed. The study protocol was approved by the institutional review committee (Ref no: 117-072/73) of Kanti Children's Hospital, Kathmandu.

Pus sample in cotton swab and aspirated pus form was inoculated on to MacConkey Agar (MA), Blood Agar (BA) and Cetrimide agar (Himedia) plates according to standard microbiological procedure (Forbes et al. 2007). Identification of *P. aeruginosa* was done by using conventional biochemical tests. Antimicrobial sensitivity testing was performed on Mueller-Hinton agar plates by Kirby-Bauer disk diffusion method as described by Clinical Laboratory Standards Institute guidelines (CLSI 2014).

ESBL detection

The isolates were screened for possible ESBL production using Ceftazidime (30µg) as per CLSI 2014 guidelines. According to the guidelines, isolates showing cefpodoxime <17mm, ceftazidime<22mm, aztreonam ≤27mm, cefotaxime <27mm, and ceftriaxone<25mm are the possible ESBL producing strains. ESBL production was confirmed among the suspected bacterial strain using combined disk (CD) assay, an increase in zone size of ≥5mm from either of the combination disk i.e. clavulanate containing disk indicated the presence of ESBL in the test organisms.

MBL detection

For the detection of production of MBL, combined disk test (CDT) was performed using two Imipenem disks (10µg), one containing 10µl of 0.1 M (292µg) anhydrous EDTA (Hi-Media, India). Disks were placed 25mm apart and an increase in zone diameter of > 4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for MBL production. These isolates were considered to be of the MBL positive phenotype (Lee et al. 2003).

RESULTS

Among 316 non repetitive pus samples processed, *P. aeruginosa* was isolated from 25(7.9%) samples (Figure 1). Maximum number of isolates were obtained from females 13(52%) and in the age group 4-5 years 8(32%).The association between culture positivity of *P. aeruginosa* isolates with age and gender was found statistically insignificant (p>0.05). Highest percentage of *Pseudomonas aeruginosa* isolates were sensitive to Polymyxin B (96%), followed by Imipenem (92%), Amikacin (88%) (Table 1).

Among 25 *P. aeruginosa* isolates, 9(36%) were ESBL producers and 2(8%) were MBL producers. Of 21 screening positive *P. aeruginosa*, 9(42.9%) were confirmed to be ESBL producers by phenotypic methods. There was no association between screening and confirmatory tests (p>0.05) (Table 2). ESBL producers were predominant in the age group 2-3 years (33.3%)(Table 3) and in male patient (55.6%) (Table 4). Out of 2 MBL producing *P. aeruginosa* 1(50%) was isolated from the age group below 2 years and male patient and 1(50%) from the age group 8-9 years and female patient. ESBL production and MBL production was found to be insignificantly associated with age and gender (p>0.05).

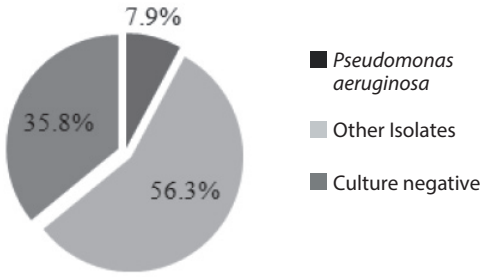


Figure 1: Distribution of *Pseudomonas aeruginosa* in pus samples

Table 1: Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotics	Susceptibility pattern		
	Sensitive, N (%)	Intermediate, N (%)	Resistant, N (%)
Amikacin	22(88)	0	3(12)
Piperacillin	2(8)	6(24)	17(68)
Piperacillin/Tazobactam	8(32)	4(16)	13(52)
Ciprofloxacin	16(64)	1(4)	8(32)
Ceftazidime	10(40)	4(16)	11(44)
Cefotaxime	5(20)	10(40)	10(40)
Imipenem	23(92)	0	2(8)
Polymyxin B	24(96)	0	1(4)

Table 2: Screening and confirmation of ESBL production

Screening	Production of ESBL				Total	P-value
	Yes	%	No	%		
Positive	9	42.9	12	57.1	21	0.102
Negative	0	0	4	100	4	
Total	**9**	**36**	**16**	**64**	**25**	

Table 3: Distribution of ESBL producing *P. aeruginosa* isolates according to patient age

Age group (Years)	ESBL Positive		Total	p-value
	No	%		
<2	2	22.2	7	0.7
2-3	3	33.3	5	
4-5	2	22.2	8	
-6-7	0	0	0	
-8-9	1	11.1	2	
10-11	0	0	1	
13-14	1	11.1	2	
Total	**9**	**100**	**25**	

Table 4: Distribution of ESBL producing isolates according to sex of patient

Sex	ESBL positive		Total	p-value
	No	%		
Male	5	55.6	12	0.57
Female	4	44.4	13	
Total	**9**	**100**	**25**	

DISCUSSION

Of the total 316 pus samples processed, *P. aeruginosa* was isolated from 25 samples. The prevalence rate of *P. aeruginosa* was found to be 7.9% which is in accordance with the study conducted by Mantravadi et al. 2015 and Upadhyay et al. 2010 that showed 7.5% and 7.4% *P. aeruginosa* growth respectively. Similar study conducted by Dash et al. 2014, Sharma et al. 2016 and Yakha et al. 2014 showed the prevalence of *P. aeruginosa* in pus as 13.0%, 22.4% and 12.1% respectively. This variation may have occurred due to type and number of pus specimen used, source of pus, use of antibiotic, climate and topographical situation of area under study, sanitation and the duration of study.

Maximum number of isolates were obtained from

females 13(52%) which is similar to the study carried out by Hassuna et al. 2015. This might be due to large number of females admitted to the hospital than the males. But most of the other similar studies showed greater isolation in males for example Al-Marzoqi and Al-Taee 2013, Biradar et al. 2016 and Sonth et al. 2015. Maximum number of isolates were obtained from the age group 4-5 years 8(32%). The study carried out by Hassuna et al. 2015 showed the higher isolation of *P. aeruginosa* from the age group below 10 years. The association between culture positivity of *P. aeruginosa* isolates with age and gender was found statistically insignificant (p>0.05).

Of 25 isolates 21 (84%) were screening positive and of 21 screening positive isolates 9 (42.8%) were confirmed to be ESBL producers which is in harmony with the study carried out by Bharti and Sharma 2014.In contrary to our study, Tsering et al. 2009 showed 32.6% screening positive for ESBL production and all the isolates were positive for ESBL production by phenotypic confirmatory tests.

The issue of ESBL and MBL production is increasing at different rates throughout the world that has become problematic in therapeutic treatment. This study showed the high prevalence of ESBL producing *Pseudomonas aeruginosa* (36%) which is in accordance with the study carried out by Ansari et al. 2016 that showed 33.1% of isolates to be ESBL producer. Similar study carried out in Nepal by Pathak and Pokharel 2015 showed 18.1% of isolates to be ESBL producer which was low as compared to this study. Similarly, the study of Poudyal et al. 2011 recorded zero percent of *P. aeruginosa* as ESBL producers. This indicated the increasing trend of ESBL production in *P. aeruginosa* in Nepal.

This study showed the prevalence of MBL producing *P. aeruginosa* to be 8% which is high as compared to the study conducted in Nepal by Mishra et al. 2012, Shrestha et al. 2011 which showed 3.3% and 2.4% of *P. aeruginosa* isolates as MBL producers respectively. Similarly, the study carried out by Ansari et al. 2016 and Khanal et al. 2013 showed 30.9% and 18.2% of *P. aeruginosa* isolates as MBL producers respectively. This study documents low prevalence of MBL producing *P. aeruginosa*. But increased use of carbapenems to treat ESBL isolates and horizontal transfer of MBL genes might lead to high prevalence of MBL in future that poses serious therapeutic challenges.

In this study, the highest percentage of ESBL isolates was isolated from the male patient (55.5%) which is in accordance with a study carried out by Anjum and Mir 2010 where the highest percentage of ESBL isolates was isolated from female. The highest percentage of ESBL isolates was isolated from the age group 2-3 years (33.3%). ESBL production was found to be insignificantly associated with age and gender (p>0.05).

Out of 2 MBL producing *P. aeruginosa* 1(50%) was isolated from the age group below 2 years and male patient and 1(50%) from the age group 8-9 years and female patient. MBL production was found to be insignificantly associated with age and gender (p>0.05).

Polymyxin B was found to be the most effective drug against *P. aeruginosa* with 96% susceptibility which is in accordance with the similar study carried out by Kumar et al. 2014, Sharma et al. 2016 and Tankhiwale 2016 that showed 94%, 95% and 94.5% susceptibility respectively. In a similar study carried out by Bhandari et al. 2012, Parajuli et al. 2014 and Patel and Garala 2014, 63.6%, 64.2% and 73% of *P. aeruginosa* isolates was sensitive toward Polymyxin B respectively which is less than our findings. In this study, Imipenem (92%), Amikacin (88%) and Ciprofloxacin (64%) were also found to be effective against Pseudomonal infections. Since Ciprofloxacin has fewer side effects and is cheaper than other drugs, it can be recommended as the drug of choice for Pseudomonal infections.

CONCLUSION

This study showed the increased prevalence of ESBL and MBL production in *P. aeruginosa* which warrants early detection in routine laboratory, immediate infection control, and antibiotic stewardship programs in order to limit the spread of ESBL and MBL positive isolates. The appearance of ESBL and MBL genes and their spread among bacterial pathogens are matters of major concern with regard to the future antimicrobial chemotherapy. Polymyxin B was found to be the most effective drug against *P. aeruginasa*.

ACKNOWLEDGEMENTS

We are thankful to all the staff of Microbiology Department of Kanti Children's Hospital for their generous help and support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Al-Marzoqi AH and Al-Taee ZM (2013). *Pseudomonas aeruginosa*: Antibiotic resistance pattern to different isolates in Al-Hillah city, Iraq. *J Nat Sci Res* **3**(3): 69-74.

Anjum F and Mir A (2010). Susceptibility pattern of *Pseudomonas aeruginosa* against various antibiotics. *Afr J Microbiol Res* **4**(10): 1005-1012.

Ansari S, Dhital R, Shrestha S, Thapa S, Puri R, Chaudhary N, Khatiwada S, and Gautam R (2016). Growing Menace of Antibacterial Resistance in Clinical Isolates of *Pseudomonas aeruginosa* in Nepal: An Insight of Beta-Lactamase Production. *BioMed Res Int*: 6437208: 8.

Bhandari S, Banjara MR, Lekhak B ,Bhatta DR and Regmi SR (2012). Multi-Drug and Pan-Drug Resistant *Pseudomonas aeruginosa*: A Challenge in Post- Antibiotic Era. *Nepal JSci Tec* **13**(2): 197-202.

Bharti and Sharma PC (2014). Prevalence of Extended spectrum beta lactamase (ESBL) producing *Pseudomonas aeruginosa* strains recovered from Human patients in Himachal Pradesh. *Ind J Basic App Med Res* **4**(1): 430-440.

Biradar A, Farooqui F, Prakash R, Khaqri SY and Itagi I (2016). Aerobic bacteriological profile with antibiogram of pus isolates. *Indian J Microbiol Res* **3**(3): 245-249.

CLSI (2014). Performance Standards for Antimicrobial Susceptibility Testing, Twenty-fourth Informational Supplement, CLSI Document M100-S24, Wayne, PA: Clinical and Laboratory Standards Institute

Dash M, Padhi S, Narasimham MV and Pattnaik S (2014). Antimicrobial resistance pattern of *Pseudomonas aeruginosa* isolated from various clinical samples in a tertiary care hospital, South Odisha, India. *Saudi J Health Sci* **3**(1): 15-19.

Hassuna NA, Mohamed AHI, Abo- Eleuoon SM and Rizk HAWA (2015). High Prevalence of Multidrug Resistant *Pseudomonas aeruginosa* Recovered from Infected Burn Wounds in Children. *American society Microbiol* **6**(4): 7.

Hirsch EB and Tam VH (2010). Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Rev Pharmacoecon Outcomes Res*. **10**(4): 441-451.

Khanal S, Joshi DR, Bhatta DR, Devkota U and Pokhrel BM (2013). *β*-Lactamase producing multidrug-resistant bacterial pathogens from tracheal aspirates of intensive care unit patients at national institute of neurological and allied sciences, Nepal. *Int Scholarly Res Notice Microbiol*.

Kumar R, Srivastva P, Rishi S, Dahiya SS, Hemwani K and Nirwan PS (2012). Detection and Antimicrobial Susceptibility Pattern of *Pseudomonas aeruginosa* isolates in various clinical samples with special reference to Metallo beta lactamase from a tertiary care hospital in Jaipur, India. *Natl J Med Res* **4**(2): 128-131.

Forbes BA, Sahm DF and Weissfeld AS (2007). Bailey and Scott's Diagnostic Microbiology 12th edition. Mosby Elsevier Publication, USA: 350-362.

Lee K, Lim YS, Yong D, Yum JH and Chong Y (2003). Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp and *Acinetobacter* spp. *J Clin Microbiol*. **41**: 4623-4632.

Mantravadi HB, Chinthaparthi MR and Shravani V (2015). Aerobic isolates in pus and their antibiotic sensitivity pattern: a study conducted in a teaching hospital in Andhra Pradesh. *Int J Med Sci Public Health* **4**(8): 1076-1079.

Mishra SK, Acharya J, Kattel HP, Koirala J, Rijal BP, Pokhrel BM (2012). Metallo-beta-lactamase Producing Gram-negative Bacterial isolates. *J Nepal Health Res Counc* **10**(22):208-213

Parajuli P, Basnyat SR, Shrestha R, Shah PK and Gurung P (2014). Identification and Antibiotic Susceptibility Pattern of Aerobic Bacterial Wound Isolates In Scheer Memorial Hospital. *JSM Microbiol* **2**(2): 1011.

Patel H and Garala RN (2014). Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolated at SSG hospital Baroda. *J Res Med Den Sci***2**(1): 84-87.

Pathak J and Pokharel N (2015). Multidrug Resistant and Extended Spectrum betalactamase (ESBL)

Isolates from different clinical specimens. *Int JSci Res Pub* **5**(9): 5.

Poudyal S, Bhatta DR, Shakya G, Upadhyaya B, Dumre SP, Buda G and Kandel BP (2011). ESBL producing multi drug resistant clinical bacterial isolates at National Public Health Laboratory, Nepal. *Nepal Med Coll J* ***13***(1): 34-38.

Sharma J, Singh S, Gill AK and Kaur A (2016). Prevalence and Antimicrobial Susceptibility Pattern of *Pseudomonas aeruginosa* Isolated from Pus Samples in a Tertiary Care Hospital, Bathinda. *Int JCon Med Res***3** (12): 3481-3483.

Shrestha S, Chaudhari R, Karmacharya SKattel HP, Mishra SK, Dahal RK, Bam N, Banjade N, Rijal BP, Sherchand JB Ohara H, Koirala J and Pokhrel BM (2011). Prevalence ofnosocomial lower respiratory tract infections caused by multidrugresistance pathologens. *JInt Microbiol* **33**(2): 1–17.

Solomon FB, Wadilo FW, Arota AA, Abraham YL (2017). *Ann Clin Microbiol Antimicrob* **16**: 29

Sonth SB, Bhurle A, Gokale S, Hadapad D and Solabannavar SS (2015). Resistance Pattern of *Pseudomonas aeruginosa* in a Tertiary Care Hospital. *Int J Curr Microbiol App Sci* **4**(10): 919-923.

Soumya S and Nagmoti Mahantesh B (2017). Co-existence of Beta Lactamases (ESBL and MBL) in *Pseudomonas aeruginosa* Isolates from Pus Samples. *Int J Microbiol Res* **9**(12): pp 981-983.

Tankhiwale S (2016). Beta-lactamases in *P. aeruginosa*: A threat to clinical therapeutics. *Curr Pediatr Res* **20**(1&2): 253-257.

Tsering CD, Shyamasree D, Luna A, Ranabir P and Takhellamban SK (2009). Extended spectrum beta-lactamase detection in gram-negative bacilli of nosocomial origin. *J Global Infect Dis* **1**(2): 87-92.

Yakha JK, Sharma AT, Dahal N, Lekhak B and Banjara MR (2014). Antibiotic Susceptibility Pattern of Bacterial Isolates Causing Wound Infection Among the Patients Visiting B & B Hospital. *Nepal J Sci Tech* **15**(2): 91-96.

Detection of Methicillin Resistant *Staphylococcus aureus* in Public Transportation of Kathmandu Valley, Nepal

Khadga Bikram Angbuhang[1*], Mukesh Neupane[1], Aditya Adhikari[1], Binita KC[1], Sabina Jha

[1]Department of Microbiology, GoldenGate International College, Kathmandu, Nepal

***Corresponding author:** Khadga Bikram Angbuhang, Department of Microbiology, GoldenGate International College, Nepal; E-mail: k.subba@goldengate.edu.np

ABSTRACT

Objectives: The purpose of this study was to assess microbial load and Methicillin Resistant *Staphylococcus aureus* from surfaces of public transport vehicle.

Methods: The surfaces of public transport vehicle were sampled by swabbing. A total of 56 samples from 28 different vehicles operating in Kathmandu valley were collected and processed according to the standard methodology. The isolates were identified by culture, biochemical tests and subjected to antimicrobial susceptibility testing by modified Kirby-Bauer disk diffusion method following CLSI 2013 guidelines. Methicillin resistant species of *Staphylococcus* were detected by the virtue of cefoxitin resistance.

Results: All 56 samples from the 28 different vehicles were found to have bacterial growth with average bacterial load of 2.47±1.22 x 10^5 CFU/cm^2. The gas vehicles were found to be the most contaminated. Out of 56 samples, 35 (25.9%) were found to be *S. aureus* growth positive 11 (31.4%) of them being MRSA.

Conclusion: The high flow of people with different health conditions in public transport makes the exchange of microorganism more significant. High bacterial load along with MRSA indicates the threats of transmission of infection among travelers. This is of a great public health concern as the mass population of different health condition is in direct exposure and is prone to get infected.

Key words: Public transport, antibiotic susceptibility testing, MRSA.

INTRODUCTION

Microbes in public area such as public transport, restaurants, schools, daycare centers can be a critical issue in public health, since they can bring a large number of people together which facilitate the transmission of microbes (Stepanovic et al. 2006; Kassem et al. 2007). This becomes a subject of prime concern when microbes are drug resistant and pathogenic. Therefore, increased attention has been paid to environmental microbes, to the numbers and strains of bacteria found in public places (Reynolds et al. 2005; Kassem et al. 2007; Otter and French, 2009).

The public transportation such as three-wheelers, mini/micro buses, buses, trolleybuses, trams, trains and ferries is mainly available for use by anyone (Scott and Bloomfield, 1990) and generally operates on fixed routes. Public transportation system has become increasingly important in urban areas due to mass transit and increased awareness to energy-saving methods of transportation (EPA, 1973; Barrero, 2008). During the travel, various components of the vehicle such as seats, handle, door handle are frequently encountered and may act as the important reservoir for transmission of different pathogenic and non-pathogenic microbes (Oranusi et al. 2016).

Staphylococcus aureus is an opportunistic pathogen often found on the skin, which causes a wide range of infections such as skin lesions, abscesses, endocarditis, septicemia, and toxic shock syndrome. It has now been a pathogen of concern due to the existence of methicillin resistant strains. Also the strains resistant to vancomycin, a drug often referred to as the "drug of

Date of Submission: November 18, 2018
Date of Acceptance: December 10, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22312

last resort", have been reported (Henriques Normark et al. 2001; Jarraud et al. 2002). Methicillin-resistant Staphylococcus aureus (MRSA) is one of the major human pathogens responsible for mild to severe life threatening infections worldwide (de Lencastre and Tomasz, 2011; ECDPC, 2012). Since the mid-1990s, MRSA has also been identified as the etiological agent of infections acquired in the community (CA-MRSA) (Naimi et al. 2003; Patel, 2009; Graves et al. 2010). Within the last 5 years, MRSA has moved from being primarily a nosocomial pathogen to one that is also found in community areas and public places (Carleton et al. 2004; Popovich et al. 2008).

Microbes in the public transportation can be significant as public health is concerned because of the cases of transfer of organisms either from individual to individual directly or by indirect means which includes transfer from individual to inanimate objects like seats, handles bar etc. and then to other individuals thereby causing infection (Ehrenkranz, 1964; Yeh et al. 2011). This study was performed to screen MRSA from the surfaces of public transport system of Kathmandu, Nepal.

MATERIALS AND METHODS

In this study, a total of 56 samples from 28 different public vehicles (Handle and seat surface of each vehicle) operating in Kathmandu valley were collected by wet swab methods as described by Yeh et al. (2011) from June to August, 2017. The area of sampling (2cm x 4cm) was marked and swabbed with sterile moistened cotton swab and was kept in sterile container having sterile buffered peptone water (1ml). Similarly, another moistened swabbed was used in the same way and then kept in a dry sterile container simultaneously. Then, it was transported to microbiological laboratory as soon as possible in cold chain condition.

The enumeration of bacteria was performed as described in Isenberg HD (2004). The sample (in buffered peptone water) was serially diluted in sterile normal saline up to 10^{-6} and enumerated by spread plate technique incubating overnight at 37°C. Another swab was cultured on selective and differential media (Mannitol Salt Agar and Blood Agar, HI-media, India), and incubated at 37°C for up to 48 hours. The isolated colonies from these media were then identified as '*Staphylococcus aureus*' with morphology, Gram staining, and catalase, oxidase, coagulase, DNase tests after subculture on NA.

Modified Kirby-Bauer disk diffusion test based on the guidelines of Clinical and Laboratory Standard Institute (CLSI, 2013) method was used to evaluate the antimicrobial susceptibility pattern of the isolates to a set of antibiotics and determination of methicillin resistance. The antimicrobial agents tested were: Chloramphenicol (C, 30µg), Gentamicin (GEN, 10µg), Erythromycin (E, 15µg), Penicillin (PEN, 10 units) and Cefoxitin (CX, 30µg) supplied from Hi-media. Methicillin resistant species of staphylococci were detected by the virtue of cefoxitin resistance on MHA as per CLSI M100-S23 guidelines (CLSI 2013).

RESULTS

All the 56 surface samples were found to be contaminated with an average bacterial load of 2.47 ± 1.22 x10^5 CFU/cm^2. The samples from the bus were found to be more contaminated (2.57 ±0.88 x10^5 CFU/cm^2) while that of the tempo were found to be the least contaminated (2.35 ± 1.84 x10^5 CFU/cm^2). The average bacterial load was found to be higher on the seat surfaces (2.50 ± 1.51 x10^5 CFU/cm^2) as compared to the handles (2.41 ± 1.93 x10^5 CFU/cm^2).

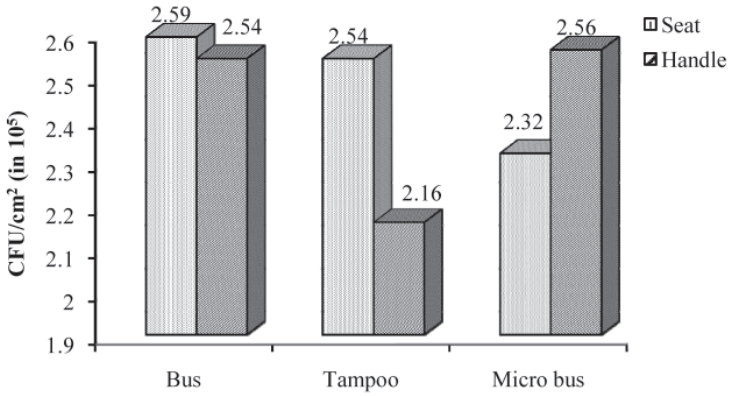


Figure 1: Average bacterial load in vehicles

The surfaces of the gas vehicles were found to be heavily contaminated (3.14 ± 1.75 x 10^5 CFU/cm^2) while that of the electric vehicles were found to be least contaminated (1.48 ± 0.64 x 10^5 CFU/cm^2). Sample-wise, the seat of the gas vehicle was found to be heavily contaminated (3.14 ± 1.75 x10^5 CFU/cm^2) while that of the electric vehicle was found to be least contaminated (1.17 ± 0.79 x10^5 CFU/cm^2).

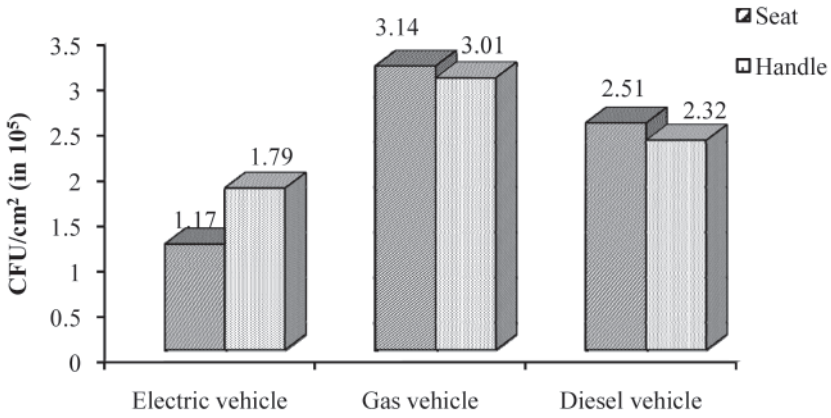


Figure 2: Average bacterial load in the surface of vehicles with respect to fuels

All the vehicles were found to harbor a diverse group of bacteria irrespective of the sample differences. A total of 135 bacteria were isolated from the study with the highest being from the surfaces of the bus 57 (42.2%). Of them, 35 (25.9%) were identified to be *Staphylococcus aureus* and 18 (13.3%) Coagulase negative staphylococci (CONS).

Table 1: Vehicle wise distribution of the bacterial isolates

S.N.	Vehicle	Number of sample	Bacterial isolates	Number	Percent
1	Tampoo	16	*S. aureus*	11	29.7
			Gram positive bacilli	16	43.2
			CONS	7	18.9
			Gram negative bacilli	3	8.1
	Sub total			37	
3	Bus	24	*S. aureus*	13	22.8
			Gram positive bacilli	28	49.1
			CONS	8	14.0
			Gram negative bacilli	8	14.0
	Sub total			57	
5	Micro bus	16	*S. aureus*	11	26.8
			Gram positive bacilli	14	34.1
			CONS	3	7.3
			Gram negative bacilli	14	34.1
	Sub total			41	
	Total			**135**	

A total of 11 (31.4%) *S. aureus* were found to be methicillin resistant (MRSA) and 34 (68.5%) isolates were methicillin susceptible strains (MSSA). Chloramphenicol was found to be the most effective antibiotic in vitro for both MRSA (11, 100%) and MSSA (34, 100%). Penicillin was found to be least effective antibiotic in vitro for both MRSA (11,100%) and MSSA (29, 85.2%).

Table 2: Antimicrobial susceptibility pattern of MSSA and MRSA

S.N.	Antibiotics	MSSA (n=34)		MRSA (n=11)	
		S (%)	R (%)	S (%)	R (%)
1	Penicillin	5(14.7)	29 (85.2)	0(0)	11 (100)
2	Erythromycin	7(20.5)	27(79.4)	3(27.2)	8(72.7)
3	Gentamycin	32(94.1)	2(5.8)	11 (100)	0(0)
4	Chloramphenicol	34(100)	0(0)	11 (100)	0(0)

DISCUSSION

As part of daily activities, many common spaces are shared with other people. This makes it possible to spread diverse microorganisms that can lead to infections. People who use public transport can pass the etiological agents of different infections to other apparently healthy people (Rusin et al. 2002). Due to the regular high flow of mass and the environmental condition of operation, public vehicles have a powerful impact on health of the consumers and the influence is growing globally. Nepal is an under-developed nation and there is no provision of systematic public transport in spite of the mass mobility (UN 2018). Public transport service was started in Kathmandu valley from 1959 and now, different- capacity vehicles such as Tempo, Micro bus, Mini bus, and large bus are in operation in Valley's road and increasing day by day (Pokharel and Acharya, 2015). The public transportation serves people of different health conditions, and become colonized with different pathogenic and non-pathogenic microbes thereby serving as the source of infection to the travelers (Dora et al. 2011). To the best of knowledge, paper regarding the microbiological condition of public transport in Nepal is not available yet. This study was aimed to assess the bacterial load, *S. aureus* and MRSA in public transport vehicle of Kathmandu Nepal.

All the vehicles were found to be colonized with different microbes with the average bacterial load of $2.47 \pm 1.22 \times 10^5$ CFU/cm^2 which indicates poor hygienic condition according to the surface hygiene guideline (BC Centre for Disease Control 2010). The physical contact of the travelling population or their clothing to the surfaces of the bus and the generation of droplets while talking, coughing or sneezing may be primarily responsible for the colonization of the microbes on the solid surface of vehicles (Chowdhury et al. 2016). Similarly, the dust generated in the streets, especially of Kathmandu Valley is also considered to be equally responsible for the microbial colonization (Gautam 2010; Sattar 2016).

The load was in harmony with the reports of Turkey (Tan and Erdoğdu, 2017) but lower than that of Chittagong, Bangladesh $3.1\text{-}23.9 \times 10^5$ CFU/4cm^2 (Chowdhury et al. 2016). The variation in the results may be attributed to the geographical diversity and the corresponding socio-economic and anthropogenic status of the population leading to crowding and ill hygienic practices (Chowdhury et al. 2016).

A total of 35 (25.9%) of *S. aureus* was detected in the study with 31.4% being methicillin resistant. A number of studies have reported varying degrees of MRSA isolates from the hand-touch surface in the public vehicles worldwide (CS et al. 2018; Iwao et al. 2012; Lutz et al. 2014; Otter and French 2009; Peng et al. 2015). The difference in pattern may be due to the geographical, health status of the living population and socio-economic differences between the sample sites (Bogomolova and Kirtsideli 2009; Jones and Harrison 2004).

Chloramphenicol and Gentamicin were found to be effective against MRSA but the increasing trends of antibiotic resistance among MRSA strains is a matter of concern since the public vehicles can act as a potential point source of infection to the apparently healthy population that may ultimately lead to serious epidemics and potential therapeutic failure (Rusin et al. 2002; Eguia and Chambers 2003; Kramer et al. 2006; Tan and Erdoğdu, 2017). Hence, it is suggestive to consider the proper measures for the sanitation and cleansing of the vehicle that may be primitive detergent washing or recent breath-safe system that may decrease the load of some pathogenic organisms (Lukasik 2009).

CONCLUSION

The presence of high bacterial load, *S. aureus* and MRSA on the surfaces of different parts of public vehicles impose the possibility of transmission of serious infection with antibiotics resistant microorganisms.

ACKNOWLEDGEMENTS

Authors are most grateful to CEO, principal, HOD and all the staffs of GoldenGate International College, Kathmandu, Nepal for their facilitation, help and support during the study period.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Barrero R (2008). Energy savings in public transport. IEEE Veh Tech **3**(3): 26-36.

BC Centre for Disease Control (2010). Enviromental hygiene monitoring: a guide for enviromental health officer. Vancouver, BC: BC centre for disease control provicinal health services authority.

Bogomolova E and Kirtsideli I (2009). Airborne fungi in four stations of the St. Petersburg Underground railway system. *Int Biodeterior Biodegradation* **62:** 156-160

Carleton H, Diep B, Charlebois E and Sensabaugh G (2004). Community-adapted methicillin-resistant Staphylococcus aureus (MRSA): population dynamics of an expanding community reservoir of MRSA. *J Infect Dis* **190**(10): 1730-1738.

Chowdhury T, Mahmud A, Barua A, Khalil M, Chowdhury R, Ahamed F and Dhar K (2016). Bacterial contamination on hand touch surfaces of public busses in Chittagong City, Bangladesh. *J Env Sci Toxic Food Tech* **10**: 48-55.

CLSI (2013). Performance Standards for Antimicrobial Susceptibility Testing: Twenty third informational supplement. *Approved Standard*. M100-S23.

CS VK, Prasad S, Patil S, Reddy A, Jha A, KG RK, VL J and *KG B* (2017). Public transport: a large scale fomite of methicillin-resistant *Staphylococcus aureus*. *Int J Res Med Sci* **6**(1): 172-176.

de Lencastre H and Tomasz A (2011). The CEM-NET initiative: molecular biology and epidemiology in alliance--tracking antibiotic-resistant staphylococci and pneumococci in hospitals and in the community. *Int J Med Microbiol* **301**(8): 623-629.

Dora C, Hosking J and Mudu P (2011). Urban transport and health. In: sustainable transport: A source book for policy-makers in developing cities. Eschborn, Germany: 1-42.

ECDPC (2012). Antimicrobial resistance surveillance in Europe 2011. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm.

Eguia JM and Chambers HF (2003). Community-acquired methicillin-resistant *Staphylococcus aureus*: epidemiology and potential virulence factors. *Curr Infect Dis Rep* **2003**(5): 459-466.

Ehrenkranz N (1964). *Person to person transmission of Staphylococcus aureus*. Quantitative characterisation of nasal carrer sprading infection. *N Engl J Med* **271**: 225-330.

EPA (1973). Transportation control strategies for the state implementation plan: city of Philadelphia., Research Triangle Park. NC: EPA.

Gautam RD (2010). Air pollution: its causes and consequences with reference to Kathmandu metropolitan city. *The Third Pole* **8**(10): 27-33.

Graves S, Kobayashi S and DeLeo F (2010). Community-associated methicillinresistant methicillinresistant *Staphylococcus aureus* immune evasion and virulence. *J Mol Med* **88**(2): 109–114..

Henriques Normark B, Novak R, Ortqvist A, Källenius G, Tuomanen E and Normark S (2001). Clinical isolates of *Streptococcus pneumoniae* that exhibit tolerance of vancomycin. *Clin Infect Dis* **32**(4): 552-558.

Isenberg HD (2004). Clinical Microbiology Procedures Handbook (Vol. 1 and 2). 2nd edition. ASM Press, USA

Iwao Y, Shizuka Y, Takano T, Higuchi W, Nishiyama A and Yamamoto T (2012). Isolation and molecular characterization of methicillin-resistant *Staphylococcus aureus* from public transport. *Microbiol Immunol* **56**(1): 76-82.

Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J and Vandenesch F (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun* **70**(2): 631-641.

Jones AM and Harrison RM (2004). The effects of meteorological factors on atmospheric bioaerosol

concentration- a review. *Sci Total Environ* **326**: 151- 180.

Kassem I, Sigler V and Esseili M (2007). Public computer surfaces are reservoirs for methicillin-resistant staphylococci. *ISME J* **1**(13): 265-268.

Kramer A, Kampf G and Schwebke I (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases* **6**: 130.

Lewis R (1995). The Rise of Antibiotic-Resistant Infections. *FDA Consum* **29**(7): 11-15.

Lukasik G (2009). H1N1 Influenza A Surface Disinfection Testing Report. St. Petersburg, Florida: BCS laboratories, Inc.

Lutz JK, Balen JV, Crawford JM, WilkinsIII JR, Lee J, Nava-Hoet RC and Hoet AE (2014). Methicillin-resistant *Staphylococcus aureu*s in public transportation vehicles (buses): Another piece to the epidemiologic puzzle. *Am J Infect Control* **47**: 1285-1290.

Naimi T, LeDell K, Como-Sabetti K and Borchard S (2003). Comparison of community and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA* **290**(22): 2976-2984.

Oranusi S, Ebelebe I and Egabadon E (2016). Diversity of commercial Intercity vehicles (Buses and Tricycle). *FUTOJNLS* **2(**1): 235-243.

Otter JA and French GL (2009). Bacterial contamination on touch surfaces in the public transport system and in public areas of a hospital in London. *Lett Appl Microbiol* **49**(6): 803-805.

Patel M (2009). Community-associated meticillin-resistant *Staphylococcus aureus* infections: epidemiology, recognition and management. *Drug* **69**: 693-716.

Peng Y, Ou Q, LinD, Xu P, Li Y, Ye X, Zhou J and Yao Z (2015). Metro system in Guangzhou as a hazardous reservoir of methicillin-resistant Staphylococci: findings from a point-prevalence molecular epidemiologic study. *Scientific Report* **5**: 1-5

Pokharel R and Acharya SR (2015). Sustainable transport development in Nepal: challenges, opportunities and strategies. *J East Asia Soc Transp Stud* **115**: 209-226.

Popovich K, Weinstein R and Hota B (2008). Are community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains replacing traditional nosocomial MRSA strains? *Clin Infect Dis* **46(**6): 787-794.

Reynolds K, Watt P, Boone S and Gerba C (2005). Occurrence of bacteria and biochemical markers on public surfaces. *Int J Environ Health Res* **15(3)**: 225-234.

Rusin P, Maxwell S and Gerba C (2002). Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of gram-positive bacteria, gram-negative bacteria, and phage. *J Appl Microbiol* 93(4): 585-592.

Sattar SA, Wright, KE, Zargar B, Rubino JR, and Ijaz MK (2016). Airborne infectious agents and other pollutants in automobiles for domestic use: Potential health impacts and approaches to risk mitigation. *Journal of Environmental and Public Health* Article ID **1548326**: 1-12.

Stepanovic S, Cirkovic I, Djukic S, Vukovic D, Svabic-Vlahovic M (2006). Public transport as a reservoir of methicillin-resistant staphylococci. *Lett Appl Microbiol* 47(4): 339-341.

Tan AS and Erdogdu G (2017). Microbiological burden of public transport vehicles. *Istanbul J Pharm* **47**(2): 52-56.

UN (2018). https://www.un.org/development/desa/dpad/wpcontent/uploads/sites/45/publication/ ldc_list.pdf. Accesed on 11-11-2018.

Yeh PJ, Simon DM, Millar JA, Alexander HF and Franklin D (2011). A diversity of antibiotic-resistant *Staphylococcus* spp. in a public transportation system. *Osong Public Health Res Perspect* **2**(3): 202–209.

In-Vitro Biofilm Detection among Uropathogens and Their Antibiogram Profile

Bijayata Shrestha[1]*, Basudha Shrestha[2], Asia Poudel[1], Binod Lekhak[3], Milan Kumar Upreti[1]

Department of Microbiology [1], GoldenGate international College, Kathmandu, Nepal

Department of Pathology[2], Kathmandu Model Hospital, Baghbazaar, Kathmandu, Nepal

Central Department[3] of Microbiology, Kirtipur, Nepal

***Corresponding author:** Bijayata Shrestha, Department of Microbiology, GoldenGate International College, E-mail: bijayatastha1@gmail.com

ABSTRACT

Objectives: The study was carried out in Kathmandu Model Hospital, Kathmandu with the aim of *in- vitro* biofilm detection among uropathogens and its correlation with antibiotic resistance.

Methods: Uropathogens (n=234) were isolated, and identified with standard microbiological techniques and further subjected to Modified Congo Red Agar Method for the biofilm detection *in-vitro*; antimicrobial susceptibility testing (10 antibiotics) was performed by Modified Kirby Bauer disc diffusion method. The MIC and MBEC values of Levofloxacin were determined by agar dilution for planktonic forms and by microdilution method for biofilm phase respectively.

Results: Among 234 urine isolates, 134(57%) were positive for *in-vitro* biofilm production and 88(37.6%) were multidrug resistant (MDR). *E.coli* was the predominant biofilm forming uropathogens. The incidence of biofilm producers was found to be independent of age-wise, gender wise and indoor-outdoor distribution of patients. The association between biofilm production and multidrug resistance among uropathogens was found statistically non-significant (p-value>0.05). The MBEC values of biofilm phase of growth were found to be greater than the MIC values for their planktonic counterparts. The MBEC values ranged from 4 to more than1024 µg/ml whereas the MIC values ranged from 0.003-16 µg/ml.

Conclusion: The results of this study suggest that biofilm detection is a critical step to fight against biofilm-involved infections. However, further studies are needed for the development of effective preventive and treatment strategies of biofilm associated UTIs to avoid recurrence and persistence.

Key words: Biofilms, UTI, MIC, MBEC, Multidrug Resistance.

INTRODUCTION

As human prefers to live in community, microorganisms that affect human life in so many ways also prefer to exist in a community. Such microbial community attached to a surface that may be biotic or abiotic, embedded in a self-produced extracellular polymeric matrix is referred to as "Biofilm" (Donlan and Costerton 2002). As per NIH (2002), about 65-80% of human infections are caused by biofilm forming organisms.

UTI is the most common infection encountered in the community as well as hospital settings and is often associated with the problems of recurrence and persistence (Soto et al. 2007; Ejrnaes et al. 2011). Chronic and recurrent infections are usually caused by biofilm associated pathogens which are recalcitrant to standard antibiotic therapy (Hancock et al. 2007; Ejrnaes et al. 2011). Bacteria in biofilms are phenotypically different from their planktonic counterparts and exhibit higher antibiotic resistance, leading to treatment failure and recovery from the infection very difficult (Choong and Whitefield, 2000). Thus, treatment of biofilm associated infections should target biofilms rather than their planktonic counterparts (Kostakioti et al. 2013).

Date of Submission: November 12, 2018 **Date of Acceptance:** December 10, 2018

Published Online: January 2019 **DOI:** https://doi.org/10.3126/tujm.v5i0.22313

The main objective of the study was to determine the proportion of biofilm producers among uropathogens and their antibiogram profile, the knowledge of which can guide towards effective management of biofilm associated UTI and help in prevention of recurrent and persistent UTI.

MATERIALS AND METHODS

Study site and population

This study was carried out in Department of Pathology of Kathmandu Model Hospital in collaboration with Department of Microbiology of GoldenGate International College. Ethical approval for the study was taken from Institutional Review Committee phect Nepal. A total of 1299 midstream urine from UTI suspected patients received for culture were included in the study.

Urine culture

Semi-quantitative urine culture was performed on CLED agar using a standardized calibrated loop (0.001ml). The agar plate was incubated at 37°C for 24 hours and then observed for colonial count. Colonial count greater than 10^5cfu/ml, was considered significant.

Isolation and identification of bacterial isolates

The isolates were identified based on the standard microbiological procedures which included colonial appearance, staining reaction and biochemical properties.

Antibiotic susceptibility testing

The antibiotic susceptibility testing was done by modified Kirby Bauer disc diffusion technique. Antibiotics were selected as per CLSI (2013) guidelines.

Screening of biofilm producers

The isolate was streaked over modified Congo Red agar and incubated at 37°C for 24 hours and observed for black coloured colonies.

Determination of MICs and MBECs

MICs of Levofloxacin for all the isolates were determined by agar dilution method (EUCAST, 2000). MBECs of Levofloxacin for the sessile form of biofilm forming isolates were determined using microdilution method, a modification of method described by Ghanwante (2012).

Quality control

E.coli ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

Data analysis

Data were analyzed using SPSS version 16. p-value less than 0.05 was considered significant.

RESULTS

Out of 1299 urine specimens, 234(18%) showed significant growth. The most commonly isolated organism from UTI patients was *E. coli*, accounting for 78.6% of the total isolates.

Table 1: Bacterial isolates among UTI patients

Bacterial isolates	Total(n=234)
E. coli	184(78.6%)
Klebsiella spp.	8(3.4%)
Proteus spp.	2(0.8%)
P. aeruginosa	2(0.8%)
A. baumannii	2(0.8%)
Staphylococcus spp.	23(9.8%)
Enterococcus faecalis	8(3.4%)
Enterobacter spp.	2(0.8%)
Citrobacter spp.	2(0.8%)
Salmonella Typhi	1(0.4%)

Out of 234 uropathogens, 57% (134) were biofilm producers among which *E.coli* (90%) constituted the highest percentage. Other organisms involved in biofilm production were *Klebsiella* spp., *Acinetobacter baumannii, Staphylococcus* spp. and *Enterococcus faecalis.*

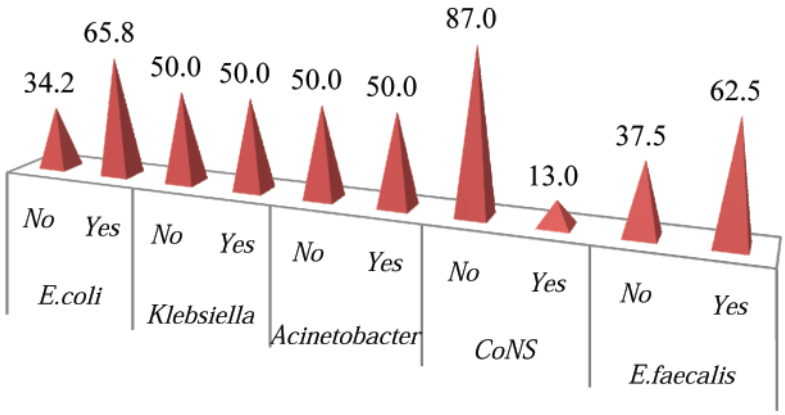


Figure 1: Biofilm producers among different uropathogens

The incidence of biofilm producers was similar among male and female patients. The percentage of biofilm producers was slightly higher among in-patients (62.5%) as compared to out-patients (56.7%). However, biofilm production among uropathogens was statistically non-significant to sex-wise and indoor- outdoor distribution of patients (p-value>0.05).

Table 2: Pattern of biofilm forming uropathogens among patients

Biofilm	Sex (p>0.05)		Patient type (p>0.05)		Total (n=234)
	Male (n=66)	Female (n=168)	Outpatient (n=210)	Inpatient (n=24)	
Producer	38(57.7%)	96(57.1%)	119(56.7%)	15(56.7%)	134
Non-producer	38(43.3%)	72(42.9%)	91(43.3%)	9(37.5%)	100

Though biofilm producers were present among patients of all age groups, the number was highest among the age group 20-30 years.

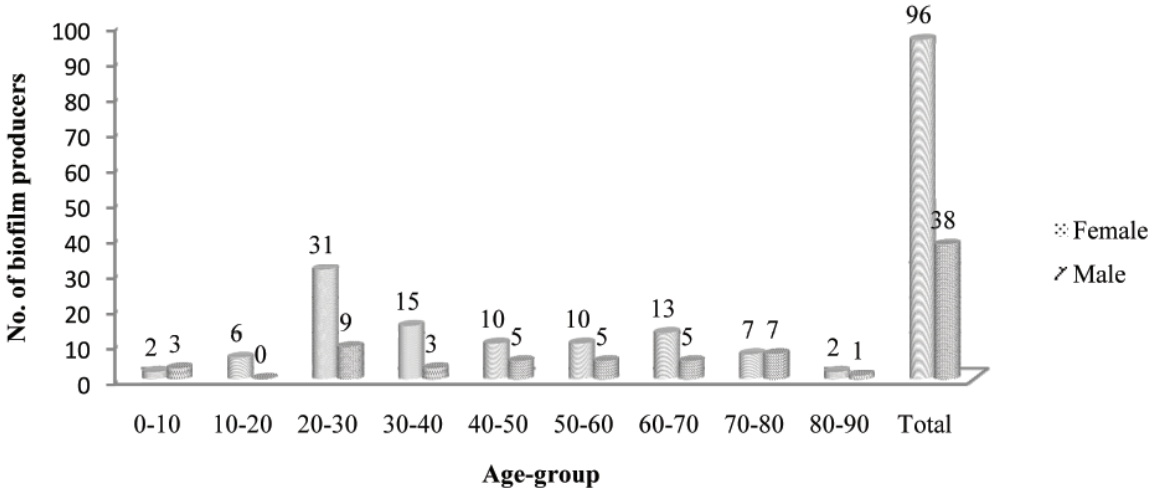


Figure 2: Occurence of biofilm producers among patients

Although, higher resistance was observed for individual antibiotics tested for biofilm producing and biofilm non- producing uropathogens, difference in antibiotic resistance pattern was not statistically significant for majority of the antibiotics tested.

Table 3: Antibiotic resistance among biofilm producing and non producing uropathogens

Antibiotic	Resistance among uropathogens		
	Biofilm Producers(n=134)	Biofilm non-producer(n=100)	p-value
AMX	105(78.5%)	73(73%)	>0.05;NS
CTX	57(42.5%)	33(33%)	>0.05;NS
NIT	3(2.2%)	11(11%)	<0.05;S
CIP	63(47%)	36(36%)	>0.05;NS
COT	61(47.4%)(n=129)	41(42.3%)(n=97)	>0.05;NS
CFM	56(44.4%)(n=126)	27(36.4%)(n=74)	>0.05;NS
LEV	61(45.5%)	32(32%)	<0.05;S
NX	5(62.5%)(n=8)	12(52.2%)(n=23)	>0.05;NS
GEN	3(37.5%)(n=8)	5(21.7%)(n=23)	>0.05;NS
E	8(100%)(n=8)	14(60.9%)(n=23)	>0.05;NS

Among 234 uropathogens, 37.6% were multidrug-resistant. No statistically significant association was observed between biofilm production and multidrug resistance among uropathogens.

Table 4: Association between biofilm production and multi-drug resistance

	Multi-drug Resistance		
Biofilm	**Yes**	**No**	**Total**
Producer	50(37.31%)	84(62.68%)	134
Non-producer	38(38%)	62(62%)	100
Total	**88(37.6%)**	**146(62.4%)**	**234**

p-value>0.05

The MIC values of Levofloxacin for the uropathogens ranged from 0.03-16µg/ml whereas the MBEC values for the biofilm producing urine isolates ranged from 4 to greater than 1024µg/ml. Dramatic increase in the inhibitory concentration on transition from planktonic to sessile forms was observed.

DISCUSSION

E.coli being the most common agent, accounted for about 78.6% cases. Diverse virulence factors such as fimbriae, hemolysin, iron uptake systems, cytotoxins, phase variation, biofilm formation, etc act as the weapons of UPEC against the host and help in the establishment of UTI (Davis and Flood 2011).

Biofilm though clinically relevant, its presence is underestimated due to the need for *in-vivo* diagnosis (Bordi and de Bentzmann 2011). Among the different screening tests available for *in- vitro* biofilm formation, Modified Congo Red agar method was employed in this study. The Congo red agar method proposed by Freeman et al. (1989) is a simple, cost- effective phenotypic method of screening of biofilm formation and does not require technical expertise, which makes it appropriate for laboratory use in a developing country like ours.

Using the MCRA (Modified Congo Red Agar) method, 57% of total urinary isolates were found to be biofilm producers. It has been reported that more than 50% of total human infections are associated with biofilm production (Costerton et al. 1987). Previous studies have also proposed the importance of bacterial biofilm formation in UTI (Chung and Whitefield 2000; Hall et al. 2014). Biofilm is one of the virulence factors of uropathogens allowing them to persist in the urinary tract (Hancock et al. 2007; Marhora et al. 2010). Biofilm provides survival advantage to pathogens through the expression of several other virulence factors, acquisition of antibiotic tolerance and an increased resistance against host immune defenses (Soto et al. 2007).

The incidence of biofilm producers among indoor patients (62.5%) was greater than outdoor patients (56.3%). However, the distribution of biofilm producers among outdoor and indoor patients was not statistically significant (p value>0.05). The debilitated health condition or the use of catheters among indoor patients might account for higher percentage of biofilm producers among indoor patients. From the total *E.coli*

isolates, 65% of UPEC were observed to produce biofilm, which is similar to the findings of Sharma et al. (2009) showing 67.5% of *E.coli* isolates as biofilm producers. It has been reported that biofilm forming UPEC are often responsible for the problem of recurrence faced in the case of UTI. As per study of Soto et al. (2007), 74 % of UPEC strains causing relapse was biofilm producers. Antimicrobial Resistance among biofilm producers appeared to be higher as compared to non-biofilm producers with reference to individual drugs except for Nitrofurantoin. The increase in antimicrobial resistance among biofilm producers is due to slow growth rate and the presence of the protective covering of exopolysaccharide which alters the penetration of antimicrobial agents through the biofilm and hinders the activity of antimicrobial agents against the bacterial cells (Hung and Henderson 2009; Lopez et al. 2010; Hall et al. 2014). The association between biofilm production and multidrug resistance among uropathogens was statistically non-significant. Similar result was obtained by Bardoloi *et al.* (2014). However, there are a number of literatures available which establish a significant role of biofilm production in multidrug-resistance (Ghanwante 2012; Sanchez et al. 2013).

In this study, MIC was determined for all the uropathogenic isolates and MBEC was determined for all the biofilm forming uropathogens. MBEC provides a more reliable method for accessing the antibiotic susceptibility of biofilm as it is targeted against biofilm mode of life (Ghanwante, 2012). The antibiotic chosen for the determination of MIC and MBEC was Levofloxacin. Levofloxacin is a broad spectrum antibiotic of Fluoroquinolone group. Fluoroquinolones are initial agents in empirical therapy for various types of UTI (McGregor et al. 2008). Moreover, fluoroquinolone are reported to have high clinical cure rates (Akram et al. 2007) as well as have activity against biofilms (Ishida et al. 1998). The MIC values ranged from less than 0.003-16 µg/ml. However, the MBEC values were found to be higher and ranged from 2-more than 1024µg/ml. This showed that very high concentration of antibiotic is required for the elimination of biofilm. Studies have revealed that concentration required to inhibit biofilm is 10-1000 times greater than required to inhibit planktonic cells (Chung and Whitefield 2000; Kostakioti et al. 2013). As long as the biofilm associated with any kind of infection is not removed, there is chance that the infection will persist or recur even after the eradication of planktonic cells after the standard antibiotic therapy as the surviving biofilm act as reservoir of pathogenic organisms (Lewis 2001). Since, very high concentration of antibiotic is required for the eradication of biofilms, which is not possible to be obtained in the human body, it is necessary to search for other therapeutic interventions for biofilm associated infections.

CONCLUSION

The distribution of biofilm forming uropathogens was independent of sex, age and indoor-outdoor distribution of patients. High prevalence of biofilm producers among uropathogens is indicative of the need for screening of biofilm as a common laboratory procedure. Increased antibiotic resistance was observed among biofilm producers as compared to non-biofilm producer strains. The drastic increase in the MBEC as compared to MIC demonstrated high antimicrobial resistance among biofilm producers.

ACKNOWLEDGEMENTS

We are grateful to Department of Pathology of Kathmandu Model Hospital and Department of Microbiology of GoldenGate International College for providing the necessary support for the completion of this research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Akram M, Shahid M and Khan AU (2007). Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in JNMC Hospital Aligarh, India. *Ann Clin Microbial Antimicrob* **6**:1-7

Bordi C and de Bentzmann S (2011). Hacking into bacterial biofilms: a new therapeutic challenge. *Ann Intensive Care* **1**: 1-8.

Choong S and Whitfield H (2000). Biofilms and their role in infections in urology. *BJU international* **86**: 935-941.

CLSI (2013). Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement, vol. 33. CLSI Document M100–S20. Wayne, PA.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M and Marrie TJ (1987). Bacterial biofilms in nature and disease. *Ann Rev Microbiol* **41**: 435-464.

Donlan RM and Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**: 167-193.

Ejrnaes K, Stegger M, Reisner A, Ferry S, Monsen T, Holm SE and Frimodt-Møller N (2011). Characteristics of *Escherichia coli* causing persistence or relapse of urinary tract infections: phylogenetic groups, virulence factors and biofilm formation. *Virulence* **2:**528-537.

EUCAST (2000). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution: EUCAST definitive document 3.1. *Clin Microbiol Infect* **6:** 509-516.

Freeman DJ, Falkiner FR and Keane CT (1989). New method for detecting slime production by coagulase negative staphylococci. *J Clinical Path* **42**: 872-874.

Ghanwate NA (2012). Biofilm eradication studies on uropathogenic *E. coli* using ciprofloxacin and nitrofurantoin. *Int J Pharm Biomed Res* **3**: 127-131.

Hall MR, McGillicuddy E and Kaplan LJ (2014). Biofilm: basic principles, pathophysiology, and implications for clinicians. *Surg Infect* **15** :1-7.

Hancock V, Ferrieres L and Klemm P (2007). Biofilm formation by asymptomatic and virulent urinary tract infectious *Escherichia coli* strains. *FEMS Microbiol Lett* **267**: 30-37.

Hung CS and Henderson JP (2008). Emerging concepts of biofilms in infectious diseases. *Missouri Medicine*. **106**: 292-296.

Ishida H, Ishida Y, Kurosaka Y, Otani T, Sato K and Kobayashi H (1998). In vitro and in vivo activities of levofloxacin against biofilm-producing *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **42**: 1641-1645.

Kostakioti M, Hadjifrangiskou M and Hultgren SJ (2013). Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* **3**: 1-23.

Lewis K (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**: 999-1007.

McGregor JC, Allen GP and Bearden DT (2008) Levofloxacin in the treatment of complicated urinary tract infections and acute pyelonephritis. *Ther Clin Risk Manag* **4**: 843-853.

Sharma M, Aparna, Yadav S, Chaudhary U (2009). Biofilm production in uropathogenic Escherichia coli. *Indian J Pathol Microbiol*. **52**: 294-298.

Soto SM, Smithson A, Martinez JA, Horcajada JP, Mensa J and Vila J (2007). Biofilm formation in uropathogenic *Escherichia coli* strains: relationship with prostatitis, urovirulence factors and antimicrobial resistance. *J Urol* **177**: 1387-1392.

Drug Susceptibility Profile of *Mycobacterium tuberculosis* Isolated from Patients Visiting National Tuberculosis Centre, Nepal

Sangita Dahal[1], Megha Raj Banjara[1], Dhruba Kumar Khadka[2], Gokarna Raj Ghimire[2], Supriya Sharma[1]*

[1] Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal
[2] National Tuberculosis Centre, Thimi, Bhaktapur, Nepal

***Corresponding author:** Ms. Supriya Sharma, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal; E-mail: suprisharma@hotmail.com

ABSTRACT

Objectives: The objective of this study was to assess drug susceptibility pattern of *Mycobacterium tuberculosis* (MTB).

Methods: This cross-sectional study was carried out among 145 clinically suspected and previously treated pulmonary tuberculosis patients visiting National Tuberculosis Centre, Bhaktapur, Nepal. After obtaining written informed consent, questionnaire was administered and sputum samples were collected from each patient. Each sample was subjected to Ziehl-Neelsen (ZN) staining and cultured on Lowenstein Jensen (LJ) medium at 37°C for 8 weeks. MTB isolates were identified by growth rate and colony morphology, confirmed by biochemical tests. Drug susceptibility testing (DST) of identified isolates was performed by proportion method.

Results: A total of 49.7% (n=72) sputum samples were positive for MTB by culture and 46.9% (n=68) were positive by ZN staining. Among culture positive isolates of MTB (n= 72), 25% (n=18) were resistant to at least one drug. The prevalence of multi drug resistant tuberculosis (MDR-TB) was 15.3% (n=11) of which 5.5% (n=4) were resistant to rifampicin (RIF) only, 1.3% (n= 1) were resistant to isoniazid (INH) only. Out of 18 resistant isolates, 61.1% (n=11) were resistant to both RIF and INH, 21.4% (n=3) resistant to INH were susceptible to RIF and 26.6% (n=4) resistant to RIF were susceptible to INH. Smoking (P=0.001) and coughing (P=0.009) were statistically significant with isolation of MTB.

Conclusion: Since the prevalence of MDR-TB was high, MDR-TB strains should be identified in order to initiate second line treatment.

Key words: TB, MDR-TB, Smoking, Nepal

INTRODUCTION

Tuberculosis (TB) is a ninth leading cause of death by an infectious disease worldwide, despite global efforts and financial investments by governments and nongovernmental organizations in disease-control programs during the past 20 years (Raviglione et al. 2012, WHO 2017). In 2016, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 6.2 million (59%) were among men, 3.2 million (31%) among women and 1.0 million (10%) among children and 1.7 million died from the disease (including 0.4 million among people with human immunodeficiency virus) (WHO 2017). Over 95% of cases and deaths occur in developing countries.

Anti-tuberculosis drug resistance is a major public health problem that threatens progress made in TB care and control worldwide (WHO 2012). Drug resistance arises due to improper use of antibiotics in chemotherapy of drug susceptible TB patients and because of insufficient diagnostic facilities (Zhao et al. 2012). Drug resistant

Date of Submission: August 23, 2018
Date of Acceptance: November 12, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22314

bacteria especially MDR-TB persists as a global public health problem (Chiang et al. 2013). MDR-TB is TB due to organism which show high level resistance to both isoniazid (INH) and rifampicin (RIF) with or without resistance to other anti-TB drugs (Ormerod 2005). The emergence of extensively drug resistant tuberculosis (XDR-TB), defined as TB resistant to INH, RIF, quinolones and at least one of three injectable second line drugs (kanamycin, capreomycin or amikacin), in every region of world has raised further alarms about the future or TB control (Marahatta et al. 2010). Globally an estimated 4.1% of new cases and 19% of previously treated cases had MDR-TB and estimated 6.2% patient with MDR-TB had extensively drug resistant TB (XDR-TB). WHO estimates that there were 600000 new cases with resistance to rifampicin (RIF), of which 490000 had MDR-TB (WHO 2017).

TB is a major public health problem in Nepal and ranks as one of the most prevalent communicable diseases throughout the country (Upadhyaya et al. 2014). About 45% of the total population is infected with TB, of which 60% are adult. Each year about 45000 people develop active TB, out of which 20500 have infectious disease and 5000-7000 people are dying every year by TB (DoHS 2014), 9.3% of new patient develop resistant to at least one drug and level of MDR-TB among new cases is 2.2% while among retreatment cases is 17.4% (NTC 2014).

Early diagnosis of tuberculosis and rapid detection of rifampicin (RIF) and isoniazid (INH) resistant is important for the early administration of appropriate therapeutic agent for the prevention of additional resistance development (Bossier et al. 2006).

MATERIALS AND METHODS

This cross-sectional study was conducted at National Tuberculosis Centre (NTC), located at Bhaktapur district, Nepal from January to July 2016. One hundred forty-five new or previously treated patients of any age and gender visiting NTC were enrolled in the study. Informed consent was taken from each patient. Early morning sputum samples were collected from patients attending NTC after taking informed consent. Each patient was instructed to collect sputum sample in wide mouth, transparent, sterile, screw capped plastic container (Tille et al. 2014). If the sputum was saliva, blood stained, less than 3 ml and contained greater than 25 epithelial cells per low power field and less than 10 pus when observed microscopically, then the specimen was rejected (Cheesbrough 2002; Lee et al. 2015). Digestion and decontamination of sputum sample was done using sodium hydroxide method (Modified Petroff''s method) in order to remove contaminants. The digested specimen was spread evenly in glass slide, air dried, heat fixed and stained by ZN staining and observed under oil immersion objective for the presence of acid fast bacilli (AFB). Approximately, 0.2 ml of each of resuspended sediment was inoculated to each of duplicate Lowenstein Jensen (LJ) media tubes and incubated at 37^{o}C. The tubes were slightly opened during incubation for about 2-3 days in order to evaporate excess water and then tightened the cap tightly. The tubes were observed after 24-48 hours to discard contaminated tubes. Then, the tubes were observed weekly up to 8 weeks. *M. tuberculosis* was identified by observing their growth rate and colony morphology (rough, tough, buff). The colonies on the LJ media were further confirmed by conventional biochemical tests such as susceptibility to para-nitro benzoic acid (PNB), nitrate reductase test, niacin test and heat labile catalase test (RNTCP 2009). Drug susceptibility testing was done by proportion method. For this, one ml of sterile distilled water (SDW) was added in a sterile glass homogenizer. One loopful of colonies was transferred to a glass homogenizer with six 3 mm glass beads and vortexed for 20-30 seconds. Four ml of sterile distilled water was added and homogenized by rotating the inner rotator. Turbidity with McFarland standard No. 1 was adjusted with sterile distilled water and 2 ml loopful of above solution was mixed with 2 ml of sterile distilled water in order to make the 10^{-2} dilution (S2). Similarly, two loopful of S2 was added with 2 ml of sterile distilled water in order to make 10^{-4} dilution (S4). One loopful each of 10^{-2} suspension was inoculated into control tube as well as set of drugs containing media. Similarly, one loopful each of 10^{-4} suspension was inoculated into two control tubes as well as set of drugs containing media.The slopes were incubated at 37^{o}C and read at 28 days and again in the 42 days (WHO 2001). The obtained data was analyzed using SPSS 16.0 software. The chi square test was used to associate pulmonary tuberculosis with habit of smoking, alcoholism, coughing, travel to foreign country and BCG vaccination. The P-value < 0.05 was considered statistically significant.

RESULTS

A total of 145 patients were enrolled in the study. Out of 145 patients, 99 (68.2%) were male. The highest percentage of samples were from 44.1% age group 20 - 40 years (44.1%) followed by age group 40 - 60 years (37.2%) and > 60 years (13.1%) while the lowest percentage was seen in age group < 20 years (5.5%). Among 145 included patients, 68 (46.9%) were positive by ZN microscopy and 72 (49.7%) were culture positive.

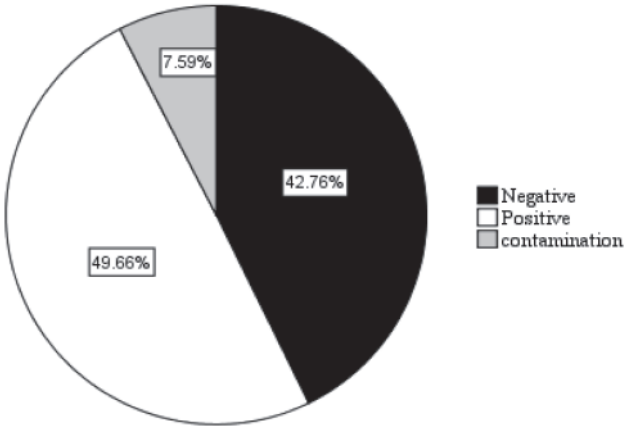


Figure 1: Distribution of culture positive, culture negative and contamination among total specimens

Considering culture as gold standard, the sensitivity and specificity of ZN staining were found to be 87.5% and 96.8% respectively.

Among four anti-tuberculosis drugs, the culture positive isolates (n=72) were found to be most susceptible to EMB and least susceptible to RIF.

Table 1: Distribution of drug resistance among culture positive isolates with four anti-tubercular drugs (n=72)

Drug	Susceptible N (%)	Resistant N (%)
RIF	57 (79.1%)	15 (20.8%)
INH	58 (80.5%)	14 (19.4%)
STR	61 (84.7%)	11 (15.2%)
EMB	70 (97.2%)	2 (2.7%)

INH= isoniazid; RIF= rifampicin; STR= streptomycin; EMB= ethambutol

Among 72 culture positive isolates of MTB responsible for PTB, 25% (n=18) were DR strains resistant to at least one drug of which 6.9% (n=5) were isolated from new patients and 18.1% (n=13) were identified from previously treated patients. In this study, it was observed that triple drug resistance was highest 7 (9.7%) followed by mono drug resistance 5 (6.9%) and double drug resistance 4 (5.5%), while quadruple drug resistance was lowest 2 (2.7%). Drug resistance pattern among MTB isolates is shown in table 2. The total prevalence of MDR-TB was 15.2% (n=11). Out of 18 resistant isolates, 61.1% (n=11) were resistant to both RIF and INH, 21.4% (n=3) resistant to INH were susceptible to RIF and 26.6% (n=4) resistant to RIF were susceptible to INH.

Table 2: Distribution of drug resistance pattern among MTB isolates (n = 72)

Drugs	Resistance to any drugs	18 (25%)
	One drug resistance	
	RIF	1 (1.3%)
	INH	4 (5.5%)
	Total	5 (6.9%)
	Two drugs resistance	
	INH + RIF	2 (2.7%)
	INH + STR	2 (2.7%)
	Total	4 (5.5%)
	Three drugs resistance	
	INH + RIF + STR	7 (9.7%)
	Four drugs resistance	
	INH + RIF + STR + EMB	2 (2.7%)
	MDR - TB	11 (15.2%)

MTB and alcoholism (P = 0.92), BCG vaccination (P = 0.238) and travel history to foreign country (P = 0.534) were not statistically significant but with the habit of smoking (P = 0.001) and coughing (P = 0.009) were found statistically significant.

DISCUSSION

In this study, cases of MTB identified by culture was in consistent with NTC report 2014 but higher than recent study of Maharjan et al. (2017). This finding was lower than in other developing countries like Bangladesh and Iran (Mottalib et al. 2011; Nasiri et al. 2014).The higher incidence of TB among man could be attributed to vulnerability of men to TB because of their mobile life style and exposure to predisposing factors like smoking, alcohol, drug abuse (Bhatta et al. 2009). The present finding was in contrast to previous study in Pakistan in which rates of notified TB cases is higher in young females (Codlin et al. 2011).

The findings of this study showed that majority of the TB patients belong to the economically active young age group of 20-60 years. This finding was consistent with the earlier finding by Bhatt et al. (2009) in which majority of TB patients were in the age group of 21-50 years, suggesting that TB is common among the economically active group having direct impact to the family and the national economy.

Comparison of ZN staining to the culture, which is regarded as gold standard, in this study showed higher sensitivity and slightly less specificity than previous study (Abdelaziz et al. 2016). The study conducted in African population with a high prevalence of HIV shows acid-fast microscopy was highly sensitive (93.1%) and specific (100%) when performed by trained technologists in a carefully controlled manner using established techniques (Sheay et al. 2009). This finding implies that the ZN staining is quite efficient in the diagnosis of pulmonary tuberculosis, but RT-PCR showed higher sensitivity and specificity as compared to ZN staining in earlier study (Upadhyaya et al. 2014). This shows tuberculosis diagnosis by PCR is highly efficient than conventional method of diagnosis.

Drug resistant cases detected in this study was less than the research conducted by Thapa et al. (2016) in German-Nepal tuberculosis project laboratory which showed 31.1% of DR cases. Maharjan et al. (2017) also noted 37.2% DR cases.The MDR strains prevalence in TB patients in this study was similar to previous studies (Thapa 2016; NTC 2016), but slightly higher than the research conducted in 2011 (7.9%) and 2015 (11.7%) (Maharjan et al. 2017; Wagley et al. 2016). This implies MDR strains prevalence in TB patients is continually increasing which poses a serious public health problem due to poor patient management, non-adherence to the prescribed regimen, irregular supply of drugs, poor quality of drugs and poor national TB control programme. INH monoresistance in this study was lower than reported from South eastern China (3.5%) (Liu et al. 2013), Kenya (12.9%) (Ndung's et al. 2012) and Mozambique (14.9%) (Nunes et al. 2008) and RIF monoresistance was higher than INH monoresistance but Rijal et al. (2005), Maharjan et al. (2017) showed

different results. The probable reason for RIF resistance might be due to broad use of RIF for the treatment of other bacterial infections.

CONCLUSION

With reference to culture as gold standard; ZN staining is quite efficient in the diagnosis of PTB, which is economically viable in low resource setting countries like Nepal. MDR-TB cases are increasing. The association of MTB infection with smoking and coughing patients were found to be statistically significant.

ACKNOWLEDGEMENTS

We would like to thank Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal and National Tuberculosis Centre, Nepal for support as well as patients for providing their samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abdelaziz MM, Bakr WM, Hussien SM and Amine AE (2016). Diagnosis of pulmonary tuberculosis using Ziehl-Neelsen stain or cold staining techniques? J *Egypt Public Health Assoc* **91**(1):39-43.

Bhatt CP, Bhatt AB and Shrestha B (2009). Nepalese people's knowledge about tuberculosis. *SAARC J Tuber Lung Dis HIV/AIDS* **6** (2): 31-37.

Brossier F, Veziris N, Truffot-Pernot C, Jarlier V and Sougakoff W (2006). Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. *J Clin Microbiol* **44**: 3659-3664.

Cheesbrough M (2002). *District laboratory practice in Tropical countries part II*. Low price edition, Cambridge University Press, India, 71-76.

Chiang CY, Van Weezenbeek C, Mori T and Enarson DA (2013). Challenges to the global control of tuberculosis. *Respirology* **18**(4): 596-604.

Codlin AJ, Khowaja S, Chen Z, Rahbar MH, Qadeer E, Ara I, McCormick JB, Fisher-Hoch SP and Khan AJ (2011). Gender Differences in Tuberculosis Notification in Pakistan. J Trop Med Hyg **85**(3): 514-517.

DoHS (2016). Annual Report 2015/2016. Government of Nepal, Ministry of Health, Department of Health Services, Kathmandu.

Lee YJ, shin S, Roh EY, Kim DK, Chung HS and Lee CH (2015). Acceptability of Sputum Specimens for Diagnosing Pulmonary Tuberculosis. J Korean Med Sci **30**(6): 733–736.

Liu Q, Zhu L, Shao Y, song H, Li G, Zhou Y, Shi J, Zhong C, Cheng C and Lu W (2013). Rates and risk factors for drug resistance tuberculosis in Northeastern China. BMC Pub. Health **13**: 1171.

Maharjan S, Singh A, Khadka DK and Aryal M (2017). Drug Resistance Pattern in Pulmonary Tuberculosis Patients and Risk Factors Associated with Multi-Drug Resistant Tuberculosis. *Biomedical Life Sci J* **5**(2): 106-117.

Marahatta SB, Kaewkungwal J, Ramasoota P and Singhasivanon P (2010). Risk factors of Multidrug Resistant Tuberculosis in central Nepal: A pilot study. *Kathmandu Uni Med* J **9**(32):392-397.

National Tuberculosis Center (NTC) (2017). Government of Nepal, Ministry of Health, Department of Health Services, Kathmandu.

National Tuberculosis Programme Nepal (2016). *Annual Report 2072/2073 (2016)*. Government of Nepal, Ministry of Health & Population, Department of Health Services, National Tuberculosis Center, Thimi, Bhaktapur.

National Tuberculosis Programme Nepal (2014). *Annual Report 2070/2071*. Government of Nepal, Ministry of Health & Population, Department of Health Services, National Tuberculosis Center, Thimi, Bhaktapur.

Ndung'u PW, Kariuki S, Ng'ang'a Z and Revathi G (2012). Resistance patterns of *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients in Nairobi. *J Infect Dev Ctries* **6**(1): 33-39.

Nunes EA, De Capitani EM, Coelho E, Panunto AC, Joaquim OA and Ramos MC (2008). *Mycobacterium tuberculosis* and nontuberculous mycobacterial isolates among patients with recent HIV infection in Mozambique. *J Bras Pneumol* **34**(10): 822-828.

Ormerod LP (2005). Multi drug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment. *British Med Bul* **73**: 17-24.

RNTC Programme (2009). *Revised National TB Control*

Programme Training Manual for Mycobacterium tuberculosis Culture & Drug Susceptibility Testing. Training Manual, Indian Council of Medical Research) and National Tuberculosis Institute, Central TB Division, Nirman Bhawan, New Delhi.

Raviglione M. Marais B, Floyd K, Lonnroth K, Getahun H, Migliori GB, Harries AD, Nunn P, Lienhardt C, Graham S, Chakaya J, Weyer K, Cole S, Kaufmann SH and Zumla A (2012). Scaling up interventions to achieve global tuberculosis control: progress and new developments. *Lancet* **379**(9829): 1902**-**13.

SAARC TB and HIV/AIDS Centre. *SAARC Training Modules for AFB Smear Microscopy and Quality Assurance in AFB Smear Microscopy. Trainer's Manual.* SAARC TB and HIV/AIDS Centre, SAARC TB and HIV/AIDS Centre, Thimi, Bhaktapur 2011.

Shea YR, Davis JL, Huang L, Kovacs JA, Masur H, Mulindwa F, Chow Y and Murray PR (2009). High Sensitivity and Specificity of Acid-Fast Microscopy for Diagnosis of Pulmonary Tuberculosis in an African Population with a High Prevalence of Human Immunodeficiency Virus. *J Clin Microbiol* **47**(5): 1553**-**1555.

Thapa G, Pant ND, Khatiwada S, Lekhak B and Shrestha B (2016). Drug susceptibility patterns of the *Mycobacterium tuberculosis* isolated from previously treated and new cases of pulmonary tuberculosis at German-Nepal tuberculosis project laboratory, Kathmandu, Nepal. *Antimicro Resis Infect Control* 5(1):30.

Tille PM (2014). *Bailey and Scotts's Diagnostic Microbiology*. 13th edition, Mosby Inc, USA, 484-512.

Upadhyaya SK, Dixit SM, Shrestha S, Dangol SD, Pokhrel D, Banjara S, Shrestha KS, Hengoju S and Dahal BK (2013). Assessment of detection of *Mycobacterium tuberculosis* in sputum samples by Real time PCR based method. J Sci Eng Technol **9**(1): 181–188.

Wagley U, Bhatta DR, Rijal KR and Ghimire G (2014). Distribution of *Mycobacterium* species present in sputum of suspected pulmonary tuberculosis patients. *SAARC J Tuber Lung Diseases HIV/AIDS* **6**(1): 19**-**23.

WHO. Global tuberculosis report 2017. Geneva, Switzerland 2017.

WHO. Global tuberculosis report 2012. Geneva, Switzerland 2012.

WHO. Guidelines on Standard Operating Procedures for Laboratory Diagnosis of HIV-Opportunistic Infections. Regional Office for South-East Asia, New Delhi 2001.

Zhao P, Li XJ and Zhang SF (2012). Social behavior risk factors for drug resistant tuberculosis in mainland China: a meta-analysis. *J Int Med Res* **40**(2): 436**-**445.

In vitro Antibacterial Activity of Organic Extracts of *Aloe barbadensis* against Multi-drug Resistant *Pseudomonas aeruginosa* Isolated from Wound Specimens

Mamata Adhikari[1], Anil Kumar Sah[2], Dev Raj Joshi[1]*

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal

[2]Annapurna Neurological Institute and Allied Science Hospital, Maitighar, Kathmandu, Nepal, Maitighar, Kathmandu

***Corresponding author:** Dr. Dev Raj Joshi, Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal; E-mail: dev.joshi@microbiotu.edu.np

ABSTRACT

Objectives: In order to investigate alternate therapeutic option, this study was carried out to assess the *in vitro* antibacterial activity of gel extract of *Aloe barbadensis* against multiple antibiotic resistant *Pseudomonas aeruginosa* isolated from wound specimens.

Methods: A total of 180 different wound specimens collected in a hospital, were subjected to isolate and identify *P. aeruginosa* by cultural methods. Antibiotic susceptibility testing was done by Kirby-Bauer disc diffusion method to screen multidrug resistant isolates. *A. barbadensis* extracts were prepared using aqueous and organic solvents and their *in vitro* inhibitory action was evaluated by agar well diffusion methods.

Results: Out of total, 38 (21.1%) of the wound specimens showed the occurrence of *P. aeruginosa*, among which 15 (39%) isolates were multi-drug resistant. Organic extracts of various concentrations (0.2 - 0.8 v/v %) inhibited 66.7% of MDR and all non-MDR (n=23) *P. aeruginosa* with zone of inhibition ranging from 9.5 ±1.0 to 21.3 ± 2.2 mm but not by aqueous extract. A positive Pearson's correlation (r=0.983) was found between antibacterial effect and concentrations of the extracts. The antibacterial activity of organic extracts was statistically associated with antibiotic resistance profile of the organism ($p<0.05$).

Conclusion: Organic extracts of *A. barbadensis* revealed variable *in vitro* inhibitory action against both MDR and non-MDR *P. aeruginosa* isolated from wound specimens. Although further confirmation is needed, aloe gel extract may be applied as an alternate treatment option.

Key words: *Pseudomonas aeruginosa*, MDR, *Aloe barbadensis*, organic extracts, antibacterial activity, wound infection

INTRODUCTION

There is an increasing incidence of *Pseudomonas aeruginosa* in wound infections (Masaadeh and Jaran 2009) because an open wound provides a moist, warm and nutritious environment perfect for microbial colonization and proliferation (Benbow 2010). *P. aeruginosa* is an opportunistic and highly resistant nosocomial pathogen. It can grow on a wide range of substrates and quickly respond to environmental alteration (Lambert 2002). It can express virulence factors and surface proteins affecting wound healing. The development of a wound infection following elective surgery or traumatic injury remains a major cause of morbidity in patients (Kim et al. 1999).

Several studies have shown the emergence of multi-drug resistant (MDR) isolates of *P. aeruginosa* associated with community acquired and nosocomial infections (Bhandari et al. 2015; Chaudhary et al. 2016; Fan et al. 2016). Ali et al. (2015) observed higher resistance of *P. aeruginosa* to the commonly used antibiotics like ofloxacin (61.3%), cefepime (57.3%), ceftazidime (53.9%) and amikacin (53.0%). It is obvious that the

Date of Submission: November 2, 2018
Date of Acceptance: December 5, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22315

widespread MDR *P. aeruginosa* threaten the life of the infected patients globally.

The search of newer antibacterial sources has been fostered because antibiotic alternatives may reduce the use of antibiotics. In other hand, 80% of the population in developing countries mainly relies on traditional herbal medicines for therapies against infections (WHO 2002) due to wide availability, easy use, low cost, minimal or no adverse effect on human health. During the past several decades, both in vivo and in vitro studies have demonstrated higher antibacterial potency of biologically active compounds extracted from medicinal plants (Malterud et al. 1993; Goudarzi et al. 2015). *Aloe barbadensis* a medicinal plant, contains over 75 potentially active constituents including vitamins, enzymes, minerals, sugars, lignin, saponins, lupeol, salicylic acids, amino cinnamonic acid, phenols and sulfur (Surjushe et al. 2008). Many of these compounds are bactericidal, virucidal, fungicidal and anti-inflammatory in nature (Hamman 2008). Furthermore, the inhibitory action of extracts of *A. barbasensis* against many gram negative pathogens including *P. aeruginosa* has been recognized (Ibrahim et al. 2011; Renisheya et al. 2012;, Abakar et al. 2017). However, its antibacterial activity against MDR *P. aeruginosa* has rarely been evaluated in Nepal.

Therefore, in this study, we tested *in vitro* inhibitory action of different organic extracts of locally available *A. barbadensis* at different concentrations against MDR and non-MDR *P. aeruginosa* isolated from various wound specimens.

MATERIALS AND METHODS

Study design and wound specimen collection: In this prospective study, 180 wound specimens including pus, postoperative wound, and burn-wound and chronic wound swabs were collected by hospital professionals from out and in-patient departments of Annapurna Neurological Institute and Allied Science Hospital, Kathmandu, Nepal in between May to November 2017.

Isolation and identification of *P. aeruginosa*: The specimens were inoculated on MacConkey agar, Blood agar and Cetrimide agar (Hi-media, India) followed by incubation at 37 °C for 24 hours. The organism was identified on the basis of the colony characteristics, pigmentation, Gram's staining, catalase, oxidase tests and standard conventional biochemical tests: indole, methyl red, Voges–Proskauer, citrate, triple sugar iron, citrate, urease, nitrate reduction and oxidative/ fermentative tests.

Antibiotic susceptibility testing and screening of MDR *P. aeruginosa*: Antibiotic susceptibility test (AST) of all *P. aeruginosa* isolates was done by Kirby Bauer disc diffusion method (Figure 1A) and interpreted according to guidelines of Clinical Laboratory Standard Institute (CLSI 2016). The antibiotics used for AST were: Amikacin (AK)-30 mcg, Gentamicin (GEN)-30 mcg, Cefotaxime (CTX)-30 mcg, Ciprofloxacin (CIP)-5 mcg, Ceftriaxone (CTR)-30 mcg and Imipenem (IPM)-10 mcg. Based on the results of AST, MDR isolates of *P. aeruginosa* were screened as the isolate was resistant to two antibiotics of different groups with different mode of action.

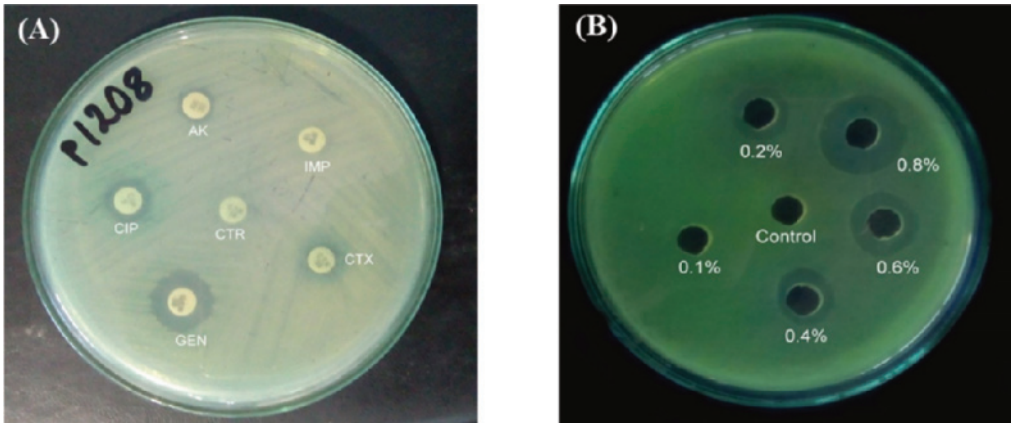


Figure 2: P. aeruginosa (P/1208) on Muller Hinton Agar plates showing antibiotic sensitivity testing (A) and antibacterial activity by DMSO extract of A. barbadensis (B). Abbreviations for antibiotics are as mentioned in the text. For in vitro antibacterial activity testing, 0% (control) to 0.8% (v/v) DMSO extracts was used. Zone of inhibitions were measured as mm.

Preparation of *A. barbadensis* gel extracts: Mature, healthy and fresh leaves of *A. barbadensis* were collected and washed in the running tap water for 10 minutes, rinsed with sterile distilled water and surface sterilized with 70% ethyl alcohol followed by 0.1% $HgCl_2$. The leaves were dissected longitudinally and the colorless parenchymatous tissue (aloe gel) was scraped out using a sterile knife. Fresh aloe gel was dried at 80 °C for 48 hours and then powdered. Ten grams of the powder was soaked in 100 ml of four different solvents (distilled water, DMSO, acetone and ethanol) separately for 24 hours. The content was filtered through Whatman filter paper (except for DMSO extract) and filtrate was evaporated to dryness. The dried powdered extract was dissolved in distilled water for further use. In case of acetone extract, extracted powder was dissolved in NaOH and neutralized with 0.15 M HCl.

Determination of antibacterial activity of *A. barbadensis* gel extract: The antibacterial activity of *A. barbadensis* gel extract against the *P. aeruginosa* isolates was tested by agar well diffusion technique (Figure 1B). Each of aloe extract was diluted with distilled water to prepare test solutions of 0.1%, 0.2%, 0.4%, 0.6% and 0.8% (v/v) concentrations. Exactly 100 µL of each test solution of each of aloe gel extracts were loaded into each of the 5 mm diameter wells on Mueller Hinton agar which were swabbed with an overnight broth culture of the organism and incubated at 37 °C for 24 hours. The respective pure solvent (acetone, ethanol and DMSO) was used as the control. In vitro inhibitory action was measured in terms of zone of inhibition (mm) around the test wells.

RESULTS

***P. aeruginosa* isolates from wound specimens and antibiotic resistance:** Out of total 180 wound specimens, 38 (21.1%) of the specimens showed the occurrence of *P. aeruginosa*. The highest numbers of the *P. aeruginosa* isolates were recovered from burn wound infections (n = 7, 35.0%) while the lowest number from pus specimens (n = 9, 13.9%) (Table 1).

Table 1: Distribution of P. aeruginosa in various wound specimens

Type of specimen	Total	Growth obtained (*P. aeruginosa*)	Percent
Burn wound swabs	20	7	35.0
Postoperative wound swabs	40	8	20.0
Chronic wound swabs	55	14	25.5
Pus specimens	65	9	13.9
Total	**180**	**38**	**21.1**

Antibiotic susceptibility testing revealed that out of total 38 isolates of *P. aeruginosa*, 15 (39.0%) isolates were designated as MDR *P. aeruginosa* (Figure 2) while others were regarded as non-MDR. The highest number of MDR *P. aeruginosa* were isolated from burn wound specimens (n=6, 85.7%) followed by postoperative wound (n = 5, 62.5%).

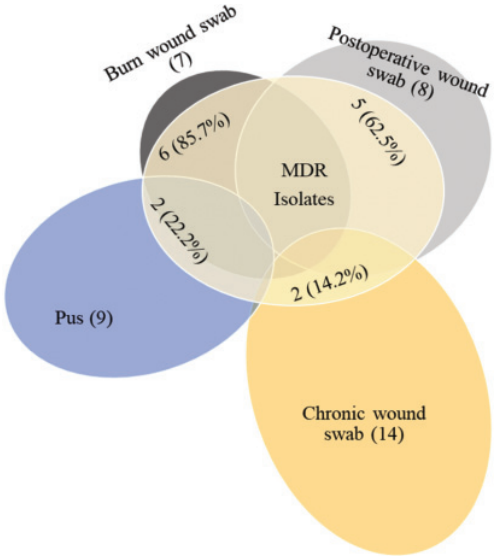


Figure 2: Venn diagram showing distribution of multidrug resistant (MDR) *P. auroginosa* isolates in different clinical samples. Parentheses in each sample represent the number of total *P. aeruginosa* isolates (N = 38). Frequencies of MDR isolates (n = 15) from each sample are expressed as number (n/N %).

***In vitro* antibacterial activity of *A. barbadensis* extracts against *P. aeruginosa*:** In order to evaluate the inhibitory potential of *A. barbadensis* against wound infecting *P. aeruginosa*, both the MDR (n = 15) and non-MDR (n = 23) isolates were tested. Production of clear inhibition zone around the agar well containing the extract was considered as 'sensitive' isolate, otherwise 'resistant' as no such zone was observed. While aqueous extract did not show any antibacterial effect, extracts with all three organic solvents; acetone, ethanol and DMSO demonstrated antibacterial effect at varying concentrations.

As demonstrated in Figure 3, among total of 38 isolates of *P. aeruginosa*, 33 (86.8%) were inhibited by at least one of organic extract of *A. barbadensis*. Out of the total 15 MDR isolates, 10 (66.7%) were found to be sensitive to aloe extracts. Interestingly, all 23 (100%) non- MDR isolates of *P. aeruginosa* were found to be sensitive to aloe extracts.

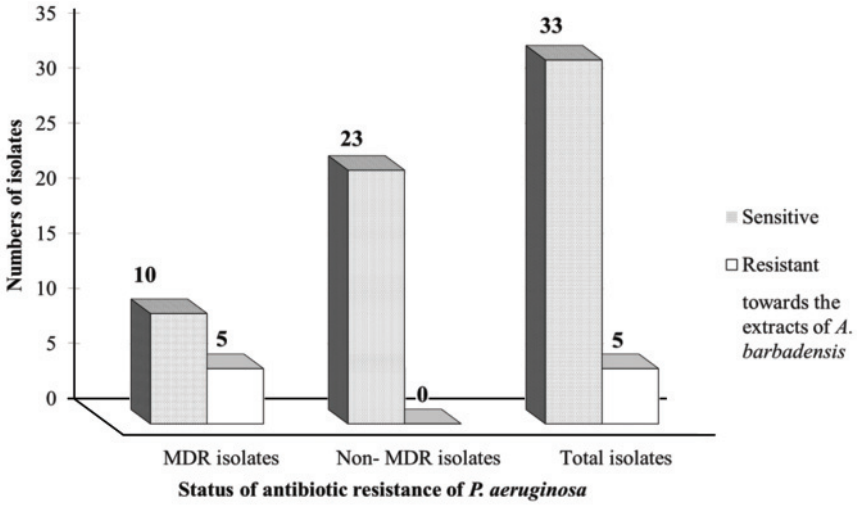


Figure 3: Bar diagram showing antibacterial activity of organic extracts of *A. barbadensis* against MDR and non MDR isolates of *P. aeruginosa*. The numbers represent the frequencies of isolates either sensitive or resistant towards at least one organic extract of *A. barbadensis*.

The maximum zone of inhibition of 21.3 ± 2.2 mm was revealed by acetone extract at concentration 0.8% against the MDR isolate of *P. aeruginosa* obtained from a burn wound while minimum zone of inhibition of 9.5 ± 1.0 mm was shown by DMSO extract at 0.2% concentration. DMSO extract of *A. barbadensis* showed increasing size of zone of inhibition as concentration was raised from 0.2% to 0.8% (Pearson's correlation coefficient, r=0.9951) against MDR isolates of *P. aeruginosa* and the linear curve was well fitted with R^2 of 0.9999 (Figure 4A). Comparatively, acetone extracts revealed highest in vitro inhibitory activity (larger zone of inhibition in all concentrations). Similar to the results against MDR isolates, non-MDR *P. aeruginosa* were also inhibited by 0.4 to 0.8% concentrations of acetone and DMSO extracts and 0.6 – 0.8% concentrations of ethanol extracts (Figure 4B). The results showed comparatively similar pattern of antibacterial activity of organic extracts of *A. barbadensis* against both the MDR and non-MDR isolates.

Association between multidrug resistance and antibacterial activity of *A. barbadensis*: Despite the similar inhibitory pattern of individual aloe extracts, we attempted to determine the association between MDR status of *P. aeruginosa* and gross sensitivity towards *A. barbadensis* applying chi-square test.

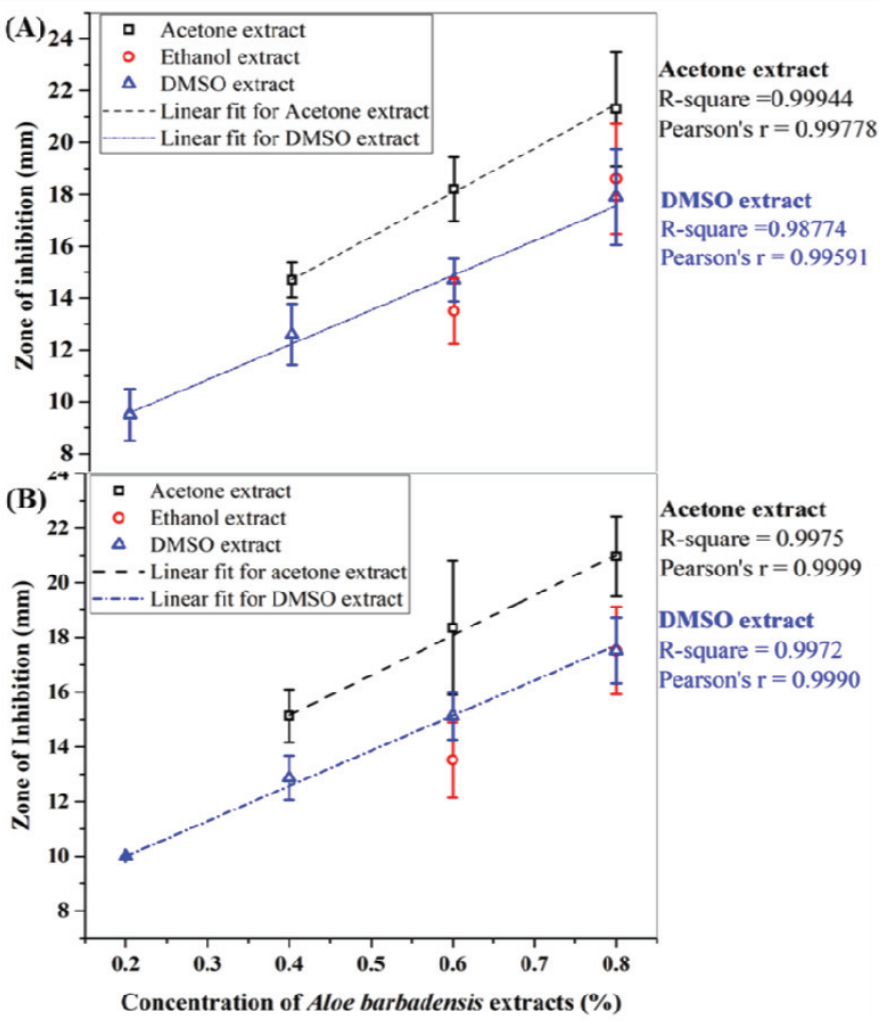


Figure 4: Antibacterial activity of *Aloe barbadensis* extracts at different concentration against multidrug resistant (A) and non-multidrug resistant (B) isolates of *P. aeruginosa*. Error bars represent the standard deviation. Pearson's r is correlation coefficient between concentration of extracts and ZOI produced by respective extracts. Since ethanol extract showed inhibition only at two concentrations, statistical analysis was not valid.

Compared to all non-MDR isolates, only two-third of MDR isolates (n = 5) of *P. aeruginosa* were sensitive towards Aloe extracts. A significant statistical association ($p < 0.05$) was revealed between MDR status and the sensitivity towards *A. barbadensis* (Table 2).

Table 2: Sensitivity of MDR isolates to *A. barbadensis*

Status of antibiotic resistance	Sensitivity to *A. barbadensis* extract		Total (%)	p-value*
	Sensitive (%)	Resistant (%)		
MDR	10 (66.7)	5 (33.3)	**15 (100.0)**	0.006
Non- MDR	23 (100.0)	0 (0.00)	**23 (100.0)**	
Total	33 (86.8)	5 (13.2)	38 (100.0)	

*Chi-square test

In order to understand which organic solvent extracts of *A. barbadensis* produce maximum inhibitory activity against *P. aeruginosa* isolates, the sizes of zone of inhibition (mm) revealed by each organic extracts at different concentrations were compared for MDR and non-MDR isolates. Though acetone extracts at all concentrations (0.4 - 0.8%) revealed higher magnitude of inhibition than DMSO and ethanol extracts, there was no significant difference in diameter of the zone of inhibition produced by different extracts for MDR and non-MDR isolates ($p > 0.05$) for all concentrations (Table 3).

Table 3: Association between drug resistance and antibacterial activity of organic extracts of *A. barbadensis* at different concentrations

Extract	Concentration	Diameter of zone of inhibition (mm) against		p-value*
		MDR	Non-MDR	
Acetone	0.8 %	21.3 ± 2.2	21.0 ± 1.5	0.129
Ethanol		18.6 ± 2.1	17.5 ±1.6	
DMSO		17.9 ± 1.9	17.5 ± 1.2	
Acetone	0.6 %	18.2 ± 1.2	18.4 ± 2.4	0.299
Ethanol		13.5 ± 1.3	13.5 ± 1.4	
DMSO		14.7 ± 0.8	15.1 ± 0.9	
Acetone	0.4 %	14.7 ± 0.7	15.1 ± 1.0	0.191
Ethanol		0	0	
DMSO		12.6 ± 1.2	12.9 ± 0.8	
Acetone	0.2 %	0	0	
Ethanol		0	0	
DMSO		9.5 ± 1.0	0	

*ANOVA

DISCUSSION

In order to investigate an alternate therapeutic option, this study was carried out to assess the antibacterial activity of *A. barbadensis* against MDR *P. aeruginosa* isolated from wound specimens. Since *P. aeruginosa* is generally a part of wound infection etiology, the target pathogen was isolated from different types of wound specimens. In this study, *P. aeruginosa* was recovered from 21.1% as key etiological agent of wound infections. However, previous studies reported the prevalence of *P. aeruginosa* as 5.6% to 44.0% (Bhatt and Lakhey 2007; Bangera et al. 2015; Rai et al. 2017). Considering variable prevalence of *P. aeruginosa* in different types of wound infections (Rajbahak et al. 2012; Bastola et al. 2017; Serra et al. 2015; Kshetry et al. 2015), we selected burn wound swab, postoperative wound swab, chronic wound swab and pus specimens for this study where the burn wound specimens contributed maximum of *P. aeruginosa* isolates (35%).

Infections caused by *P. aeruginosa* are often severe and life threatening (Ahmed et al. 2008) and are difficult to treat because of its limited susceptibility to antimicrobial agents and the high frequency of the emergence of antibiotic resistance during therapy (Niederman, 2001; Paladino et al. 2002). Because we found high prevalence of MDR *P. aeruginosa* in wound infections (39.5%) which is higher than the study of Bangera et al. (2015) with resistance against the most of aminoglycoside, fluoroquinolone and beta-lactams (Rajbahak et al. 2012), we argue that there may be increasing trend of antibiotic resistance of *P. aeruginosa* causing wound infection. The high resistance of *P. aeruginosa* may be due to the intrinsic resistance like chromosomally encoded β-lactamase, biofilm formation, energy dependent efflux (Hancock 1997) and acquired resistance by mutational events leading to over-expression of endogenous β-lactamases or efflux pumps, diminished expression of specific porins and target site modifications. Acquired resistance is also due to acquisition of resistance genes which mainly refers to transferable β-lactamases and aminoglycoside-modifying enzymes.

In order to assess the in vitro inhibitory activity of *A. barbadensis*, we analyzed if there is relation among concentration, types of extraction solvents and antibacterial activity of the *A. barbadensis*. We found that organic extracts with acetone, ethanol and DMSO extracts gave zone of inhibition in certain concentrations but not by aqueous extract. It may be due to partial solubility or insolubility of active compound in aqueous solution (non-polar nature and active compound). Broadly, the magnitude of inhibition was obtained in the pattern of acetone > DMSO > ethanol extracts (0.4 - 0.8%). The results of this study corroborated with Ibrahim et al. (2011) that reported better antimicrobial activity of the acetone extract as compared to ethanol and aqueous extracts. In this study, the maximum zone of inhibition (21.3 ± 2.2 mm) by acetone extract at 0.8% concentration was found which was to be similar (21 mm by acetone extract) to the study of Abakar et al. (2017) for P. aeruginosa suggesting that acetone could be the best organic solvent for extraction. Various extracts showed different zone of inhibition which may be attributed to different solubility of various compounds found in *Aloe barbadensis*. More polar substances extract more of the compounds embedded within the plant cells

(Karpagam and Devaraj 2011). Our experiment showed that, the inhibitory action increases proportionately with the increment of concentration of the extracts corroborating with results of Renisheya et al. (2012).

Among MDR isolates, two-thirds of the isolates were sensitive to aloe extract while all non-MDR isolates were found sensitive. The antibacterial activity of extract was found statistically associated with antibiotic resistance profile of the organism ($p<0.05$). This indicates the multidrug resistant *P. aeruginosa* were less susceptible to aloe extract. Irrespective of the different wound specimens, the zone of inhibition was nearly similar for all isolates of *P. aeruginosa*. However, in a particular concentration of all extracts, there was no significant difference in diameter of the zone of inhibition for MDR and non- MDR isolates ($p > 0.05$).

From this study it is clear that *A. barbadensis* demonstrates inhibitory activity against *P. aeruginosa* indicating its therapeutic potential. Lawrence et al. (2009) identified the compounds with maximum antibacterial activity against *P. aeruginosa* as p-coumaric acid, pyrocatechol and cinnamic acid. Pyrocatechol denatures proteins and disrupts cell membranes (Cowan 1999). Cinnamic acid inhibits glucose uptake and ATP production in the resting cells of bacteria (Kouassi and Shelef 1998). Similarly, p-coumaric compound is reported to increase the lag phase of the microorganism (Baranowski et al. 1980) and is also able to inhibit the enzymatic activity of the microorganisms (Weir et al. 2004). However, in this study the active compounds were not identified. So it is recommended for further research that isolation of key bioactive compound of Nepalese variety of *A. barbadensis* would be useful asset in this regard.

CONCLUSION

Both the MDR and non-MDR isolates of *P. aeruginosa* recovered from wound specimens were inhibited in vitro by organic extracts of *A. barbadensis*. However, comparison study showed slightly higher frequency of non-MDR isolates was sensitive towards organic extracts of *A. barbadensis*. Acetone extract had comparatively better inhibitory activity and magnitude of *in vitro* inhibition was positively correlated with concentration of extracts. Though further confirmation is necessary, in the light of the multiple antibiotic resistance problems, this study showed that *A. barbadensis* may be used as an alternate therapeutic option for the treatment of *Pseudomonas* caused infections more particularly wound infections.

ACKNOWLEDGEMENTS

The authors acknowledge University Grants Commission, Nepal for the partial financial support and Annapurna Neurological Institute and Allied Science Hospital, Nepal for laboratory facilities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abakar HOM, Bakhiet SEA and Abadi RSM (2017). Antimicrobial activity and minimum inhibitory concentration of Aloe vera sap and leaves using different extracts. *J Pharmacogn Phytochem* **6(3)**: 297-303.

Ahmed BED, Magda EN, Rawia B and Amr ES (2008). Pseudomonas aeruginosa exotoxin A: its role in burn wound infection and wound healing. *Egypt J Plast Reconstruct Surg* **32**: 59-65.

Ali Z, Mumtaz N, Naz SA, Jabeen N and Shafique M (2015). Multi-drug resistant *Pseudomonas aeruginosa*: a threat of nosocomial infections in tertiary care hospitals. *J Pak Med* **65(1)**: 12-16.

Bangera D, Shenoy SM and Saldanha DRM (2015). Clinico-microbiological study of *Pseudomonas aeruginosa* in wound infections and the detection of metallo-β-lactamase production. *Int Wound J* **13**: 1-4.

Baranowski JD, Davidson PM, Nagel CW and Branen AL (1980). Inhibition of *Saccharomyces cerevisiae* by naturally occurring hydroxycinnamates. J. *Food Sci* **45**: 592-594.

Bastola R, Parajuli P, Neupane A and Paudel A (2017). Surgical site infections: distribution studies of sample, outcome and antimicrobial susceptibility testing. *J Med Microb Diagn* **6(1)**: 1-7.

Benbow M (2010). Wound swabs and chronic wounds. *Practice Nurse* **39(9)**: 27-30.

Bhandari P, Thapa G, Pokhrel BM, Bhatta DR and Devkota U (2015). Nosocomial isolates and their resistant pattern in ICU patients at National Institute of Neurological and Allied Sciences, Nepal. *Int J Microbiol* (572163): 1-6.

Bhatt CP and Lakhey M (2008) The distribution of pathogens causing wound infection and their antibiotic susceptibility pattern. *J Nepal Health Res Counc* **5**: 22-26.

Chaudhary AK, Bhandari D, Amatya J, Chaudhary P and Achrya B (2016). Metallo β-lactamase producing gram-negative bacteria among patients visiting Shahid Gangalal National Heart Centre. *Austin J Microbio Res* **2**: 208-212.

Clinical and Laboratory Standards Institute (CLSI) (2016). Performance standards for antimicrobial

susceptibility testing; 26th informational supplement. CLSI document M100-S26. Wayne, PA: Clinical and Laboratory Standards Institute.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin Microbiol Rev* **12(4)**: 564-582.

Fan X, Wu Y, Xu M, Xu ZP, Kudinha T, Bazaj A, Kong F and Xu YC (2016). Diverse genetic background of multi-drug resistant *Pseudomonas aeruginosa* from mainland China, and emergence of an extensively drug-resistant ST292 clone in Kunming. Sci Rep **6**: 1-8.

Goudarzi M, Fazeli M, Azad M, Seyedjavadi SS and Mousavi R (2015). Aloe vera gel: effective therapeutic agent against multidrug-resistant Pseudomonas aeruginosa isolates recovered from burn wound infections. *Chemother Res Practice* 2015 (639806): 1-5.

Hamman JH (2008). Composition and application of *Aloe vera* leaf gel. *Molecules* **13**: 1599-1616.

Hancock REW (1997). The bacterial outer membrane as a drug barrier. *Trends Microbiol* **5**: 37-42.

Ibrahim M, Srinivas M and Lakshmi Narasu M (2011). Phyto-chemical analysis and antimicrobial evaluation of *Aloe vera* gel against some human and plant pathogens. *Asian J Curr Chem* **1(1)**: 1-11.

Karpagam T and Devaraj AR (2011). Studies on the efiicacy of Aloe vera on antimicrobial activity. *IJRAP* **2(4)**: 1286-1289.

Kim HS, Kacew S and Lee BM (1999). In vitro chemopreventive effect of plant polysaccharides (Aloe barbadensis Miller, Lentinus edodes, Gandoderm lucidum, and Coriolus vesicolor). *Carcinogenesis* **20**: 1637-1640.

Kouassi Y and Shelef, LA (1998). Inhibition of *Lysteria monocytogenes* by cinnamic acid: possible interaction of the acid with cysteinyl residues. *J Food Safety* **18(3)**: 231-242.

Kshetry AO, Lekhak B and Raghubanshi BR (2015). Antibiogram of bacteria isolated from wound exudates. *Int J Biol Med Res* **6(2)**: 4997-5002.

Lambert PA (2002). Mechanisms of antibiotic resistance patterns of gram negative isolates in a tertiary care hospital of Nepal. *Asian J Pharma Clin Res* 7: 30-33.

Lawrence R, Tripathi P and Jeyakumar E (2009). Isolation, purification and evaluation of antibacterial agents from *Aloe vera*. *Braz J Microbiol* **40**: 906-915.

Malterud KE, Farbrot TL and Huse AE (1993). Antioxidant and radical scavenging effects of anthraquinones and anthrones. *Pharmacology* **47**: 77-85.

Masaadeh HA and Jaran AS (2009). Incident of *Pseudomonas aeruginosa* in post- operative wound infection. *Am J Infect Dis* **5(1)**: 1-6.

Niederman MS (2001). Impact of antibiotic resistance on clinical outcomes and the cost of care. *Crit Care Med* **29**: 114-120.

Paladino JA, Sunderlin JL, Price CS and Schentag JJ (2002). Economic consequences of antimicrobial resistance. *Surg Infect* (Larchmont) **3**: 259–267.

Rai S, Yadav UN, Pant ND, Yakha JK, Tripathi PP, Poudel A and Lekhak B (2017). Bacteriological profile and antimicrobial susceptibility patterns of bacteria isolated from pus/wound swab samples from children attending a tertiary care hospital in Kathmandu, Nepal. *Int J Microbiol* 2017 (2529085): 1-5.

Rajbahak S, Shrestha C, Shrestha J and Singh A (2014). Bacteriological changes of burn wounds with time and their antibiogram. *Sci Rep* **12**: 70-76.

Renisheya JJMT, Johnson M, Nancy BS, Laju RS, Anupriya G and Renola JJET (2012). Anti-bacterial and antifungal activity of *Aloe vera* gel extract. IJBAR **3**: 184-187.

Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, Amato B, Gallelli L and de Franciscis S (2015). Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Expert Rev Anti Infect Ther* **13(5)**: 605-613.

Surjushe A, Vasani R and Saple DG (2008). Aloe vera: a short review. *Indian J Dermatol* **53**: 163-166.

Weir TL, Park SW and Vivanco, JM (2004). Biochemical and physiological mechanisms mediated by allelochemicals. *Curr Opinion Plant Biol* **7**: 472-479.

World Health Organization (WHO) (2002). *Traditional medicine strategy (2002–2005). WHO/EDM/TRM/2002.1.* Geneva, Switzerland: World Health Organization.

Methicillin Resistant *Staphylococcus aureus*: Prevalence and Antibiogram in Various Clinical Specimens at Alka Hospital

Kushal Shahi[1], Komal Raj Rijal[1]*, Nabaraj Adhikari[1], Upendra Thapa Shrestha[1] Megha Raj Banjara[1], Vijay K Sharma[2], Prakash Ghimire[1]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal

[2] Institute of Medicine, Tribhuvan University, Maharajgunj, Kathmandu, Nepal

***Corresponding author:** Dr. Komal Raj Rijal, Lecturer, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal; E-mail: rijalkomal@yahoo.com

ABSTRACT

Objectives: This study aimed to study the prevalence and antibiotic susceptibility pattern of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from clinical specimen.

Methods: During the study period (April-September, 2013), 754 various clinical samples collected from patients visiting at Alka Hospital were cultured for isolation of *S. aureus*. The isolates were characterized as *S. aureus* by their morphology on Gram staining, growth characteristics and coagulase production. Screening of methicillin-resistant *S. aureus* was determined using cefoxitin disk as recommended by Clinical Laboratory Standard Institute. All isolates were subjected to antimicrobial susceptibility testing by modified Kirby Bauer disc diffusion methods.

Results: Total 109 (14.4%) isolates were confirmed as *S. aureus* and 36 (33.0%) *S. aureus* isolates of them were screened as methicillin resistant *S. aureus*. Maximum percentage (63.9%) of methicillin resistant *S. aureus* were comprised of pus specimens. Highest percentage (47.6%) of MRSA was isolated from the age group of above 60 years. Maximum percentage of MRSA (63.9%) was detected in admitted patients. Majority of MRSA isolates were observed to be multidrug resistant. All 36 isolates of MRSA were sensitive to vancomycin. Beside vancomycin, ceftriaxone (83.3%) found to be most effective drug for the MRSA isolates.

Conclusion: The emergence of drug resistance and its dissemination in MRSA is worrisome. So we need to develop newer agents as well as slow down the spread of resistant strains by various control measures.

Key words: Antibiogram, *S. aureus*, MRSA.

INTRODUCTION

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the eminent public health concerns of the 21st century as result of antibiotic pressure. During the past four decades, Methicillin-Resistant *S. aureus* (MRSA) has evolved from a controllable nuisance into a serious public health concern (Singh et al. 2014). MRSA is one of the potent pathogens causing a variety of infections ranging from relatively benign skin diseases to life-threatening infections such as pneumonia, meningitis, endocarditis and septicemia (Baorto 2014). MRSA was first reported in 1961in United Kingdom, just one year after launch of methicillin and outbreaks of MRSA infections were reported in Europe soon thereafter (Davies and Davies 2010). Later it has been emerged as one of leading nosocomial pathogens for past 50 years and emerged in the community as well (Gopalakrishnan and Sureshkumar 2010). MRSA accounts more than 50% of nosocomial infection (Venkatesh et al. 2014). MRSA infections are a global problem across health economy as it has been associated with significant morbidity, mortality, a poorer outcome

Date of Submission: November 12, 2018
Date of Acceptance: December 8, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22316

and higher costs (Soltani et al. 2010). Fighting MRSA involves re-enforcement of infection control measures as well as rational use of antimicrobials. Community-associated cases of MRSA (CA-MRSA) were reported starting in the late 1990s (DeLeo et al. 2010). CA-MRSA infections were first described in pediatric patients only but now have become a significant public health threat. CA-MRSA posses *Mec* (SCC*mec*) types IV and V, that are resistant to fewer antimicrobial agents and exhibit enhanced virulence. CA-MRSA infections typically occur as skin or soft tissue infections but can develop into more invasive and serious infections. CA-MRSA tends to occur more in conditions where people are in close physical contact such as long-term care facilities (Raygada and Levine 2009).

In MRSA, the horizontally acquired *MecA* gene encodes a penicillin-binding protein (PBP2a), which has low affinity to all β-lactams, responsible for resistance. The *MecA gene* complex also contains insertion sites for plasmids and transposons that facilitate the acquisition of resistance to other antibiotics (Dominguez 1997). Resistance to multiple antibiotics among MRSA isolates is very common and has posed a serious therapeutic challenge becoming a problem of global extent. MRSA is of serious concern because of resistance to many antimicrobials that are used on a regular basis in hospitals limiting therapeutic options and increasing cost of treatment. Presently MRSA isolates have been uniformly susceptible only to glycopeptides, considered as last resort for these strains. Clinical isolates of MRSA with reduced susceptibility to glycopeptides were first described in Japan in 1997 (Hiramatsu et al. 1997). Vancomycin resistant *S. aureus* is not widely seen even though a low-level resistance to vancomycin is being reported. The prolonged hospitals stay and indiscriminate are the possible predisposing factors of MRSA emergence. Asymptomatically colonized healthcare workers are the major sources of MRSA in the hospital environment (Orji et al. 2012).

MRSA are prevalent throughout the world. In US about 40% of *S. aureus* infections acquired are found to be methicillin-resistant (Zaoutis TE 2013). The percentage of hospitals isolating MRSA in the developed countries has increased from 2% in the 70's to 30% in the 90's (Gordon 1993). MRSA is now endemic in Nepal. The growing problem in the Nepalese scenario is that MRSA prevalence is rapidly increasing with the time. The incidence of MRSA in Nepal varied from 11.76% in 1999 reported by Lamichhane to 60% in 2010 observed by Khanal and Jha carried out in a tertiary-care hospital in Eastern Nepal. These studies clearly show the need of efficacious and rapid infection control measures. The present study provides a hospital level initiative to understand emerging trends of antimicrobial resistance among local MRSA isolates and provides a platform to initiate epidemiological studies for MRSA infections. The current status of antimicrobial susceptibility profile of local MRSA isolates is essential for the selection of appropriate therapy for the management of infections. Data from such study can be utilized to formulate cost effective empirical therapy and make better hospital infection control policies. A hospital level study helps to know the best treatment options available for MRSA infected patients.

MATERIALS AND METHODS

Study population

This was a prospective descriptive study carried out at Alka hospital, Lalitpur. Participants were the patients visiting Alka hospital. All clinical specimens were obtained from participants for study.

Conventional microbiological tests

All clinical specimens collected aseptically were processed and cultured using standard microbiological procedures. Isolated colonies from the pure culture were identified by performing the standard conventional biochemical tests. Susceptibility tests of the different clinical isolates towards various antibiotics were performed by modified Kirby-Bauer M02-A9 disk diffusion method using Mueller Hinton Agar (MHA). MRSA isolates in pure culture were preserved in 20% glycerol containing tryptic soya broth and kept at -70°C until subsequent tests were performed (CLSI 2007).

Detection of methicillin resistant *Staphylococcus aureus* isolates

The methicillin resistant *Staphylococcus aureus* isolates were screened using cefoxitin disk (30µg). The screened isolates were subjected to antimicrobial susceptibility testing by modified Kirby Bauer disc diffusion methods for the determination of current antimicrobial susceptibility pattern of local MRSA isolates (CLSI 2007).

RESULTS

MRSA profile in different specimens

Out of total 754 various clinical samples processed and cultured for isolation of *Staphylococcus aureus*, 109 (14.4%) isolates were confirmed as *S. aureus*. Out of them 36 (33.0%) isolates were screened as methicillin resistant *Staphylococcus aureus*. Maximum percentage (63.9%) of methicillin resistant *S. aureus* strains were comprised of pus specimens (Table 1).

Table 1: Distribution of MRSA in different specimens

Specimens	*S. aureus*, n(%)	MRSA	MSSA
Pus	77 (70.6)	23 (63.9)	54 (73.9)
Urine	13 (11.9)	6 (16.7)	7 (9.6)
Sputum	8 (7.3)	5 (13.9)	3 (4.1)
Ear swab	4 (3.7)	0	4 (5.5)
Plural fluid	3 (2.8)	1 (2.8)	2 (2.8)
Other	4 (3.7)	1 (2.8)	3 (4.1)
Total	109 (100)	36 (33.03)	73 (66.97)

Distribution of MRSA isolates among outdoor and indoor patients in different age groups

Out of total 36 MRSA strains isolated, 14 (38.9%) strains were isolated from outpatients whereas 22 (61.1%) strains of MRSA were isolated from admitted patients. The highest percentage of MRSA (27.8%) was observed in the age group of above 60 years (Table 2).

Table 2: MRSA among outdoor and indoor patients in different age groups

Age (Years)	Outpatients		Indoor patients	
	S. aureus n(%)	MRSA	S. aureus	MRSA
<10	1 (2.2%)	0	10 (15.9%)	4 (18.2%)
11-20	6 (13%)	4 (28.5%)	7 (11.2%)	1 (4.6%)
21-30	11 (23.9%)	1 (7.1%)	13 (20.6%)	3 (13.6%)
31-40	2 (4.3%)	1 (7.1%)	5 (7.9%)	1 (4.6%)
41-50	5 (10.9%)	2 (14.3%)	13 (20.6%)	5 (22.7%)
51-60	10 (21.8%)	2 (14.3%)	5 (7.9%)	2 (9%)
>60	11 (23.9%)	4 (28.7%)	10 (15.9%)	6 (27.3%)
Total	**46 (42.2%)**	**14 (38.9%)**	**63 (57.8%)**	**22 (61.1%)**

Antibiotic susceptibility pattern of isolates

All 36 strains of MRSA were sensitive to vancomycin. Beside vancomycin, ceftriaxone found to be most effective for the MRSA strains (75%), followed by levofloxacin (69.4%), tetracycline (66.7%), amikacin (66.7%) and ciprofloxacin (63.9%). Similarly, most resistant drug among the MRSA strains was amoxycillin (91.6%) and cloxacillin (88.8%) (Table 3)

Table 3: Antibiotics resistance pattern of MRSA isolates

Antibiotics	*S.aureus*, n(%)	MRSA, n(%)
Amoxycillin	62 (56.9)	33 (91.6)
Ciprofloxacin	35 (32.1)	13 (36.1)
Erythromycin	39 (35.8)	17 (47.2)
Levofloxacin	52 (22.9)	11 (30.6)
Cloxacillin	50 (45.9)	32 (88.8)
Cefoxitin	36 (33)	36 (100)
Amikacin	34 (31.2)	12 (33.3)
Vancomycin	0	0
Ceftriaxone	24 (22)	9 (25)
Chloramphenicol	31 (28.4)	15 (41.6)
Tetracycline	27 (24.8)	12 (33.3)

DISCUSSION

MRSA has emerged as a serious threat to public health worldwide. It has added to the burden of patient by prolonging hospital stay and increasing morbidity, mortality rate and cost. Present study showed prevalence rate of MRSA to be 33.03%. This finding is consonent with the finding of Fayomi et al. (2009), in this study which is carried out in Ido-Ekiti, Nigeria; they found 31% MRSA. The result obtained is in agreement with the findings of Vidya et al. (2010), who reported 29.1% MRSA isolates in Mangalore, South India. Likewise, our finding is similar with the finding of Mir (2013), he reported the prevalence of MRSA to be 32.2% in Pesawar, Pakistan. In Nepal, these findings also synchronize with the findings of Sapkota (2006), who reported 31.1% MRSA and Thapa (2004) reported 29.23% MRSA. On the contrary, report shown by Rajbhandari (2002), has alarmingly high incidence of MRSA infection (54.9%). Our outcome is belied with the findings of Rijal et al. (2008), who reported 75.5% MRSA in a study conducted in Pokhara Valley. Similar study done in western parts of Nepal by Tiwari et al. also had shown alarmingly high rate of MRSA isolate (69.1%) in 2009 which the authors has attributed to indiscriminate use of antibiotics and its accessibility in these areas (Tiwari et al. 2009). Above studies show considerable variations between institutions, often in the same geographical areas, 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 hospital and, not less important, the clonal and often epidemic nature of these microorganisms (Rijal et al. 2008). This study showed maximum percentage of *S aureus* (70.6%) and MRSA (63.9%) isolated from pus ascertaining the role of the organism as cause of pyogenic infection. This is similar to the study done in Nepal, India and Pakistan (Shrestha et al. 2009). This study observed highest percentage (47.6%) of MRSA was isolated from the age group of above 60 years. This might be due to the high dose of medication because of difficulties in infection treatment in that group. Furthermore, the higher vulnerability of such age group can be correlated with the reduced immune system in that group of patients. This result is consonant with finding of Duran et al. (2012). Higher percentage of MRSA was isolated from admitted patients that suggest high antibiotic pressure in hospital and hence they are more prone to be MRSA. This is harmony with report of Adeleke and Olarinde (2013). However in remote regions of Nepal where availability and use of antibiotics is limited, the prevalence of MRSA was observed to be low, reported by Subedi and Brahmadath in 2005.

Analysis from previous studies revealed a relationship between methicillin resistance and resistance to other antibiotics. This study showed that all MRSA isolates were significantly less sensitive to antibiotics. Homogeneous insusceptibility to beta-lactams like amoxycillin and cloxacillin, resistant MRSA was also observed in our study. This may be due to presence of intrinsically developed beta-lactamase in MRSA strain. It also showed the high resistance to erythromycin as such antibiotics are usually used at random to cure generalized and pyogenic infection. Antimicrobials such as amikacin and tetracycline with resistance less than 35% could be used against of MRSA infection. But due to their mode of action, have limited use for empirical therapy of MRSA related infection. Resistance to ceftriaxone was observed to be 25% in this study. Limiting its indiscriminate use and doing antibiotic susceptibility testing, it could be considered for empirical therapy for MRSA infection in this setting. The multi-drug resistant has become in methicillin-resistant *S.aureus* strains. It has added burden to hospital personal in infection control and has limited therapeutic option. In this study, majority of MRSA isolates were MDR. Studies conducted in eastern and western part of Nepal also reported MDR-MRSA to be as high as 65-78%. Though these MDR strains are not found with additional virulence properties, multidrug resistance only restricts the options available to treat infections caused by this organism (Kumari et al. 2008). Vancomycin, a glycopeptide seems to be the only antimicrobial agent which showed 100% effectiveness through all parts of Nepal and may be used as the drug of choice for treating multidrug resistant MRSA infections, it should be preserved for life threatening infection. But its toxic side effects like renal impairment and high cost has limited its use. When vancomycin is

considered for treatment, in vitro susceptibility testing is most owing to emergence of Vancomycin resistant *Staphylococcus aureus* (VRSA) in various parts of world (Sakoulas and Moellering 2008).

CONCLUSION

Existence of MRSA isolates is a serious matter of concern. Moreover, drug resistance in MRSA at study area is worrisome in the current therapeutic scenario as majority of MRSA isolates were multidrug resistant. These findings call for urgent attention on regular surveillance in antibiotic profile of *Staphylococcus* isolates. Vancomycin still remains the drug of choice for MRSA infection; it should be preserved for life-threatening MRSA infection. Ceftriaxone promises to be the best for the treatment of MRSA isolates in the study area in vitro.

CONFLICT OF INTEREST

The authors don't have any conflict of interest.

REFERENCES

Baorto EP (2014). *Staphylococcus aureus* infection. *Medscape* **2**: 50-59.

Clinical Laboratory Standard Institute (2007). Performance standards for antimicrobial susceptibility testing; Seventeenth informational supplement-Clinical Laboratory Standard Institute M100-S17 **27**: 1.

Davies J and Davies D (2010). Origin and evolutions of antimicrobial resistance. *Microbiol Mol Biol Rev* **74**(3): 417-433.

DeLeo FR, Otto M, Kreiswirth BN and Chambers HF (2010). Community-associated methicillin resistant *Staphylococcus aureus*. *Lancet* **375**: 1557-1568.

Domínguez MA, Liñares J and Martín R (1997). Molecular mechanisms of methicillin resistance in *Staphylococcus aureus*. *Microbiologia* **13**(3): 301-308.

Gopalakrishnan R and Sureshkumar D (2010). Changing trends in antimicrobial susceptibility and hospital acquired infections over an 8-year period in a tertiary care hospital in relation to introduction of an infection control programme. *J Assoc Physicians India* **58** (Suppl): 25-31.

Gordon J (1993). Clinical significance of methicillin sensitive and methicillin resistant *Staphylococcus* in UK Hospitals and the relevance of povidone iodine in their control. *Postgrad Med J* **69** (3): 106-116.

Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y and Kobayashi I (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350**: 1670-1673.

Kumari N, Mohapatra TM and Singh YI (2008). Prevalence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in a Tertiary-Care Hospital in Eastern Nepal. *J Nepal Med Assoc* **47**: 53-56.

Orji I, Nworie A, Eze UA, Agberotimi IO, Okereke EC and Azi SO (2012). The prevalence and antimicrobial susceptibility profile of methicillin resistant *Staphylococcus aureus* isolates from clinical specimens in a tertiary hospital, south east Nigeria. *Continental J Pharm Sci* **6**(1), 23-29.

Raygada JL and Levine DP (2009). Managing CA-MRSA Infections: Current and emerging options. *Inf Med* **26**: 42-44.

Rijal KR, Shrestha N, Pahari N, Shrestha B, Paudel B, Nepal A, Ghimire P and Rijal B (2008). Methicillin resistant *Staphylococcus aureus* in patients visiting Western region hospital. *JIOM* **30**(1): 21-25.

Sakoulas G and Moellering RC Jr (2008). Increasing antibiotic resistance among methicillin-resistant *Staphylococcus aureus* strains. *Clin Infect Dis* **46**(5): 360-367.

Shrestha B, Pokhrel BM and Mohapatra TM (2009). Phenotypic Characterization of nosocomial isolates of *Staphylococccus aureus* with reference to MRSA. *J Infect Dev Ctries* **3**(7): 554-560.

Singh H, Atray M and Modi PK (2014). Antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus aureus* (MRSA) isolates in tertiary care center at southern Rajasthan. *Int J Pharm Sci Res* **5**(2): 607-611.

Soltani R, Khalilia H, Rasoolinejadb M, Alireza AbdollahicAandGholamiaK(2010).Antimicrobial susceptibility pattern of *Staphylococcus aureus* strains isolated from hospitalized patients in Tehran, Iran. *Iranian J Pharm Sci* **6**(2): 125-132.

Tiwari HK, Das AK, Sapkota D, Sivrajan KK and Pahwa VK (2009). Methicillin resistant *Staphylococcus aureus*: prevalence and antibiogram in a tertiary care hospital in western Nepal. *J Infect Dev Ctries* **3**(9): 681-4

Venkatesh VN, Shwetha DC, Ravish KM, Sudhindra KS and Srinivasa S (2014). MRSA study of antimicrobial susceptibility pattern of methicillin-resistant *Staphylococcus aureus* at tertiary care hospital in south India. *J Biomed Pharma Res* **3**: 25-28.

Zaoutis TE (2013). Rates of serious MRSA infections in children increased about 10% per year since 2005. *Am Acad Pedria* **34:** 22-28.

Poor Quality of Treated Water in Kathmandu: Comparison with Nepal Drinking Water Quality Standards

Sujan Maharjan[1], Tista Prasai Joshi[1]*, Sujen Man Shrestha[1]
Environment and Climate Study Laboratory, Nepal Academy of Science and Technology, Lalitpur, Nepal

***Corresponding author**: Dr. Tista Prasai Joshi, Nepal Academy of Science and Technology Lalitpur, Nepal; E-mail: tista.prasai@nast.gov.np, tistaprasai@gmail.com

ABSTRACT

Objectives: In order to evaluate the quality assurance of drinking water in Kathmandu valley, this study analyzed selected physiochemical and microbial parameters of treated water samples and compared with Nepal Drinking Water Quality Standards (NDWQS).

Methods: Treated water samples were collected from all over the Kathmandu valley and analyzed in terms of physicochemical and microbiological parameters over the period of one year from July 2017 to July 2018. The physio-chemical parameters of water samples were performed according to standard methods for the examination of water and waste water. The total coliforms were enumerated by standard membrane filtration technique.

Results: We report that microbiological aspect of treated water was the major problem as 66% of the water samples crossed the guideline value for total coliform count. Above 92% of jar water samples, 77% of tanker water samples and 69% of filtered water samples had the total coliform count exceeding the NDWQS. Moreover, 20% of bottled water was contaminated by coliform bacteria. Iron and ammonia content were found to be higher than the guideline values in 16% and 21% of the total treated water samples respectively. Analyzing the types of treated water samples showed that 35% and 15% of tanker water samples had higher ammonia and iron content respectively, and the same parameters were higher in 23% and 19% in the filtered water samples respectively than the standard criteria recommended by NDWQS.

Conclusion: The treated water samples exceed the standard values set by NDWQS and hence had poor quality. The presence of faecal pollution indicating coliform bacteria was the key problem for treated drinking water of Kathmandu valley. Therefore, monitoring and proper treatment of water should be conducted to prevent dissemination of waterborne diseases.

Key words: Ammonia, coliform, iron, treated water, water quality, NDWQS

INTRODUCTION

Water pollution is a worldwide problem and poses a serious threat to human life. For most Nepalese, obtaining sufficient water is a greater concern than obtaining safe water. Department of Water Supply and Sewerage (DWSS) reported that around 86% of the Nepalese population has access to basic water supply facility as of mid – 2015 (DWSS 2015). However, the quality of supplied water is questionable as of 2016/2017 report by Department of Health Service (DoHS) showed 23,742 cases of water borne diseases among inpatients in Nepal and out of which 270 cases resulted in death. The leading water borne disease was Typhoid fever causing 115 fatalities (DoHS 2016).

Those at greatest risk of waterborne disease are infants and young children, people who are debilitated and the elderly, especially when living under unsanitary conditions. So the water quality guidelines describe reasonable minimum requirements of safe practice to protect the health of consumers. No single water quality assurance approach is universally applicable, and the nature and form of drinking-water standards

Date of Submission: November 2, 2018
Date of Acceptance: December 5, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22319

may vary among countries and regions (WHO 2017).

The drinking water quality of Kathmandu Metropolitan area has been degraded physically, chemically and microbiologically (Prasai et al. 2007). The drinking water sources of stone spouts, taps and tube wells are contaminated with total coliform. The pH, ammonia, turbidity, electrical conductivity and arsenic level are also deviated from the WHO guidelines and Nepal standard for drinking water (Bajracharya et al. 2007). Enteric bacteria are found in biofilm in drinking water distribution system of Kathmandu valley (Shakya et al. 2012). Different treatment methods are used by most of the households in Kathmandu valley to tackle this problem. Among the various treatment methods used, boiling and using a ceramic filter are the most common ones (Shrestha et al. 2016; Shrestha et al. 2018).

Many countries with water scarcity and poor quality drinking water depend on packaged, bottled, and treated water as an alternative of public water supply systems (Dindarloo et al. 2015). Household level water treatment industry has experienced significant growth over the past several years (Chaidez and Gerba 2004). These household level treatment plants are specially designed to remove a broad range of contaminants in drinking water, including colour, odour, iron and microbial contaminants. The treatment systems used include channel through activated carbon, distillation, reverse-osmosis, ultra-filtration, membrane filters and UV disinfection. However, it is possible that contaminants occurrences can occur at the different stages of production of drinking water treatment systems (Smeti et al. 2009).

The intense increase in the drinking of bottled and packaged water has been prompted by users concern over increasing water pollution (Warburton 1993). The use of costly methods applying reverse osmosis and UV radiation are getting popular in Nepal (Lantagne and Clasen 2012). Commercially available processed jar water is extensively used by the public, however, the quality and safety of bottled jar water from human health perspective is questionable. In a research on jar water quality in Nepal reported the water was not safe for human consumption due of the presence of coliform bacteria (Budhathoki 2010). In the context of Nepal, the water suppliers should abide by the directives of National Drinking Water Quality Standards, for maintaining drinking water quality parameters (NDWQS 2005). To ensure effective treatment in terms of pathogen removal at the water treatment system, a microbial risk assessment needs to be performed (George et al. 2015). For the safe drinking water, its physicochemical and microbiological parameters should meet the minimum requirements of drinking water quality standards. The main aim of the research study was to investigate the physicochemical and bacteriological parameters in the treated water from the Kathmandu valley.

MATERIALS AND METHODS

The experiments were conducted in the Environmental and climate study laboratory, Nepal Academy of Science and Technology (NAST). The water samples were examined for their physiochemical and microbiological quality in order to explore the contamination problems. The samples were quickly analyzed for physiochemical and total coliform count test on the arrival to the laboratory. If immediate analysis was not possible, it was stored at 4°C to avoid changes until analysis. Temperature and pH were analyzed by pH meter (EC-210 Rocker Scientific Co.). Electrical conductivity was measured by Conductivity meter (HI 8633 HANNA). Turbidity was measured by nephelometer (HI 98713 ISO Turbidimeter HANNA). For the chemical parameters, hardness and chloride were analyzed by EDTA and Argentometric titration respectively. Iron was analyzed by phenanthroline spectrophotometric method (6715 UV/Vis Spectrophotometer JENWAY). Arsenic (QUANTOFIX® Arsenic 10 (Macherey-Nagel Germany)), ammonia (*VISOCOLOR® alpha* Ammonium (Macherey-Nagel Germany)) and nitrate (*VISOCOLOR® alpha* Nitrate (Macherey-Nagel Germany)) were detected by colorimetric method. Total coliform counts were performed using the standard membrane filtration (MF) technique. The 100 mL water sample was filtered using 0.45 mm pore size, 47 mm diameter filter membrane as described by APHA (2005). Membrane filters were placed onto m-Endo agar at 37 °C and bacterial colonies were enumerated by colony counter after 24 hours.

RESULTS

Throughout the year 2017, 243 water samples from different sources such as filtered water, jar water, tanker water, and bottled water were analyzed for physicochemical and bacteriological parameters. Out of 243 samples, majority of samples 175 (72%) were from Lalitpur district, followed by 55 (23%) from Kathmandu and 13 (5%) from Bhaktapur districts as shown in Figure 1.

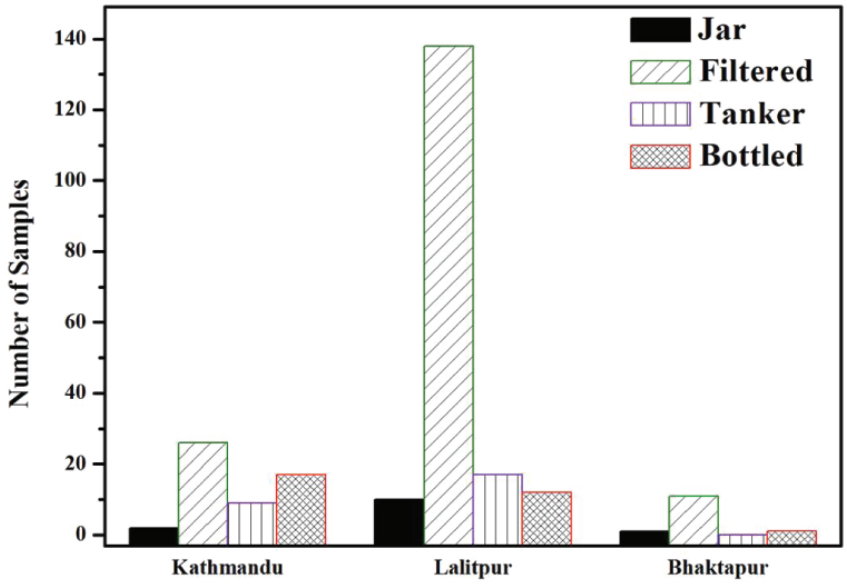


Figure 1: Treated water samples collected from Kathmandu valley

Among the treated water samples, variation of pH, presence of ammonia and iron content were the key problem in chemical constituents. While the presence of coliform in treated drinking water was a major problem in the Kathmandu valley. Out of 243 treated sample water, 160 (66%) samples contains coliform contamination. 50 (21%), 39 (16%), 29 (12%), 6(2.4%) and 4(1.6%) treated water samples exceeded the national guideline value for ammonia, iron, pH, turbidity and nitrate respectively as shown in Figure 2.

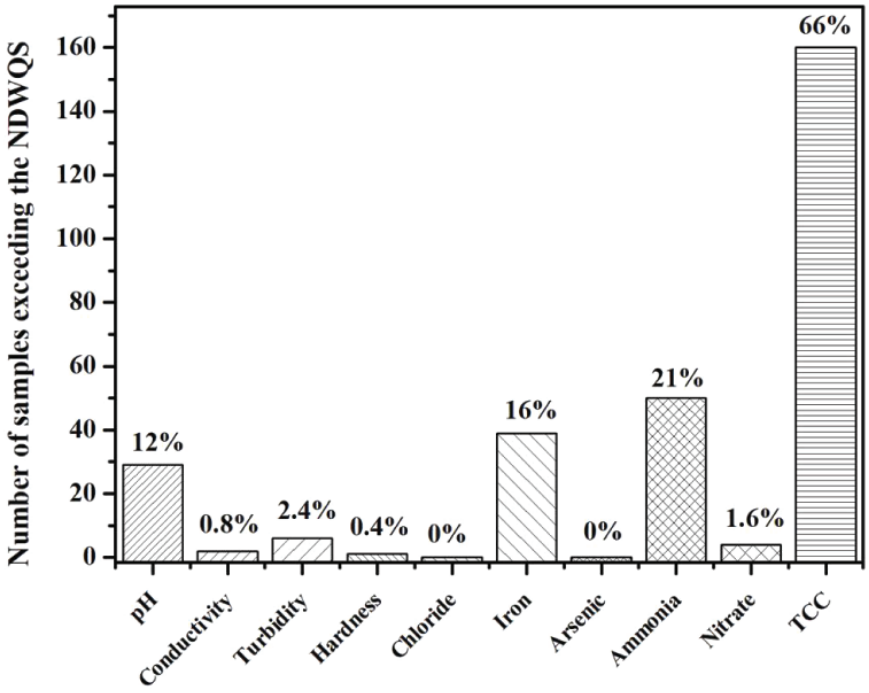


Figure 2: Frequency of treated water samples crossing the NDWQS 2005 values

The treated water was grouped as filtered water, jar water, bottled and tanker water. The tanker water is widely used for the household purpose inside the Kathmandu valley. During this study, the tanker water and filtered water had exceeded the most parameters limits of NDWQS 2005. The total number of samples exceeding the different parameters of the treated drinking water obtained from the Kathmandu valley is given in Figure 3.

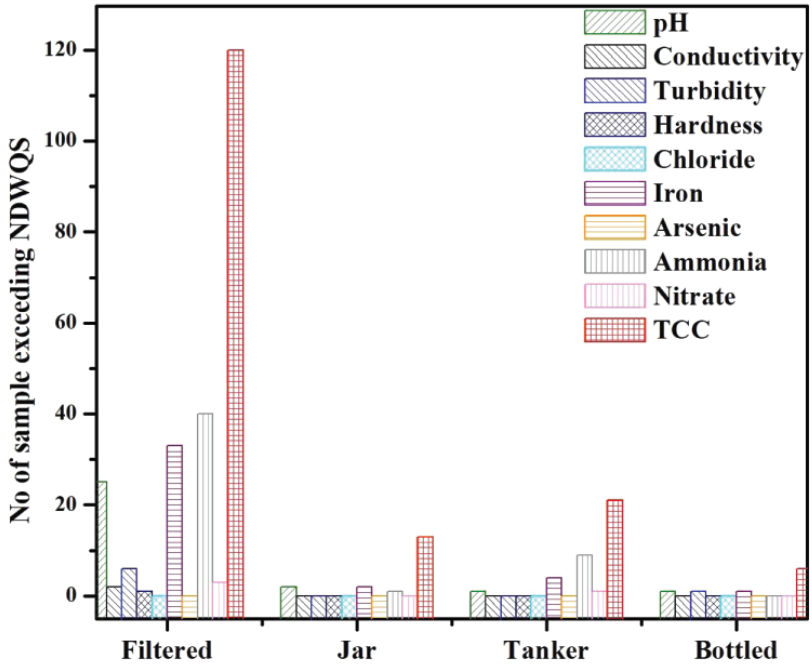


Figure 3: Frequency distribution of treated water samples crossing the NDWQS value

The study revealed that 14% of filtered water sample had pH value beyond the NDWQS 2005. In this study, concentration of iron (Fe) in filtered water samples (19%) and tanker water samples (15%) were higher than the standard limit of NDWQS. Ammonia concentration in both filtered water samples (23%) and tanker water samples (35%) were higher than NDWQS limit. For microbial assessment, 69% of filtered water samples exceeded the guideline value for total coliform count. Above 92% of jar water samples and 77% of tanker water samples exceeded the guideline value for total coliform count. While in case of bottled water, 20% of samples crossed the guideline value for total coliform count.

DISCUSSION

The main goal of this study was to evaluate the selected quality parameters of treatment claimed water available in Kathmandu valley so that the efficacy of the water treatment or post-treatment contamination could be assessed. For this we tested four types of treatment claimed drinking water samples including filtered, tanker, jar and bottled waters available in the valley. The measurements of quality parameters were judged based on National drinking water quality standards of Nepal.

The pH value for filtered water samples was beyond the standard limit at the time of analysis. Although pH is not directly related to health risk it is very important in disinfection process applying chlorine. When the pH exceeds 8, disinfection is less effective while low pH is acidic and is corrosive to pipes (WHO 2017). Some filtered water samples had turbidity higher than NDWQS value which questions the efficiency of filters used in removing the turbidity. Two of the filtered water samples had higher conductivity than the standard limit. Electrical conductivity is a measure of total ion content of water. The presence of salts and contaminants with waste waters increase the conductivity of the water. All the treated water samples had their hardness and chloride within the standard at the time of analysis except one filtered water sample which exceeded the hardness limit of 500 mg/L. Hardness in water is caused by a variety of dissolved polyvalent metallic ions, predominantly calcium and magnesium cations. Hardness is the traditional measure of the capacity of water to react with soap, hard water requiring considerably more soap to produce a lather. Bottled and packaged waters might be naturally mineralized or naturally soft or demineralized. Thus, the mineral consumption from drinking-water and cooking water may vary widely, depending upon location, treatment and water source (WHO 2017). In this study, filtered and tanker water samples had iron content higher than the NDWQS value. Iron contamination can occur due to the excessive corrosion of iron pipes mainly due to the oxidation by dissolved oxygen to form a precipitate of iron (III) (Shrestha and Lama 2014). Usually high iron concentrations may not constitute a direct health risk but this could have a bad impact on odor and taste (Smedley et al. 1995).

The results are found to be consistent with the various other studies conducted, which found 48% in Bhaktapur municipality, 26% in Madhyapur Thimi and 15% of treated water samples from Kathmandu valley were contaminated with iron (Diwakar et al. 2008; Jayana et al. 2009; Koju et al. 2014). All the tested treated water samples were free from arsenic contamination. In this study, filtered and tanker water samples showed the presence of ammonia higher than the NDWQS value. Ammonia originates mainly from the metabolic, agricultural and industrial processes and can be an indicator of the possible bacterial, sewage and animal waste pollution (Shrestha and Lama 2014). Similar studies conducted on treated water samples and untreated drinking water samples from variety of sources showed that 9% and 5.17%, respectively of the water samples exceeded the ammonia guideline limit (Koju et al. 2014; Diwakar et al. 2008). Previous study has reported 11% of the total drinking water samples of Madhyapur, Thimi area crossed the guideline value for ammonia (Jayana et al. 2009). Nitrate was also observed in filtered (three) and tanker (one) water samples beyond the guideline value. However, nitrate content was observed to be within the permissible level (Jayana et al. 2009; Koju et al. 2014). Nitrate contamination may cause large scale health effects through drinking-water exposure. Nitrate contamination of water might be due to the sewage and agricultural runoff. It is difficult to remove nitrate and disinfection may convert it to more toxic form. Moreover, there is a risk factor for methaemoglobinaemia caused by excess nitrate/nitrite exposure to infants of 3–6 months of age (WHO 2017).

According to WHO guidelines (2017) and NDWQS (2005), the number of total coliforms should not be observed in 100 mL of drinking water. In this study, most of the treated water samples showed coliform contamination which makes it unsafe for drinking purpose. Our analysis of jar water marketed in Kathmandu valley revealed that 92% of jar water samples were heavily contaminated with coliform bacteria and unsatisfactory for drinking purpose. In tanker water samples, 77% of samples crossed the guideline value for total coliform count. While in case of bottled water or processed drinking water sample available in the market, 20% of sample crossed the guideline value for total coliform count.

Previous studies reported 36% of treated water samples contained coliform bacteria indicating possible contamination of faecal origin (Koju et al. 2014). While in another study conducted in Dharan municipality Nepal, all the tap water samples and most of the bottled drinking water samples were found to be contaminated with one or more than one type of indicator organisms (Pant et al. 2016). The presence of total coliform was 26.32% in drinking jar water in Bangladesh which indicated that some of the drinking jar water samples were of poor quality which may increase the risk of water-borne diseases (Mina et al. 2018). It has been speculated that the occurrence of coliform bacteria in treated water samples implies that the treatment capacities is insufficient for the water samples. Contamination by microbial pathogens is the most direct risk, and specific regulations for private drinking water suppliers should be strictly regulated. Thus, contamination of drinking water is a major public health problem in a developing country like Nepal. Hence, there is a need for a thorough assessment of relevant physiochemical and microbiological parameters along the entire chain from the drinking water treatment.

CONCLUSION

This study revealed considerable microbial contamination of the jar water, filtered water, tanker water and bottled water. Furthermore, ammonia concentration was highest in tanker water followed by filtered water and jar water. Bottled water was safer than any other treated water used for drinking purposes with some risk of coliform contamination. Our outcomes make significant contribution to the understanding of the interconnection of water pollution and its direct effect for public health. Therefore, treated water may not always be of good quality as is perceived. For this reason, it is recommended that water for human consumption is appropriately treated for bacterial contamination before consumption. The results of these analyses indicated the need to identify the critical control points along the production stages to minimize the possible risk.

ACKNOWLEDGEMENTS

The authors are grateful to Nepal Academy of Science and Technology for providing the laboratory facilities. Thanks to all people who brought water samples at NAST research laboratory. We would like to appreciate all the help and efforts provided by the Environment Lab members especially Mr. Sujit Dahal and Ms. Smrita Acharya for their help and support.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

APHA (2005). Standards methods for the examination of water and waste water 21th Edition. American Public Health Association, American Water Works Association, Water Pollution Control Federation. Published by the American Public

Health Association, Washington DC.USA

Bajracharya A, Yami K, Prasai T, Basnyat S and Lekhak B (2007). Assessment of drinking water quality of Kathmandu metropolitan areas. *NJST* **8**: 113-118.

Budhathoki R (2010). Analysis of the physico-chemical and bacteriological parameters of bottled water available in Kathmandu valley. Dissertation submitted to Tribhuvan University.

Chaidez C and Gerba G P (2004). Comparison of the microbiologic quality of point of-use (POU)-treated water and tap water. *Int J Env Health Res* **14(4)**, 253 – 260

Dindarloo K, Ghaffari HR, Kheradpisheh Z, Alipour V, Ghanbarnejad A, Fakhri A and Goodarzi B (2015). Drinking water quality: comparative study of tap water, drinking bottled water and point of use (PoU) treated water in Bandar-e-Abbas, Ira. *Desalin Water Treat* **57** 1-7.

Diwakar J, Yami KD and Prasai T (2008). Assessment of drinking water of Bhaktapur municipality area in pre-monsoon season. *Sci World* **6**: 94.

DoHS (2016). Annual report of department of health services. Ministry of Health and Population, **23**.

DWSS (2015). Annual progress review. Department of Water Supply and Sewerage, Kathmandu.

George J, An W, Joshi D, Zhang D, Yang M and Suriyanarayanan S (2015). Quantitative microbial risk assessment to estimate the health risk in urban drinking water systems of Mysore, Karnataka, India. *Water Qual Expo Health* **7:** 331-338.

Jayana BL, Prasai T, Singh A and Yami KD (2009). Assessment of drinking water quality of madhyapur-thimi and study of antibiotic sensitivity against bacterial isolates. *NJST* **10**: 167-172.

Koju NK, Prasai T, Shrestha SM and Raut P (2014). Drinking water quality of Kathmandu valley. *NJST* **15**: 115-120.

Lantagne DS and Clasen TF (2012). Use of household water treatment and safe storage methods in acute emergency response: case study results from Nepal, Indonesia, Kenya, and Haiti. *Env Sci Tech* **46**: 11352-11360.

Mina SA, Marzan LW, Sultana T and Akter Y (2018). Quality assessment of commercially supplied drinking jar water in Chittagong city, Bangladesh. *Applied Water Sci* **8**:24.

National Drinking Quality Standards and Directives (NDWQS) (2005). Implementation directives for national drinking water quality standards. Government of Nepal, Ministry of Physical planning and Works, Kathmandu.

Pant ND, Poudyal N and Bhattacharya SK (2016). Bacteriological quality of drinking water sources and reservoirs supplying Dharan municipality of Nepal. *Annals Clinical Chem Lab Med* **2**: 19-23.

Prasai T, Lekhak B, Joshi DR and Baral MP (2007). Microbiological analysis of drinking water of Kathmandu valley. *Sci World* **5**: 112-114.

Shakya P, Joshi TP, Joshi DR and Bhatta DR (2012). Evaluation of physicochemical and microbiological parameters of drinking water supplied from distribution systems of Kathmandu municipality. *NJST* **13**: 179-184.

Shrestha KB, Thapa BR, Aihara Y, Shrestha S, Bhattarai AP, Bista N, Kazama F and Shindo J (2018). Hidden cost of drinking water treatment and its relation with socioeconomic status in Nepalese urban context. *Water* **10**: 607.

Shrestha S, Aihara Y, Kondo N, Rajbhandari S, Bhattarai A, Bista N, Kazama F, Nishida K, Timilsina HP and Shindo J (2016). Household water use in the Kathmandu valley: a dry season survey. Interdisciplinary Center for River Basin Environment (ICRE), Kofu Yamanashi, Japan.

Smedley P, Edmunds W, West J, Gardner S and Pelig-Ba K (1995) Health problems related to groundwater in the Obuasi and Bolgatanga areas, Ghana. British Geological Survey Technical Report WC/95/43, Natural Environment Research Council Polaris House, North Star Avenue Swindon, 122.

Smeti EM, Thanasoulias NC, Lytras ES, Tzoumerkas PC and Golfinopoulos SK (2009). Treated water quality assurance and description of distribution networks by multivariate chemometrics. *Water Res* **43**: 4676-4684.

Warburton DW (1993). A review of the microbiological quality of bottled water sold in Canada. Part 2. The need for more stringent standards and regulations. *Can J Microbiol* **39***:* 158-168.

World Health Organization (2017). Guidelines for drinking-water quality: first addendum to the fourth edition. Licence: CC BY-NC-SA 3.0 IGO.

Intestinal Parasitic Infection among the School Children of Kathmandu, Nepal

Chetana Dahal[1], Puja Katwal[1], Anju Thapa[1], Deepa Sharma[1] and Rama Khadka[1]*

[1] Padmakanya Multiple Campus, Baghbazar, Kathmandu, Nepal

***Corresponding author**: Rama Khadka; Padmakanya Multiple Campus, Bagbazar, Kathmandu, Nepal; E-mail: khadkarama2072@gmail.com

ABSTRACT

Objectives: The study was conducted to determine the intestinal parasitosis among the school children of Kathmandu, Nepal.

Methods: This study was carried out from February to May 2018. A total of 194 stool samples were collected from school going children of age 6 years to 14 years old and processed in Padma Kanya Microbiology Laboratory. The questionnaire accompanying the queries related to the study were filled. The Formal-ether sedimentation technique was used for the detection of parasites.

Result: Among 194 total cases, 12.4% (24/194) children were infected with intestinal parasites where female were highly infected (70.8%) of the age group 9-11 years (58.3%). The most common protozoan and helminthes parasite detected in this study was *E. histolytica* (33.3%, 8/24) and *Taenia* spp. (16.7%, 4/24) respectively. Intestinal parasitic infection was high in non-vegetarian children (83.3%, 20/24) than vegetarian children (16.7%, 4/24). In symptomatic cases, the intestinal parasitic infection (66.7%, 16/24) was found to be higher than asymptomatic cases (33.3%, 8/24). Due to lack of sanitation condition, the parasitic infection was found in higher in public school children (66.7%, 16/24) as compared to private school children (33.3%, 8/24). The children who don't wash hands with soap before meal (87.5%) and not taking anti helminthic drugs (95.8%) were more infected with parasitic infection. Further, children using direct tap water (45.9%) for drinking purpose were highly infected with parasitic infection.

Conclusion: The intestinal parasitic infection among school children was found closely related to their health hygiene, sanitary condition, water consumption and other activities.

Key words: Intestinal parasites, school children, formal-ether sedimentation technique, Kathmandu.

INTRODUCTION

Intestinal Parasitic Infections (IPIs) continue to be a major cause of morbidity in developing countries and are among the most common infections worldwide. Protozoal infections and Soil Transmitted Helminths (STHs) are the predominant causative agents of IPIs. The widespread nature and global impact of these infections is revealed by the fact that infections by STHs have been included as 'Neglected Tropical Diseases' (NTDs) in the initiative taken by WHO (2016). It is estimated that some 3.5 billion people are affected, and that 450 million are ill as a result of the parasitic infections, the majority being children (WHO 2013). The most common cause of parasitic infections in school going children ultimately leading to impaired physical and mental development (Halliez 2013; Sehgal 2010).

Intestinal protozoan infection and helminthic infection rank the third and the fourth respectively in Nepal (Agrawal et al. 2012). The survey that has been carried in Kathmandu valley and northern Kathmandu valley among school children has shown the prevalence ranging from 13.9% (Shakya et al. 2012) to 15% (Pandey et al. 2015) and it reaches peak level during rainy season. So, the capital city is also highly endemic for intestinal helminthes infections due to the poor sanitary conditions and unplanned urbanization (Adhikari et al. 2007). Similarly, a study of intestinal

Date of Submission: September 30, 2018
Date of Acceptance: December 7, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22320

parasitosis conducted among school-going children in Dadeldhura district showed low prevalence of 31.13% (Tiwari et al. 2013) and Rupandehi district showed high prevalence as 60% (Khanal et al. 2016). The Most common intestinal parasites reported from school going children in Nepal are *A. lumbricoides*, *H. nana*, Hookworm, *T. trichiura, Entameba histolytica* and *G. lamblia* (Khanal et al. 2016; Pandey et al. 2015; Shakya et al. 2012; Tiwari et al. 2013). These parasites are associated with diverse clinical manifestations such as malnutrition, iron deficiency anemia, mal absorption syndrome, intestinal obstruction, and mental and physical growth retardation (Gyawali et al. 2009).

Consequently, a resolution for intestinal parasitic infection had been passed by the World Health Assembly in 2001 for control of morbidity due to STHs through mass administration of antihelminthics in school children in developing countries (Pullan 2010). Several studies have dealt with the prevalence and risk factors associated with IPIs and have mostly concluded that the spectrum of IPIs vary according to geographical location and poor hygiene and low socio-economic conditions along with inadequate medical facilities and lack of access to safe drinking water are the common risk factors (Bisht et al. 2011; Wani et al. 2007). Therefore, this study was undertaken to study the distribution of IPIs and related risk component in school going children of Kathmandu valley.

MATERIALS AND METHODS

A total of 194 stool samples were collected from different schools of Kathmandu valley. The population targeted for research purpose was school going children from age groups 6-14 years**.** The time duration for the research work was February to May 2018. Before sample collection, a short and simple introduction classes about parasites were given to the children. The plastic containers were distributed among the children with their name, sample number, date and time of collection labeled to it. Then, they were advised aseptically collect the sample, not to contaminate the stool with water, soil, urine etc. The questionnaire accompanying the queries related to the study were filled there with the children capable of giving answers while others were provided with the questionnaire to be filled by their parents. On the next day, containers containing samples along with remaining questionnaires were collected from children. The samples were brought to the laboratory and macroscopic followed by microscopic observation of the samples were done. Macroscopic Examination of all stool samples were examined macroscopically for presence of blood, mucus, adult worms, segments of tapeworm, and larvae. The consistency of stool as formed, loose or watery or soft with colour were noted. For microscopic observation, concentration technique (Formal-ether sedimentation method) was done and iodine mount was used for slide preparation and observed first under low power (10x) followed by higher power (40x) under microscope. The result was recorded and analysis was done using SPSS (version 21) and chi-square test was used for data analysis.

RESULTS

Distribution of parasites in the stool samples

Among total of 194 stool samples collected from school going children, the prevalence of intestinal parasites was found to be 12.4% (24/194) in which protozoan parasites was found higher (66.7%, 16/24) compared to helminthic parasites (33.3%, 8/24). The most common protozoan parasite and helminthic parasite were *E. histolytica* (33.3%, 8/24) and *Taenia* spp. (16.7 %, 4/8) respectively.

Table 1: Distribution of parasites in the stool samples

Parasites	Total	Percentage
G. lamblia	5	20.9
E. histolytica	8	33.3
B. hominis	2	8.3
B. coli	1	4.2
Total protozoa	16	66.7
A. lumbricoides	2	8.3
Hookworm	2	8.3
Taenia spp.	4	16.7
Total helminths	8	33.3
Total parasites	**24**	**100**

Gender-wise distribution of parasitic infection

A total 194 stool samples were collected from 105 (54.1%) females and 89 (45.9%) males for the detection of intestinal parasites. Among the positive 24 samples, female had higher prevalence rate of parasitic infection (70.8%, 17/24) than male (29.2% 7/24) without statistical significant difference (P> 0.05).

Table 2: Gender-wise distribution of parasitic infection

Gender	Total samples N (%)	Positive cases (N)	Percentage	P-value
Male	89 (45.9)	7	29.2	P> 0.05
Female	105 (54.1)	17	70.8	
Total	**194 (100**	**24**	**100**	

Age-wise distribution of parasitic infection

In this study, parasitic infection was highest among children of age group 9-11 years (58.3%, 14/24) followed by 12-14 years (25.0%, 6/24) and 6-8 years (16.7%, 4/24). It was found that, there was no significant difference between parasitic infection and age group.

Table 3: Age-wise distribution of parasitic infection

Age group	Total samples (N)	Positive cases (N)	Percentage	P-value
6-8	53	4	16.7	
9-11	101	14	58.3	P> 0.05
12-14	40	6	25	
Total	**194**	**24**	**100**	

Parasitic infections in relation to dietary status of patients

Among 24 positive stool samples, parasitic infection was high among non-vegetarian children (83.3%, 20/24) than vegetarian (16.7%, 4/24). There was no significant difference between vegetarian and non-vegetarian diet and parasitic infection.

Table 4: Dietary-wise distribution of parasitic infection

Veg/Non-veg diet	Total samples (N)	Positive cases (N)	Percentage	P-value
Vegetarian	41	4	16.7	P>0.05
Non-vegetarian	153	20	83.3	
Total	194	24	100	

Distribution of parasitic infection with sources of drinking water

In this study, out of 24 positive samples, children using direct tap water had highest prevalence of parasitic infection (45.9%, 11/24) followed by direct jar water (20.8%, 5/24) and other treated water. The source of drinking water was significantly associated with parasitic infection.

Table 5: Distribution of parasitic infection with sources of drinking water

Sources of drinking water	Total samples (N)	Positive cases(N)	Percentage	P-value
Direct Tap water	32	11	45.9	
Boiled Tap water	23	2	8.3	
Direct Jar water	92	5	20.8	P <0.05
Boiled Jar water	25	1	4.2	
Boiled Pump water	11	2	8.3	
Boiled Well water	11	3	12.5	
Total	**194**	**24**	**100**	

Distribution of parasitic infection with type of school

Among 24 intestinal parasites positive samples, parasitic infection was found higher in students studying in public school (66.7%, 16/24) compared with private school (33.3%, 8/24) with no statistical significance difference.

Table 6: Distribution of parasitic infection with type of school

Type of school	Total samples (N)	Positive Cases (N)	Percentage (%)	P-value
Public	100	16	66.7	
Private	94	8	33.3	P>0.05
Total	**194**	**24**	**100**	

Distribution of parasitic infection with hand washing habit with soap

Among the total of 24 intestinal parasites positive samples, parasitic infection was found higher in students who don't wash hands with soap before meal (87.5%, 21/24) than who wash hands before meal (12.5%, 3/24) with no statistical significant difference.

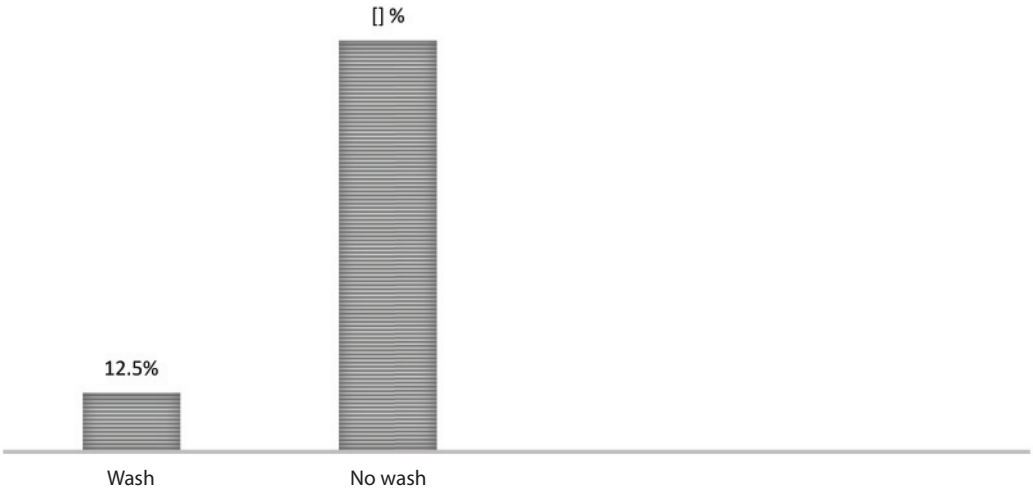


Figure 1: Distribution of parasitic infection with hand washing habit with soap

Distribution of parasitic infection with clinical symptoms

Clinical symptoms of gastroenteritis include: fever, vomiting, nausea. Out of 24 positive samples, parasitic infection was seen highest among symptomatic cases (66.7%, 16/24) than asymptomatic cases (33.3%, 8/24). The significant association of clinical symptoms with parasitic infection was observed.

Table 8: Distribution of parasitic infection with clinical symptoms

Clinical symptoms	Total samples (N)	Positive cases	Percentage (%)	P-value
Symptomatic	22	16	66.7	
Asymptomatic	172	8	33.3	P <0.05
Total	**194**	**24**	**100**	

Distribution of parasitic infection with anti-helminthic drugs

In this study, the parasitic infection was found to be higher in children who didnot taking anti helminthic drugs (95.8%, 23/24) than children taking anti-helminthic drugs (4.2%, 1/24 with no statistical significant difference.

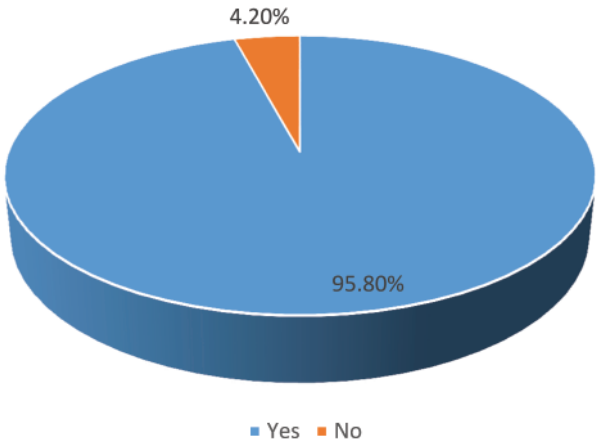


Figure 2: Distribution of parasitic infection with anti-helminthic drugs

DISCUSSION

In this study, the intestinal parasites positive was found to be 12.4 % (24/194) among the school children of Kathmandu valley. The finding was also in agreement with the study done by Shakya et al. (2012) as 13.9%. Similarly, the study done by Pandey et al. (2015) reported 15% of the intestinal parasitosis among school going children in Northern Kathmandu. However, a bit higher prevalence rate has been reported from different parts of Nepal were 17.6% by Khanal et al. (2016); and 16.5% by Tandukar et al. (2015). The higher prevalence rates were reported as 31.7% and 31.13 % (Kidane et al. (2014); Tiwari et al. 2013). Among recent study, the highest prevalence rate of intestional parasites was reported as 60% among school going children of Rupandehi districts (Khanal 2016). Similarly the study done in Durg, Chhattisgar, India reported 31.2 % of intestinal parasites (Chandi and Lakhani 2018). 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 improved hygiene habits of children and also anti-helminthic drugs given to the children.

The number of protozoan parasites was found to be higher (66.7%, 16/24) as compared to helminthic parasites (33.3%, 8/24). Other reports from Nepal have also shown the higher prevalence of protozoan infection than the helminthic infection by Tandukar et al. (2013) as 81.5% and Pradhan et al. (2014) as 73.9%. The most common protozoan and helminth parasite detected in this study was *E. histolytica* (33.3% 8/24) and *Taenia* spp. (16.7%, 4/24) respectively. Similarly, Pandey et al. (2015) reported the most common parasite was *E. histolytica* (33.3%) among protozoan infection but *A. lumbricoides* (20.0%) were the most helminth. In the study done by Chandi and Lakhani (2018) from India, helminthes was dominating by Protozoa which reported *E. histolytica* (38.5%) and *A. lumbricoides* (19.2%) were commonest intestional parasites. However the study done by Tiwari et al. (2013) reported *H. nana* (46.56%) and *G. lamblia* (7.47%) were the most common protozoa and helminth respectively. The study done in Rupandehi, Nepal, Khanal et al. (2016) reported the highest number of parasites were *A. lumbricoides* (105/130). The higher rate of protozoal infection may be due to the presence of farming land contaminated with fecal matter resulted due to open defecation, lack of public awareness and use of contaminated drinking water and resistant to chlorine by the cyst form of the protozoal parasites. Low prevalence of helminths in this study can be attributed to the nationwide bi-annual integrated deworming as well as vitamin A supplementation programme and implementation of mass drug administration in which single dose of albendazole is given.

Among the positive 24 samples, female had higher prevalence rate of parasitic infection (70.8%, 17/24) than male (29.2%, 7/24).The study was in support 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%)). The finding was found to be against some study done by Yadav et al. (2016) (male=61.8%, female 53.8%). However, Khanal et al. (2016) showed the equal prevalence among boys and girls. These differences indicated that the association of gender with parasitic infection differ from one community to another and might to socio-behavioral activities (Khanal et al. 2016).

Intestinal parasitic infection was found to be highest among children of age group 9-11 years (58.3%, 14/24) followed by 12-14 years (25.0%, 6/24) and 6-8 years (16.7%, 4/24) with no statistical significant difference. In the study done by Yadav et al. (2016), the highest number of parasitic infection was seen between age group 6 -10 years (62.8%) followed by age group below 6 years (60.1%) and was found to be statistically significant. The highest number of cases belonged to age group of 11-15 years (42.8%) and 13-15 years (70%) were reported by Tandukar et al. (2013) and Khanal et al. (2016). The higher prevalence among 9-11 years age group might be due to the carelessness of the children towards their personal hygiene and engagement of this age group in different types of games in polluted environment. Most children of this age group are fascinated towards street food and drinks which may be important predisposing factors for high prevalence of parasitic infection in this age group (Khanal et al. 2016).

According to types of food consumption, the intestinal parasitic infection was high among non-vegetarian children (83.3%, 20/24) than vegetarian (16.7%, 4/24) with no statistical significant difference. Further, *Taenia* spp. was found to be higher among the helminths in non-vegetarian group. A study conducted by Sah et al. (2013) among the school children of Itahari, Nepal reported the intestinal protozoan parasites was found significantly higher among vegetarian group (36%) than non-vegetarian group (16%) with was contrast to the present study. The higher prevalence of parasitic infection in non-vegetarian might be due to properly unwashed and uncooked meat and meat products whereas the consumption of unwashed fruits and vegetables appeared to be the source of infection for vegetarian.

The highest infection rate of intestinal parasites was reported in children using direct tap water (45.9%, 11/24) followed by using of direct jar water (20.8%, 5/24). Sources of drinking water was significantly associated with parasitic infection. The finding was in agreement with the study done by Tandukar et al. (2013; 2015) where the rate of infection was significantly higher (29.4%) in children using unboiled (direct tap) water for drinking purpose. The higher infection in direct tap water cases might be due to the sewage contamination in the pipelines of the tap due to the leakage in pipelines with contain parasites leading to infection. Further, water and sewage pipelines in Kathmandu valley are placed parallel which creates high risk on contamination of sewage to drinking water pipelines ((Adhakari et al. 2007) Jar water being pre-treated helps in preventing the transmission of the infection. However, improper treatment of jar water may lead to entry of parasites in the body.

The sanitary condition and surrounding environment of school play an important role for parasitic infection among student. The public school (66.7%, 16/24) showed higher intestinal parasitic infection compared with private school (33.3%, 8/24) with no statistical significance difference. In the study done by Tandukar et al. (2013), intestinal parasites were found more common in the children of public school (73.3%) and lower in private school (7.7%) and moderate in community school (19.0%). The higher positive rate among public school children might be due to low socio-economic status, poor hygienic habits and lack of sanitation prevailing in the school.

The personal hygiene practices are important for protection from many diseases. The parasitic infection was found higher in students who don't wash hands with soap before meal (87.5%, 21/24) than who wash hands before meal (12.5%, 3/24) with no statistical significant difference. Similar findings was obtained from the study done by Tandukar et al. (2013), the intestinal parasitic infection was found higher in those children who did not follow hand washing practice (47.5%). Children playing in outdoor environment get in contact with parasites and not washing hands before meal leads to the entry of parasites in the body.

The intestinal parasitic infection was seen highest in symptomatic cases (66.7%, 16/24) than asymptomatic cases (33.3%, 8/24). The significant association of clinical symptoms with parasitic infection was observed. This finding was in agreement with the study done by Yadav et al. (2016) which revealed that the occurrence

of parasitic infection in symptomatic children was 98.16% and in asymptomatic children was 47.98% and found statistically significant. This may be due to the symptoms such as nausea, abdominal discomfort, diarrhea and fever are the prime indicator of parasitic infestation. For the treatment of helminthic infection, anti-helminthic drug is used. So, in this study, the parasitic infection in children in relation of taking anti helminthic drugs within six months was studied and found more infection in children not taking anti helminthic drugs (95.8%, 23/24) than children taking anti-helminthic drugs (4.2%, 1/24) with no statistical significant difference. This showed the importance of periodic administration of anti-helminthic drug used by children is effective.

CONCLUSION

Intestinal parasitic infection is still major public health problem. Although, the study showed the decreasing trend of helminthes but protozoan still remain severe infection causing intestinal parasites. The parasitic infection among school children was found closely related to their health hygiene, sanitary condition, water consumption and other activities. So, it is necessary to develop the effective prevention and control strategies of parasitic infections in different populations.

ACKNOWLEDGEMENTS

We are thankful to Dr. Sabina Singh Shrestha, Assistant Campus Chief of Science and Mr. Achyut Ram Pradhananga, B .Sc programme co-ordinator of Padma Kanya Multiple Campus, Bagbazar, for providing the platform to conduct this project work. We would like to express our deepest regards and gratitude to Mr. Ramkrishna Ranjitkar and other lab staffs for providing us equipment's during the study. We are thankful to all the school children and their parents/guardians who participated in this study.

REFERENCES

Adhikari N, Bomjhan R, Khatri DB, Joshi DR, Dhakal P and Lekhak B (2007). Intestinal helminthic infections among school children in Kathmandu valley. *Journal of Nepal Health Research Council* **5** (1): 17-21.

Agrawal PK, Rai SK, Khanal Lk, Ghimire G, Banjara MR and Singh A (2012). Intestinal parasitic infections among patients attending Nepal Medical College Teaching Hospital, Kathmandu, *Nepal Med Coll J* **14**:80-83.

Bisht D, Verma AK and Bharadwaj HD (2011). Intestinal parasitic infestation among children in a semi-urban Indian population. *Trop Parasitol* **1**:104-107.

Chandi DH and Lakhani SJ (2018). Prevalence of Parasitic Infections among School Children in Bhaili, Durg Chhattisgarh, India. *Int J Curr Microbiol App Sci* **7**(9): 1919-1925

Gyawali N, Amatya R and Nepal HP (2009). Intestinal parasitosis in school going children of Dharan municipality, Nepal. *Trop Gastroenterol* **30**: 145-147.

Halliez MCM and Buret AG (2013). Extra-intestinal and long term consequences of *Giardia duodenalis* infections. *World J Gastroenterol* **19** (47):8974-8985.

Khanal LK, Upadhaya S, Lamichane P, Karn S, Pokhrel S and Pahwa (2016). Enteric parasitic infections among school children at Rupandehi. *Journal of Universal College of Medical Sciences Nepal* **4** (14): 31-34.

Kidane E, Kebede MS and Desta M (2014). Prevalence of intestinal parasitic infections and their associations with anthropometric measurements of school children in selected primary schools, Wukro Town, Eastern Tigray, Ethiopia. *Int J Curr Microbiol App Sci* **3** (3): 11-29.

Pandey S, Lama A and Shrestha RB (2015). Intestinal parasitic infection among school children of northern Kathmandu. *Asian Pac J Dis* **5**: 89-92.

Pullan RL, Smith JL, Jasrasaria R and Brooker SJ (2014). Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. *Parasites & vectors* **7**(37):01-09.

Sah RB, Paudel IS, Baral R, Poudel P, Jha N and Pokharel PK (2013). A study of prevalence of intestinal protozoan infections and associated risk factors among the school children of itahari, eastern region of Nepal. *Journal of Chitwan Medical College* 3(**3**): 32-36.

Sehgal R, Reddy GV, Verweij JJ and Rao AVS (2010). Prevalence of intestinal [5] parasitic infections among school children and pregnant women in a low socio-economic area, Chandigarh, North India. *Reviews in Infection* **1**(2):100-103.

Shakya B, Shrestha S, Madhikarmi NL and Adhikari R

(2012). Intestinal parasitic infection among school children. *J Nepal Health Res Counc* **10** (20):193-196.

Tandukar S, Ansari S, Adhikari N, Shrestha A, Gautam J, Sharma B, Raj Bhandari D, Gautam S, Nepal HP and Sherchand JB (2013). Intestinal parasitosis in school children of Lalitpur district of Nepal. *BMC Research Notes* **6**:449-451.

Tandukar S, Sherchan JB, Thapa P, Malla D and Bhandari D (2015). Intestinal Parasite Infection among School Going Children in Kathmandu Valley. *Austin J Pediatr* **2** (2): 1022.

Tiwari BR, Chadhury R, Adhikari N, Jayaswal SK, Poudel TP and Rijal K (2013). Prevelence of intestinal parasitic infections among school children of Dadeldhura District. *JHAS* 3: 14-16.

Wani SA, Ahmad F, Zargar SA, Ahmad Z, Ahmad P and Tak H (2007). Prevalence of intestinal parasites and associated risk factors among school children in Srinagar city, Kashmir, India. *J Parasitol* **93**:1541-1543.

WHO (2013). Health report, intestinal Parasites: Burdens and Trends. Available from: https://apps.who.int/ctd/intpara/burdens.htm.

WHO (2016). Health report, disease covered by NTD Department. Available from: http://www.who.int/neglected_diseases/diseases.htm.

Wilson ME (1998). Giardiasis In Public Health & Preventive Medicine. 14th ed. Pp: 10.

Yadav K and Prakash S (2016). Study of Intestinal Parasitosis among School Children of Kathmandu Valley, Nepal. *Asian Journal of Biomedical and Pharmaceutical Science* **6**:40-47.

Bacteriological Quality Analysis of Milk Available in Local Market of Janakpurdham, Nepal

Nagendra Prasad Yadav[1]*, Rakesh Kumar Yadav[2], Bishal Pokharel[3]

[1]Department of Microbiology, Model Multiple College, Janakpur

[2]Department of Pharmacology, Janaki Medical College, Janakpur

[3]Department of Community Medicine, Janaki Medical College, Janakpur

***Corresponding author**: Nagendra Prasad Yadav, Department of Microbiology, Model Multiple College, Janakpur, Nepal; E-mail: nagendrayadav2073@gmail.com

ABSTRACT

Objectives: To determine the microbial quality of milk available in the market of Janakpurdham, Nepal.

Methods: Total 20 samples of milk were collected from the market and processed for MBRT test as per standard protocol. Reduction time test for each sample of the milk was recorded in a specified format and analysed statistically.

Results: Out of 20 samples, 2 (10%) samples were found of excellent quality, 3 (15%) were of good quality, 6 (30%) were of fair quality and 9 (45%) were of poor quality. Unprocessed milk was found to be highly contaminated in comparison to the processed milk.

Conclusion: Unprocessed milk was found to be highly contaminated and not fit for the human consumption.

Key words: Milk, MBRT, microbial quality

INTRODUCTION

Milk may be defined in various ways. Chemically speaking, milk is a complex fluid in which more than 100 separate chemical compounds have been found. Its major components are water, fat, lactose, casein, whey proteins, and minerals (or ash) in amounts varying with the milk of various species of animals (JP et al. 1994). However, for any given species, the range of values for the constituents of milk is fairly constant. From a physiological standpoint, milk is the secretion of the normally functioning mammary gland of the females of all mammals, which is produced for some time following parturition for the nourishment of the young of the species during the initial period of growth. In terms of physical chemistry, milk is an opaque, whitish fluid of multi disperse phases. The true solution contains lactose, vitamins, acids, enzymes, and some inorganic salts. The colloidal phase contains casein, calcium phosphate, and globular proteins. Fat exists in the form of an oil-in-water type of emulsion, with fat globules varying from 0.1 to 22 µm in diameter (Wong et al. 1988).

As a food ingredient or consumed by itself, milk provides an excellent nutritional profile in the human diet. Nutrition experts consider milk an exceptionally complete food because it contains significant levels of required nutrients such as protein, fat, carbohydrates, minerals, and several vitamins. Low-fat and no fat milks are increasingly popular in fat-reduced and fat-free food formulations (FAO 1993). Worldwide, milk of the cow is by far of more commercial importance than milk of any other mammal. In the United States, the term "milk" legally refers to cow's milk. Milk from other species is labelled to indicate the type: sheep's milk, goat's milk, etc. Milk is the whole, clean lacteal secretion of one or more healthy cows properly fed and kept, excluding that obtained within 15 days before calving and three to five days after. Colostrum,

Date of Submission: August 8, 2018
Date of Acceptance: December 10, 2018
Published Online: January 2019
DOI: https://doi.org/10.3126/tujm.v5i0.22321

the milk secreted immediately after giving birth, is not considered milk from a legal standpoint. The U.S. Public Health Service's definition of Grade A milk is "the lacteal secretion practically free from colostrum, obtained by complete milking of one or more healthy cows, which contains not less than 8.25% milk solids-not-fat (MSNF) and not less than 3.25% milk fat (Singh et al. 1997; Harper et al. 1996).

Milk is a good medium for the growth of microorganisms. These active growing microorganisms reduce the oxidation reduction potential of the milk medium due to the exhausted oxygen by the microorganism. Normally the milk is contaminated with organisms such as *Staphylococcusaureus, Streptococcuspyogenes, Pseudomonas* etc. Contaminated milk is one of the important sources for transmission of diseases from animals to humans. The main reason for this contamination is the improper handling of milk. Normally milk is contaminated during the milking process by the microorganisms present in the exterior surface of the animals, pipelines such as udder and adjacent areas. Unsterilized dairy utensils such as milking machines, milk cans are also a good source of contamination by the microorganisms (Walstra and Jenness 1993). The formation of methylene blue reductase is thus becoming a popular tool for determining the quality of the milk. The principle of methylene blue reduction test depends on the fact that the colour imparted to the milk by adding a dye such as methylene blue will disappear more or less quickly which depends on the quality of the milk sample to be examined. Methylene blue is a redox indicator that loses its colour under the absence of oxygen and is thought to be reduced. The depletion of oxygen in the milk is due to the production of reducing substance in the milk due to the enhanced rate of bacterial metabolism. The dye reduction time refers to the microbial load in the milk and the total metabolic reaction of the microorganism (Aurand and Woods 1984).

MATERIALS AND METHODS

The samples of milk were collected from different areas of Janakpur market. Sample collections were lasted from May to June, 2017. Sampling was performed according to standard protocol for the examination of milk. All milk samples were collected from 3 different sources comprising of 5 cows milk, 6 buffalo milk and 9 DDC milk. Total 20 samples of milk were collected aseptically and processed for MBRT test as per standard protocol. Reduction time test for each sample of the milk was recorded in a specified format and analysed statistically.

RESULTS

A total of 20 samples were examined physically and then proceed for the MBRT test. Out of 20 samples, 2 (10%) samples were found excellent quality, 3 (15%) were of good quality, 6 (30%) were of fair quality and 9 (45%) were of poor quality. Among these 20 samples, 6 samples were of processed milk, 5 samples were of unprocessed/ raw milk and 9 samples were of Diary Development Corporation milk.

Table 1: MBRT table of processed milk

Samples	Reduction time (hrs)	Quality
Cow milk	3.5	Good
Cow milk	6.0	Excellent
Buffalo milk	4.0	Good
Cow milk	2.5	Fair
Buffalo milk	0.5	Poor
Buffalo milk	1.5	Fair

Table 2: MBRT table of unprocessed milk

Sample	Reduction time (Hrs)	Quality
Cow milk	2.0	Fair
Buffalo milk	0.75	Poor
Buffalo milk	1.5	Fair
Buffalo milk	0.5	Poor
Cow milk	½ hrs	Poor

Table 3: MBRT of DDC milk

Sample	Reduction Time (hrs)	Quality
DDC milk	3.5	Good
DDC milk	8.0	Excellent
DDC milk	1.5	Fair
DDC milk	2 .0	Fair
DDC milk	2.5	Fair
DDC milk	1.5	Fair
DDC milk	2.5	Fair
DDC milk	2	Fair
DDC milk	2	Fair

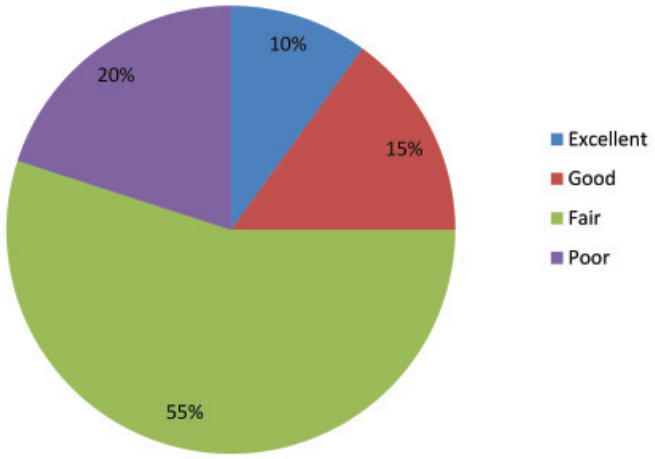


Figure 1: Percentage of quality of milk available in different areas of Janakpur market

DISCUSSION

Milk is the important source of protein. It help us to develop the brain sharpens our memory, make us powerful. Though nowadays people, for their selfish motive of earning money they mix harmful chemicals in the milk just to increase its qualitquantity. Thus milk quality is lowered and human immune system is also lowered and are suffering from many diseases (NDC 1993). The methylene blue reduction test is based on the fact that the color imparted to milk by the addition of a dye such as methylene blue will disappear more or less quickly. The removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism cause the color to disappear. Oxygen is consumed by the bacteria the greater the number of bacteria in milk, the quicker will the oxygen be consumed, & in turn the sooner will the color disappear. Thus the time of reduction is taken as a measure of the number of organism in milk (Aurand and Woods 1984).

In this study, 20 milk samples from different local areas of janakpur tested. Out of 20 samples 2(10%) were of excellent, 3 (15%) were good, 6 (30%) were fair and 9 (45%) were of poor quality. Among these 20 samples, 6 samples were of processed milk, 5 samples were of unprocessed/raw milk and 9 samples were of DDC milk.

CONCLUSION

The quality of milk was found very poor in raw milk. The quality of DDC milk i.e. pasteurized was good and it is suitable for human consumption. Milk samples which were not processed found contaminated. Thus to prevent from the milk borne diseases pasteurized milk should be consumed.

ACKNOWLEDGEMENTS

The authors are thankful to Model Multiple College for providing laboratory facilities. Thanks are due to the farmers for providing milk sample for this study.

REFERENCES

Abele CA and Ahuja CH (1945). The methylene blue reduction test as a measure of estimating the bacterial content of milk to determine its suitability for pasteurization or as a basic of grading. J *Milk and Food Tech*.

Aurand LW and Woods AE (1959). Role of xanthine oxidase in the development of spontaneously oxidized flavor in milk. *J Dairy Sci* **4:** 111.

BS. 1741. part 11, pearson's composition and analysis of foods 9th edn 1991, page 549.

FAO (2003). Manual of food quality control, 14/8, page 8/15. 1479 (part 1)1961 (Reaffirmed 2003) methods of test for dairy industry-Rapid examination of milk. Bureau of Indian standard, New Delhi.

Harper WJ and Hall CW (1976). Dairy technology and engineering. AVI publishing CO west port CT.

IS (1166-1986) (1991). Specification for condensed milk –modified lane and eynon volumetric method. Bureau of Indian standard, New Delhi, Pearson's composition and analysis of foods, 9th Edn.

IS 1479-1961 (2003). Methods of test for dairy industry– Rapid Examination of milk. Bureau of Indian standard, New Delhi.

IS 1479-1961 (1997). Methods of test for dairy industry– chemical analysis of milk. Bureau of Indian standard, New Delhi.

ICMR (1990). Method of analysis for adulterants and contamints in foods, page 28.

Roy NK and Sen DC (1991). Rapid analysis of milk. In Textbook of practical dairy chemistry vol1. Chemical analysis of fluid milk, Kalyani publisher, New Delhi, India.

National Dairy Council (1993). Knowledge of milk and other fluid dairy products. The Council, Rosemont I.

Singh H, Mccarthy OJ, and Luccey JA (1997). Physicochemical properties of milk. In advantage, vol3. PF Fox, Ed. Chapman and Hall .

Varnum AH and Sutherland JP (1994). Milk and milk products champman and Hall New York.

Walstra P and Jenness R (1984). Dairy chemistry and physics. John Wiley New York.

Wong NP, Jenness R, Keeney M and Marth EH (1988). Fundamentals of dairy chemistry, 3rded. Van Nostrand Reinhold, New York.

Tribhuvan University Journal of Microbiology

VOL. 5, NO. 1 2018 ISSN: 2382-5499 (Print), ISSN 2661-6076 (Online)

Corresponding address:
Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu
Phone : 00977-1-4331869
E-mail : cdm1990@microbiotu.edu.np
URL: www.microbiotu.edu.np

ISSN 2661-6076 (ONLINE)
9 772661 607003

ISSN 2382-5499 (PRINT)
9 772382 549002