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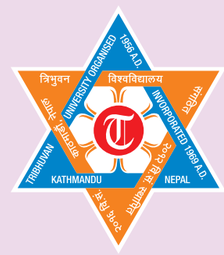

Central Department of Microbiology

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INTRODUCTION

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

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

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THE EDITORIAL PROCESS

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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.

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

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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: The conclusion should be based on results.

(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.

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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.

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

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.

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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'.

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

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).

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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.

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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.

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

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

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.

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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)

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

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

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EDITORIAL

Microorganisms for Improved Crop Production and Better Human Health in Nepal

In 2018, Nepal's 27.6% gross domestic product is contributed by agriculture. The agricultural practices in the country are traditional and productivity is not an optimum level as expected. Nepal imports agricultural products of about Rs. 80 billions from the other countries. Current agricultural practices in Nepal depend on chemical fertilizers and pesticides. These chemicals have deleterious effects on nutritional value of crops and farmers and to consumers. Further, these chemicals in agriculture have resulted adverse effect on ecology, environmental contamination, accumulation of these toxic compounds in soil. These have led a demand for technologies of improving both the quantity and quality of agricultural products. To meet the demand of the growing population, the productivity need to be increased significantly. There are very limited industries in Nepal producing biopesticides, microbial biofertilizers that can be used for increased agricultural production. Biofertilizers, biopesticides, and biocontrol agents are the alternatives to chemical agents. Microorganisms applied to soil or plant improve the productivity. These microorganisms are natural and widely used to control pests and protect human health. Microbial inoculants such as bacteria, fungi, virus and algae which are environment-friendly and can be used in sustainable manner. Therefore, farmers should be made aware of these microbial technologies and academic sector should be involved to develop these agents. Government sector, academia and industry collaboration in public private partnership model could help to move these activities further.

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

Screening of Potent Arsenic Resistant and Plant Growth Promoting *Bacillus* species from the Soil of Terai Region of Nepal

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ABSTRACT

Objectives: To isolate arsenic resistant *Bacillus* spp. and to determine plant growth promoting activities.

Methods: Eighteen soil samples were collected from the agricultural soil of Terai region of Nepal. Selective isolation of *Bacillus* species was done by heating the soil at 80 ^{o}C for 15 minutes before the isolation. Nutrient agar was used as an isolation medium. Screening of arsenic resistant *Bacillus* species was done using nutrient agar supplemented with 100 ppm sodium arsenate and sodium arsenite. For plant growth promoting activity; IAA production was detected taking 0.1% tryptophane and measuring absorbance at 540 nm, NH_3 production was tested by Nessler's reagent and phosphate solubilization activity was detected by growing colonies on Pikovskaya's agar. Sugar assimilation test was performed to identify the isolates. Most potent arsenic resistant isolate was identified by 16S rRNA gene sequencing.

Results: Among 54 randomly selected isolates, 42 were found to be Gram-positive rod-shaped, spore-forming while 12 isolates were Gram-negative bacteria. The isolates $IN_1$2a, $M_1$2a and $BG_3$4a showed growth on 100 ppm sodium arsenite containing NA. Only isolate $M_1$2a tolerated up to 1000 ppm and 15000 ppm of sodium arsenite and sodium arsenate respectively, while other isolates could not grow above 400 ppm sodium arsenite. The isolates $IN_1$2a and $M_1$2a were able to produce IAA and solubilize phosphate while $BG_3$4a could not. Both the isolates $IN_1$2a and $M_1$2a were able to utilize the sugars glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and xylose. Based on the 16S rRNA gene sequencing, isolate $M_1$2a was identified to be *Bacillus flexus* with highest similarity of 99.2%.

Conclusion: Arsenic resistant and plant growth promoting *Bacillus* spp. was isolated from the agricultural soil of Terai region of Nepal.

Key words: Soil, Arsenic resistant *Bacillus*, Plant growth promotion, Bioremediation

INTRODUCTION

Arsenic (As) is a poisonous heavy metalloid present in the soil as well as in the water as arsine (III), elemental arsenic (0), arsenite (III) and arsenate (V). It has been reported that, arsenite and arsenate forms are poisonous to environment as well as human health (Dey et al. 2016). The activity that leads to As pollution are mining, smelting, ore processing, and utilization of arsenic-based pesticides or herbicides, and arsenic contaminated water when irrigated posed heavy contamination of soil, especially in cultivated land ecosystem (Shagol et al. 2014). Arsenic consumption beyond a threshold level i.e. 0.05 ppm pose a serious health risk to humans (Dey et al. 2016). Exposure

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of arsenic to body parts cause cancer. nervous and cardiovascular problems (Ghosh et al. 2011), weight loss, loss of appetite, weakness, lethargy and easily fatigued limits the physical activities and working capacities, chronic respiratory disorder, gastrointestinal disorders like anorexia, nausea, pain in abdomen, enlarged liver and spleen as well as anemia (Dey et al. 2016).

In Nepal, safe drinking water supply is one of the major issues. Groundwater is the foremost source of drinking water in Terai region of Nepal which is usually contaminated with arsenic (Shakya, et al. 2012). Such groundwater when irrigated in crop land increases the concentration of As in crop fields and soil get contaminated. It leads to severe threats for bio amplification by entering into the food chain (Mallick et al. 2014). According to Nepal standard and World Health Organization (WHO) the concentration of arsenic in drinking water are 50µg/L and 10µg/L respectively. Nawalparasi district is placed as a prone to the Arsenic problem where the arsenic concentration is significantly beyond the safe limit (Smith et al. 2009).

Moreover, the presence of Arsenic and its forms in the environment has developed a many bacteria Arsenic resistance mechanisms like arsenite methylation, arsenite oxidation, etc. (Mallick et al. 2014). Currently the detoxification of arsenic by using bacteria has become an interest due to environmental issues immersed by other conventional chemical processes (Banerjee et al. 2013). Bacteria play a significant role in the biochemical cycle of arsenic and changes to different oxidation states with different solubility, mobility and toxicity (Banerjee et al. 2013). The important enzyme i.e. arsenic oxidase is present in the protoplasm of arsenic oxidizing bacteria which oxidizes arsenite to arsenate (Dey et al. 2016). Some bacteria such as *Bacillus subtilis, Deinococcus indicus*, *Pseudomonas fluorescens, Thermus aquaticus, Thermus thermophilus, Yersinia enterocolitica, Bacillus arsenicus* have efficiently removed soluble and particulate forms of metals, especially from dilute solutions via bioaccumulation (Pepi et al. 2011).

Bacillus species are becoming interest due to its role in wide variety of fields such as bioremediation, enzyme production, plant-growth-promoting (PGP) traits, organic acid production etc. (Poudel et al. 2016). Till now, there are limited research findings on Arsenic resistant and plant growth promoting Bacillus species regardless of their tremendous applications in Nepal. The main propose of this study is to explore the arsenic resistant *Bacillus* species having plant growth promoting traits so as to minimize Arsenic pollution and increase the yield of crops. Furthermore, potent *Bacillus* strains could be applicable as a bio fertilizer for sustainable bioremediation in agriculture.

MATERIALS AND METHODS

Sample collection, isolation and screening of arsenic resistant bacterial species

Twenty soil samples were collected from Terai region of Nepal. About 20 g of soil samples was collected in a polythene bag and processed for isolation. For the selective growth of spore forming Bacillus species, 10 g of soil sample was mixed with 100 mL of 0.85% saline solution and placed in 80°C water bath for 10 minutes (Travers et al. 1987). It was serially diluted and spread on to nutrient agar plates. All the plates were incubated at 37°C for 48 h. Bacterial colonies were sub-cultured onto Nutrient Agar plates supplemented with different concentration of sodium arsenite (up to 800 ppm) and incubated. The media plates, after incubation, were observed for growth of bacteria colonies. The colonies that showed growth were sub-cultured on nutrient media and incubated. After incubation, these colonies were subjected to further tests and studies (Selvi et al. 2014). Silver nitrate test was performed in NA plates supplemented with sodium arsenite. A single line streak (perpendicular) of the screened organism was drawn on the agar surface and incubated at 37˚C for 48 h. After incubation, the plates were flooded with 0.1 M silver nitrate solution and observed for brownish precipitate (Simeonova et al. 2004).

Phenotypic characterization of arsenic-resistant bacteria

The bacterial isolates that tolerated arsenate and arsenite concentration were selected and characterized by the morphological and biochemical features (Indole production, MR-VP test, Citrate utilization, Oxidase test, Catalase test, Starch hydrolysis, Gelatin hydrolysis, Triple sugar iron test, Mannitol salt agar, Urea hydrolysis test, Dextrose, Sucrose, Maltose, Rhamnose, Arabinose and Sorbitol tests).

Effect of arsenic on bacterial growth

Growth of arsenic resistant bacterial strains was determined in NB medium. From an overnight pure culture, 1% inoculum was added to 50 ml of NB medium supplemented with 200 ppm, 400 ppm, 600

ppm, 800ppm, 1000 ppm sodium arsenite. The cultures were incubated at 37 °C in an orbital shaker at 120 rpm for 72 h. The growth of the isolate was monitored by measuring optical density at OD 600 nm using spectrophotometric method.

Effect of NaCl and pH in the growth of arsenic resistant isolates

The freshly prepared culture was inoculated into the 5 mL of sterile NB and incubated at 37°C for 24 h. The optical density of the culture broth was measured at 600 nm using spectrophotometric method. The pH of the broth was maintained by using 1N NaOH and 1N HCl. The NaCl concentration in the broth ranged from 1-8%.

Determination of plant growth promoting (PGP) activities

The Arsenic resistant *Bacillus* species were tested for PGP activities based on whether the isolates is capable to solubilize phosphates, produce indole acetic acid, siderophores and NH_3. Phosphate solubilization activity was examined by growing isolates in modified Pikovskaya's medium with 0.5% of tricalcium phosphate (TCP) and incubated at 30°C for 5 days. Quantitative analysis of IAA was performed using the method of Loper et al. (1985) at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 mg/ml). Isolates were grown for 48 h on their respective media at 37°C. Fully-grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of per chloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink color indicated IAA production. For the ammonia production, freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 28°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color indicated a positive test for ammonia production.

Molecular identification of potent arsenic resistant *Bacillus* species

Genomic DNA was extracted by using phenol-chloroform assay method. DNA Amplification of the 16S rRNA gene was performed using the following universal primer sets: 8f (5′ AGA GTT TGA TCC CTC AG 3′) and 1492r (5′ GGT TAC CTT GTT ACG ACTT 3′). The amplification conditions were as follows: 30 cycles of DNA denaturation at 98°C for 10 s, primer annealing at 55°C for 5 s, and elongation at 72°C for 1 min. Polymerase chain reaction products was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. Sequence homology was compared with 16S rRNA gene sequences available in the DDBJ/EMBL/GenBank DNA database using the FASTA algorithm (http://www.ddbj.nig.ac.jp/), and all reference sequences was obtained through the Ribosomal Database Project II (http://rdp.cme.msu.edu/). Sequences were aligned using CLUSTAL W ver.2.01 (http://clustalw.ddbj.nig.ac.jp/) and phylogenetic tree was constructed using MEGA ver.7 by neighbor-joining method with bootstrap values calculated from 1,000 replications.

RESULTS

In total, 54 colonies were randomly selected and sub-cultured on NA media. Among this, 42 isolates were Gram positive rods and 12 isolates were Gram negative. Twelve isolates were excluded for further studies. Forty-two isolates showed moist, flat, irregular, and slightly convex colonies on Nutrient agar and were endospore forming rods (Table 1). Among these, 6 isolates had terminal spores, 14 had sub-terminal, and central spore was seen in 22 isolates. *Bacillus* spp. were isolated from soil having pH ranging from 5.2-6.3.

Table 1: Position of endospores in bacterial isolates

Location	pH of soil	Total no. of isolates	Endospore position		
			Terminal	Sub-terminal	Central
Sarlahi	5.6	3	1	2	-
Biratnagar	5.9	4	1	3	-
Saptari	6.1	9	-	1	8
Palpa	6.3	11	2	5	4
Parsa	5.3	2	1	-	1
Bara	5.8	2	-	-	2
Yangjakot	5.7	2	-	1	1
Birgunj	5.2	5	1	-	4
Nawalparasi	6.0	4	-	2	2

In total, only three isolates were able to resist 100 ppm and 1500 ppm of sodium arsenite and sodium arsenate respectively. These isolates were subjected to biochemical tests (Table 2).

Table 2: Biochemical tests of bacterial isolates

Test	Isolate		
	$IN_1$2a	$M_1$2a	$BG_3$4a
Catalase	Negative	Negative	Negative
Oxidase	Positive	Positive	Positive
O/F	Fermentative	Fermentative	Fermentative
Indole	Negative	Negative	Negative
MR	Positive	Positive	Positive
VP	Negative	Negative	Negative
Citrate	Negative	Negative	Negative
Urease	Negative	Negative	Negative
TSIA	Acid/Acid	Acid/Acid	Acid/Acid
Motility	Motile	Motile	Motile
H_2S	Negative	Negative	Negative

Two isolates $M_1$2a and $IN_1$2a were able to oxidize arsenite to arsenate whereas isolate $BG_3$4a was not able to do so (Figure 1). So, BG34a was not tested for PGP activities.

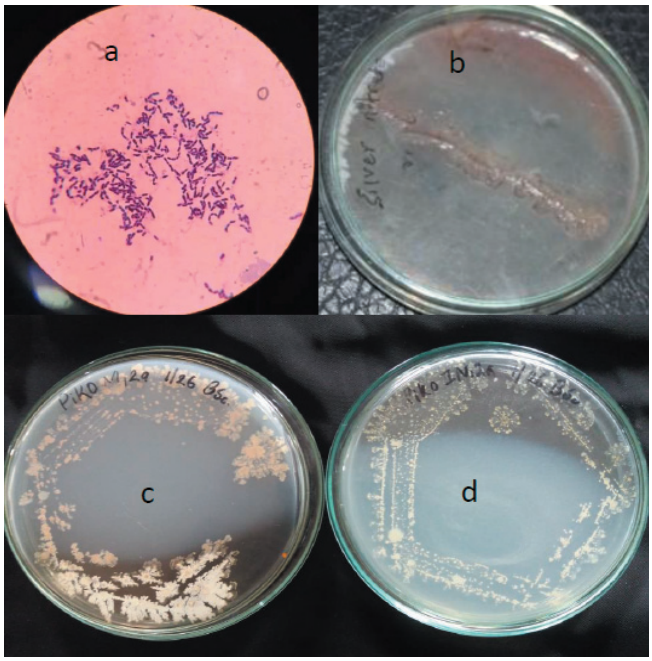

Figure 1: Typical features of Isolates; Gram stain of isolate $M_1$2a (a), Detoxifiction of arsenite to arsenate by isolate $M_1$2a (b), Phosphate solubilization activity of isolates $M_1$2a (c) and $IN_1$2a (d).

Table 3 describes the IAA production by isolates $IN_1$2a and $M_1$2a. Maximum absorbance of 0.105 was observed in $IN_1$2a culture broth containing tryptophan at 0.05 g/L and the lowest value of -0.015 was seen at concentration of 0.22 g/L. In Isolate $M_1$2a, absorbance of 0.423 was observed at 0.5 g/L of tryptophan and lowest of -0.02 at 0.25 g/L of tryptophan.

Table 3: IAA production ability of the isolates after 24 h of incubation at 37°C

Organism	Concentration of tryptophan (g/L)	Absorbance
$IN_1$2a	0.05	0.105
	0.12	0.047
	0.18	0.03
	0.22	-0.015
	0.25	-0.014
$M_1$2a	0.05	0.423
	0.12	0.190
	0.18	-0.007
	0.22	-0.012
	0.25	-0.02

Tested isolates ($IN_1$2a and $M_1$2a) could not produce ammonia after addition of reagent. Both the Isolates $M_1$2a and $IN_1$2a were able solubilize phosphate in Pikovskaya's agar (Figure 1).

Isolates $IN_1$2a and $M_1$2a were able to ferment sugars like glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and xylose. Based on the sugar assimilation pattern, test isolates could be *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. brevis*, *B. stearothermophilus.*

Table 4: Sugars assimilation pattern of isolates

Organism	Sugars	Result	Possible organisms
$IN_1$2a	Glucose	Positive	*Bacillus subtilis*
	Fructose	Positive	*Bacillus licheniformis*
	Lactose	Positive	*Bacillus pumilus*
	Sucrose	Positive	*Bacillus brevis*
	Galactose	Positive	*Bacillus stearothermophilus*
	Mannose	Positive	
	Mannitol	Positive	
	Maltose	Positive	
	Xylose	Positive	
$M_1$2a	Glucose	Positive	*Bacillus subtilis*
	Fructose	Positive	*Bacillus licheniformis*
	Lactose	Positive	*Bacillus pumilus*
	Sucrose	Positive	*Bacillus brevis*
	Galactose	Positive	*Bacillus stearothermophilus*
	Mannose	Positive	
	Mannitol	Positive	
	Maltose	Positive	
	Xylose	Positive	

As describe in Table 5, only isolate M12a was able to tolerate the sodium arsenite up to 1500 ppm.

Table 5: Growth of isolates on different concerntration of sodium arsenite containing NA media after incubation at 37 °C, 48 hours

Isolates	Concentration of Sodium arsenite (ppm)	Absorbance (λ=610 nm)
$M_1$2a	**0**	**1.723**
	200	1.649
	400	1.140
	600	1.323
	800	0.923
	1000	0.351
	1500	0.124
$IN_1$2a	0	1.213
	200	1.132
	400	0.762
	600	0.000

16Sr RNA gene sequence analysis result indicated that the isolate $M_1$2a showed 99.2% similarity with *Bacillus flexus*. The phylogenetic analysis clearly showed the isolate grouped to *Bacillus* species (Figure 2).

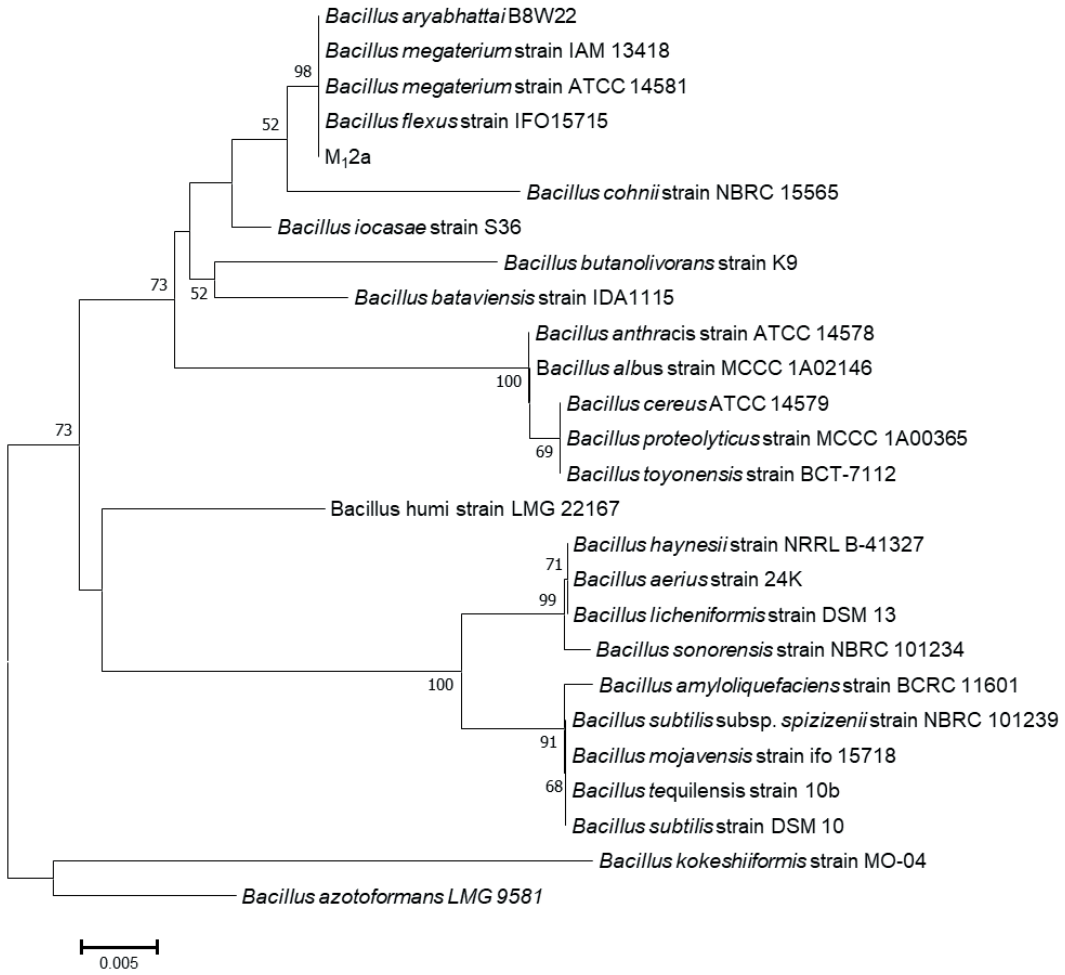

Figure 2: Neighbor-joining phylogenetic tree of isolate $M_1$2a and other closely related reference strains based on 16S rRNA gene sequences.

The growth pH of the isolate $M_1$2a and $IN_1$2a ranged from 5.0 - 9.0. The optimum pH for the growth of isolate $M_1$2a and $IN_1$2a was 7.0. Similarly, the optimum NaCl concentration for the growth of isolate $M_1$2a and $IN_1$2a was 2% (Figure 3).

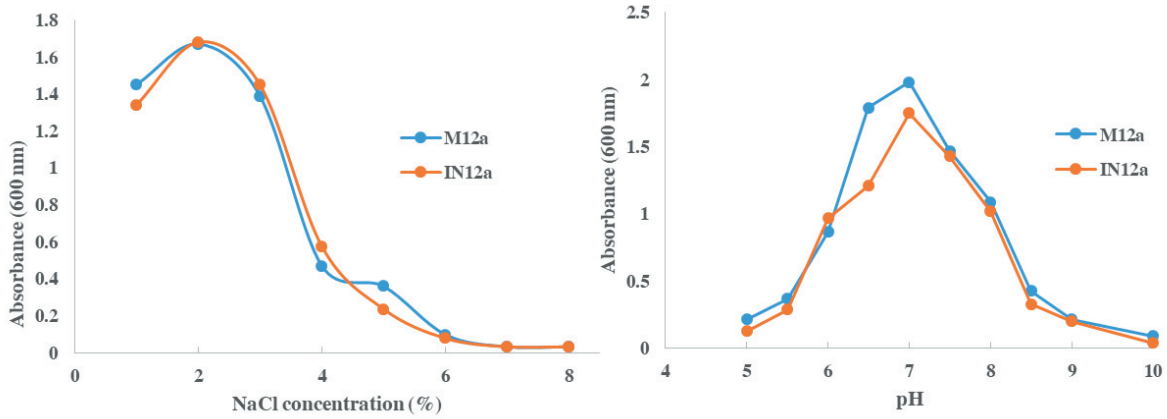

Figure 3: Growth of arsenic resistant isolates on different pH values and NaCl concentrations

DISCUSSION

From the results it can be observed that the pH of the soil was slightly acidic which is suitable for the growth of the most of plants (Kumar et al. 2019). In addition, some useful microorganisms can tolerate acidic pH and are applicable for crop enhancement (Kumar et al. 2019). Soil is the largest deposit of heavy metals such as arsenic and their compounds. These compounds may be harmful to other components of soil such as animals and plants. However, a solution to this problem can also be found in soil in the form of microorganisms that can utilize and degrade these harmful metals. Mostly the soil inhabitant microbes such as *Bacillus* species have been reported by many researchers (Schallmey et al. 2004; Radhakrishnan et al. 2017). In this study, Gram positive, endospore forming rod-shaped bacteria was isolated and tentatively identified as genus *Bacillus*. Travers et al. (1987) have reported the isolation of *Bacillus* species from various soil samples.

Isolation of arsenic resistance *Bacillus* species have been reported previously (Satyapal et al. 2016; Shakya et al. 2012; Selvi et al. 2014; Dey et al. 2016). Arsenic resistance *Bacillus aryabhattai* was isolated from the Indian soil and water samples (Singh et al. 2016). In this study, forty-two isolates were found to be Gram positive spore forming rod shaped bacteria. Shakya et al. (2011) also reported techniques of identification of *Bacillus* spp. based on cultural, morphological, and biochemical characteristics. On screening of these isolates for arsenic tolerance, 3 isolates produced colonies on Nutrient agar. The resistance was determined by inoculating the isolates on NA supplemented with sodium arsenite (Selvi et al. 2014). Colony formation in NA indicated the tolerance of arsenite and could possibly determine the toxic arsenite is utilized and converted to non-toxic forms. Only the arsenic tolerance isolates were vertically streaked on arsenic-supplemented NA and incubated. After incubation, the plates were flooded with freshly prepared silver nitrate solution which cause formation of yellowish brown precipitate which is suggestive of metabolic activity on the arsenite in the medium. This test confirmed the utilization of arsenic by the isolates $IN_1$2a and $M_1$2a. Selvi et al. (2014) also reported similar precipitation seen in media plates supplemented by arsenic and flooded with $AgNO_3$.

The arsenic resistance isolates were then tested for plant growth promoting activities. Indole acetic acid production was measured highest when the concentration of tryptophan was 0.05%. At higher concentration of tryptophan, there was low accumulation of IAA. This might be due to inhibitory effect of tryptophan against growth of *Bacillus* species. Ahmad et al. (2005) reported a contradictory result where IAA production increased with the increase in concentration of tryptophan in the medium when inoculated with *Psuedomonas* and *Azotobacter* isolates. The difference in results may be due to difference in type of microbes and their sensitivities to differing compounds. None of the isolates tested were capable of producing ammonia, both isolates were able to

solubilize phosphate in the medium. As a result, a clear zone of hydrolysis was observed around the fully developed colonies of isolates $IN_1$2a and $M_1$2a when grown on Pikovskaya's agar (Figure 1). According to Kitpreechavanich et al. (2016) *Bacillus* spp. have the ability to produce a clear zone around their colonies on Pikovskaya's agar by solubilizing phosphate in the medium.

Arsenic resistance isolates $IN_1$2a and $M_1$2a were able to assimilate all of the sugars tested. *Bacillus* spp. are well characterized and are able to assimilate the sugars as shown in Table 4. According to Bergey's manual of determinative bacteriology (1957) probably the isolates could be *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. brevis*, *Geobacillus stearothermophilus*.

Isolates $M_1$2a tolerated up to 1000 ppm of sodium arsenite and 15000 ppm sodium arsenate, which is the highest reported in Nepalese soil. In addition, mild growth was observed even up to 1500 ppm of sodium arsenite. However, further studies are necessary in this regard. The isolates showed growth from acidic to alkaline pH and at high concentration of NaCl, which is a typical characteristics of many *Bacillus* species (Poudel et al. 2016). Based on the 16S rRNA gene sequencing, high resistance isolate M12a was identified as *Bacillus flexus*. To our knowledge, this is the first report of isolation of arsenic resistant and plant growth promoting *Bacillus flexus* in Nepalese soil. These tests provide evidence that the isolates $IN_1$2a and $M_1$2a could be useful for the preparation of effective biofertilizer having PGP activity and bioremediation behavior. However, further testing is required to determine more characteristics of the isolates and also their best possible use in bioremediation of arsenic.

CONCLUSION

This is the first report to show the isolation of arsenic resistant and plant growth promoting *Bacillus flexus* in Nepalese soil. The result indicated that the isolates could be useful for the preparation of effective biofertilizer having PGP activity and bioremediation behavior. However, other experiments on arsenic resistant genes are necessary at the molecular level to understand the whole mechanism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahmad F, Ahmad I and Khan MS (2005). Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Turkish Journal of Biology* **29**(1)**:** 29-34.

Banerjee M, Banerjee N, Bhattacharjee P, Mondal D, Lythgoe PR, Martínez M, Pan J, Polya DA and Giri AK (2013). High arsenic in rice is associated with elevated genotoxic effects in humans. *Scientific Reports* **22**;3: 2195.

Bergey DH, Breed RS, Murray EGD and Hitchens AP (1957). Bergey's manual of determinative bacteriology (pp. 122-414). Baltimore: Williams & Wilkins.

Dey U, Chatterjee S and Kumar N (2016). Isolation and characterization of arsenic-resistant bacteria and possible application in bioremediation. *Biotechnol Rep* **10**: 1–7.

Ghosh P, Rathinasabapathi B and Ma LQ (2011). Arsenic-resistant bacteria solubilized arsenic in the growth media and increased growth of arsenic hyperaccumulator Pteris vittata L *Bioresour technol* **102**(19)**:** 8756-8761.

Kitpreechavanich V, Hayami A, Talek A, Chin CFS, Tashiro Y and Sakai K (2016). Simultaneous production of L-lactic acid with high optical activity and a soil amendment with food waste that demonstrates plant growth promoting activity. *J Biosc Bioeng* **122**(1): 105-110

Kumar A, Kumari M, Swarupa P and Shireen (2019). Characterization of pH Dependent Growth Response of Agriculturally Important Microbes for Development of Plant Growth Promoting Bacterial Consortium, *J Pure Appl Microbiol* **13**(2): 1053-1061.

Loper JE, Haack C and Schroth MN (1985). Population dynamics of soil pseudomonads in the rhizosphere of potato (*Solanum tuberosum* L.). *Appl Environ Microbiol* **49**(2): 416-422.

Mallick I, Hossain ST, Sinha S and Mukherjee SK (2014). Brevibacillus sp. KUMAs2, a bacterial isolate for

possible bioremediation of arsenic in rhizosphere. *Ecotoxicol Environ Safety* **107**: 236-244.

Pepi M, Volterrani M, Renzi M, Marvasi M, Gasperini S, Franchi E and Focardi SE (2007). Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello Lagoon, Italy, and their characterization. *J Appl Microbio* **103**(6)**:** 2299-2308.

Poudel P, Tashiro Y and Sakai K (2016). New application of *Bacillus* strains for optically pure L-lactic acid production: general overview and future prospects. *Biosc Biotechnol Biochem* **80**(4): 642-654.

Radhakrishnan R, Hashem A and Abd_Allah EF (2017). *Bacillus*: a biological tool for crop improvement through bio-molecular changes in adverse environments. *Front Physiol* **6**: 8:667

Satyapal GK, Rani S, Kumar M and Kumar N (2016). Potential Role of Arsenic Resistant Bacteria in Bioremediation: Current Status and Future Prospects. *J Microb Biochem Technol* **8:** 256-258.

Schallmey M, Singh A and Ward OP (2004). Developments in the use of Bacillus species for industrial production. *Canadian Journal of Microbiology* **50**(1)**:** 1-17

Selvi MS, Sasikumar S, Gomathi S, Rajkumar P, Sasikumar P and Govindan S (2014). Isolation and characterization of arsenic resistant bacteria from agricultural soil, and their potential for arsenic bioremediation. *Int J Agric Policy Res* **2:** 393-405.

Shagol CC, Krishnamoorthy R, Kim K, Sundaram S and Sa T (2014). Arsenic-tolerant plant-growth-promoting bacteria isolated from arsenic-polluted soils in South Korea. *Environ Sci Pollut Res* **21**(15): 9356-9365.

Shakya S, Pradhan B, Smith L, Shrestha J and Tuladhar S (2012). Isolation and characterization of aerobic culturable arsenic-resistant bacteria from surface water and groundwater of Rautahat District, Nepal. *J Envion Manage* **95**: S250-S255.

Singh N, Gupta S, Marwa N, Pandey V, Verma PC, Rathaur S and Singh N (2016). Arsenic mediated modifications in *Bacillus aryabhattai* and their biotechnological applications for arsenic bioremediation. *Chemosphere* **164**: 524-534.

Smith E, Kempson I, Juhasz AL, Weber J, Skinner WM and Gräfe M (2009). Localization and speciation of arsenic and trace elements in rice tissues. *Chemosphere* **76**(4): 529-535.

Travers RS, Martin PA and Reichelderfer CF (1987). Selective Process for Efficient Isolation of Soil *Bacillus* spp. *Appl Environ Microbiol* **53**(6): 1263–1266.

Evaluation of Antimicrobial Activity and Synergistic Effect of Spices against Few Selected Pathogens

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ABSTRACT

Objectives: The main objective of this study was to evaluate antimicrobial activity of ethanolic extract of spices along with determination of its synergistic effect against few selected pathogens.

Methods: In this study, ethanolic extract of 5 different spices; *Zingiber officinale* (Ginger), *Allium sativum* (Garlic), *Curcuma longa* (Turmeric), *Capsicum annum* (Chili) and *Allium cepa* (Onion) were obtained by using Soxhlet apparatus. The ethanolic extract was concentrated by evaporation and different concentrations of extract were prepared in Dimethy Sulphoxide (DMSO) solvent. Test organisms included mainly pathogens i.e. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*. The antimicrobial activities of the extracts were determined by well diffusion technique both individually and in combination. On the other hand, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was determined by serial dilution technique. The result were interpreted on the basis of the fact that the growth occurs in positive control and other tubes with inadequate amount of extract whereas the lowest concentration of agent that inhibits growth of organism, detected by lack of visible turbidity by inhibition of 99% is designed as the MIC. The MBC is identified by determining the lowest concentration of extract solution that reduces the viability of the initial bacterial inoculum by a predetermined reduction such as ≥99.9%. Likewise, for determination of Fractional Inhibitory Concentration Index (FICI), two extracts were combined along with standardized inoculum of bacterial strain. Tubes without visible turbidity were streaked on agar plate and observed for 99.9% killing.

Results: All the tested extract of spices were found effective against *S. aureus* and *K. pneumoniae* only. The highest zone of inhibition (ZOI) was found in chili extract (ZOI=26 mm) against *S. aureus* whereas lowest zone of inhibition was found in garlic extract against *K. pneumoniae* (ZOI=12mm). Similarly, highest ZOI was produced by combined extract of both Turmeric and Ginger (ZOI= 26 mm). Turmeric extract was found to be effective against *S. aureus* (MIC value = 62.5 mg /ml and MBC value = 31.25 mg/ml) and *K. pneumoniae* (MIC value 125 mg/ml and MBC value = 62.5 mg/ml). The Fractional Inhibitory Concentration (FIC) values of combined extract suggested synergistic and additive effect (0.5<FIC<1). Chili and ginger were effective with FIC value of 0.25.

Conclusion: To recapitulate, the extract of spices can be used to prevent the pathogenic organism.

Key words: Dimethyl Sulphoxide, Minimun Inhibitory Concentration, Minimum Bactericidal Concentration, Fractional Inhibitory Concentration Index

INTRODUCTION

Spices are indispensable components of Nepalese cuisines since ancient times and are considered medicinal purposes for several centuries due its extensive antimicrobial and antioxidant property. The activity of herbs and spices are not only limited to boosting flavor, but also recognized for their preservative and medicinal value (Panpatil et al. 2013). Food-borne illness caused by consuming food and

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beverages contaminated with bacteria, fungi can cause symptoms that range from upset stomach to more serious symptoms such as diarrhea, fever, vomiting, abdominal cramp, dehydration. Fifty years of increasing uncontrolled use of chemical antimicrobials have created a situation leading to an ecological imbalance and enrichment of multiple multi-resistant pathogen microorganisms (Aktug and Karapinar 1986). The burgeoning concern about safety of foods has recently led to the development of natural antimicrobials to control food borne pathogens. Addition of spices in foods imparts flavor and pungent stimuli and more importantly its natural products and naturally derived components shows antimicrobial property.

Ginger, a member of family Zingerberaceae, is an erect perennial plant growing from one to three feet in height; its stem is surrounded by sheathing bases of the two ranked leaves. Fresh ginger has been used for cold induced diseases, nausea, asthma, cough, colic heart palpation, dyspepsia, loss of appetite and rheumatism (Tyler 2002). Garlic (*Allium sativum*), belongs to Alliaceace, comprise numerous discrete cloves whereas leaves and stems are sometimes eaten, particularly whole immature and tender. It is claimed to help prevent heart disease including antherosclerosis, high cholesterol, high blood pressure and to improve immune system as well as protection against cancer (Maryland 2006). The onion (*Allium cepa*) belongs to Liliaceae family consisting of herbaceous plant part and edible bulb part; rich in proteins, carbohydrate, sodium, potassium and phosphorus (Cox et al. 2000). They are effective against common cold, heart disease, diabetes, osteoporosis, coughs and sore throat (Juven et al. 1994). Turmeric (*Curcuma longa*) is a member of the ginger family Zingiberceace. Various sesquiterpenes and curcuminoids have been isolated from the rhizome of *C. longa*, attributing a wide array of biological activities, anti-inflammatory, wound healing, anticancer and antibacterial (Sandur et al. 2007). The chili pepper (*Capsicum annum*), a member of night shade family, Solanaceae, is a diploid, facultative self-pollinating and closely related to potato, tomato, eggplant (UN FAOSTAT 2014). Capsaicin, a well-studied chemical component of *Capsicum* species has already demonstrated a high degree of biological activity affecting nervous, cardiovascular, digestive system (Britto et al. 2009). Chemical analysis has demonstrated that capsicum fruits contain relatively high concentration of several essential nutrient including vitamin C up to 6 times the concentration of orange (Sagdic et al. 2003)

Therefore, the investigation of antimicrobial properties of spices used as food additives to control the growth of food-borne pathogens may give useful results. But the main obstacle for using spices and medicinal plants as food preservatives is that their high concentration is required in food to inhibit the microbial growth as well as oxidation. This high concentration of spices and herbs causes negative organoleptic effects i.e. alter the taste, color, odor and texture of food and limit their use in food preservatives system as well as to develop safe and potent antimicrobial and antioxidant food preservatives from them (Burt 2004).

Several researchers have investigated the individual effects of essential oils of spices and medicinal plants on antimicrobial activity against food borne bacteria as well as antioxidant activity. Study performed by Maharjan et al. (2012) compares the sensitivity of some human pathogenic bacteria to various spice extract viz. essentials oils, acetone and methanol extracts by agar well diffusion method. Of the different spices tested clove, ajowan and cinnamon were found to possess relatively higher antimicrobial activities. The MBC value ranged from 0.39 to 25mg/ml. The lowest MBC value was given by essential oil of cinnamon against *E. coli, S. aureus and S.*Typhi. In contrast to these studies, this research mainly focuses on the spices that are used in food items in regular basis and focuses on synergistic interaction of selected spices against selected pathogens as their combination effects on both antimicrobial and antioxidant activities seem to be dubious. This approach may increase the antimicrobial and antioxidant efficacy at sufficiently low concentration by taking their advantages of possible synergistic interaction which may reduce their adverse side effects as well as negative organoleptic effect in food and facilitate their use in food preservation system (Foster 2004) .Therefore, the main aim of the study is to shed some light on the antimicrobial potency of spices used in Nepal with main regard to synergistic activity of spices.

MATERIALS AND METHODS

Collection of spices: Five different fresh spices viz., ginger, garlic, turmeric, chili, and onion free from disease were collected from various places of Lalitpur. The study was carried out in Microbiology laboratory of Pinnacle College, Lagankhel, Kathmandu.

Soxhlet extraction with 96% ethanol: Thoroughly

washed dried leaves of 5 selected spices were dried under shade at room temperature for four days. The dried samples were cut into pieces by means of plant cutter and subjected to grinding. 25 gm of obtained dried powder was subjected to soxhlet extractor along with 150ml of 96% ethanol followed by filtration using Whatmann filter (No 1). For removal of ethanol from the extract, it was placed on evaporating dish placed over Bunsen burner. Finally, dense extract was diluted in 10% Dimethyl Sulphoxide (DMSO) to obtain standard working solution (Rajendhran 2008).

Preparation and standardization of inoculum: The Gram positive (*S. aureus*) and Gram negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa*) precultured in nutrient broth and incubated for about 2 hours. The turbidity of inoculum was adjusted by using Mc farland standard as a reference. The tubes were compared with turbidity of 0.5 Mc farland solution (1-2* 10^8 cfu/ml) (Tandukar et al. 2017).

Phytochemical screening: Phytochemical screening was carried out on ethanolic extract of spices for detecting its chemical composition especially for tannins (5% $FeCl_3$), flavonoids (1% NH_3), terpenoids (0.5 chloroform) and alkaloids (dil HCl + Mayer's reagent) were employed during the study (Byadgi 2018).

Determination of antimicrobial activity: Antimicrobial activity of spices against the selected organism was determined by Agar well diffusion technique under aseptic condition. 20ml of sterilized molten Muller Hinton Agar was poured into sterile petri plates; after solidification, fleshly prepared inoculum was swabbed on respective plates with the aid of sterile cotton swab. By using sterile cork borer no 6, wells (diameter-7mm) were made in inoculated media plates which was finally filled with 50µl of working solution of different spices. The diameter of zone of inhibition was measured after incubation at 37^0 C for 24 hours (Dingle et al. 2009).

Determination of MIC and MBC: The crude extract which showed antimicrobial and DMSO solution were subjected to two-fold serial dilution method by Finegold and Baron (2014) to determine MIC and MBC. For each bacterium, a set of dry screw capped test tubes were taken and labeled as 1,2,3,4,5,6,7,8,9,10,11 – Tube no .1 taken as positive control (2ml plant extract) and Tube no. 11 as negative control (Nutrient broth). By mixing nutrient broth and plant extract followed by process of homogenization and dilution, the concentration of tube no 4, 5, 6, 7, 8, 9 and 10 becomes 125, 62.5, 31.25, 15.63, 7.81 and 1.92 mg/ml which is further added with 50µl of 4hrs culture of microorganism. All the tubes were incubated at 37^0 C for 24 hrs and observed turbidity by comparing with positive negative control. For MBC calculation, the tubes were sub cultured on nutrient agar plate and incubated at 37^0 C for 24 hrs. Then they were examined for the growth of bacteria.

Determination of synergistic activity: For detection of synergistic activity, combination of extract – Ginger & Garlic, Ginger & Chili, Turmeric & Ginger, Tumeric & Chili and Garlic & Onion – was prepared by mixing 2ml of each extract. Same procedure was performed as done for detection of antimicrobial activity of spices individually (Al-Mahmood 2009).

Determination of fractional Inhibitory concentration index (FICI): FIC index was determined by Multiple combinations bacterial testing (MCBT); two extracts were combined in test tube along with standardized inoculum of the bacterial cultured. Wells without visible turbidity was sampled by streaking a 10µl aliquot on agar plate, incubating for a day and observing 99.9% killing (Singh 2015).

RESULTS

A total of 6 different spices (Ginger, Gralic, Turmeric, Onion and Chili) were included in this study, collected from local houses of Lalitpur area. The crude extracts of spices were tested against altogether four pathogens.

Under phytochemical screening, tannins were present in all spices except garlic whereas flavinoids were present only in turmeric and onion. Furthermore, terpenoids were present in garlic, turmeric and onion only whereas the alkaloids were present in all spices except turmeric.

Table 1: Phytochemical screening of crude ethanolic extract of spices

Phytochemical test	Reagent used	Spices	Observation
Tannins	5% FeCl3	Ginger	+
		Garlic	-
		Turmeric	+
		Chili	+
		Onion	+

Phytochemical test	Reagent used	Spices	Observation
Flavonoids	1% NH3	Ginger	-
		Garlic	-
		Turmeric	+
		Chili	-
		Onion	+
Terpenoids	0.5ml	Ginger	-
	Chloroform	Garlic	+
	& 1ml conc	Turmeric	+
	H2SO4	Chili	-
		Onion	+
Alkaloids	Mayer's	Ginger	+
	Reagent	Garlic	+
		Turmeric	-
		Chili	+
		Onion	+

All the four selected pathogens were tested with specific antibiotics by using Kirby-Brauer disk diffusion method. According to Clinical and Laboratory Standard Institute (CSLI 2014), six different sets of antibiotics were used to determine antibiotic susceptibity pattern of selected pathogens. Among them *E. coli* was seen resistant to most of antibiotic except Gentamicin (ZOI = 18mm). *S. aureus* was found highly sensitive to ceftriaxone with ZOI of 22mm but resistant to Amoxyclav and Bacitracin only. Similarly, *K. pneumoniae* was highly sensitive to Gentamicin (ZOI = 23mm) and resistant to Ceftriaxone whereas Ciprofloxacin was found most effective against *P. aeruginosa* (ZOI = 35mm).

Table 2: Antibiotic susceptibility pattern of selected test organisms

Test organisms	Antibiotic disc	Symbol	Concentration	Diameter of ZOI(mm)	Inference
S. aureus	Chloramphenicol	C	10mcg	21mm	Sensitive
	Gentamicin	GEN	10mcg	18mm	Sensitive
	Amoxiclav	AC	30mcg	12mm	Resistant
	Ceftriaxone	CTR	30mcg	21mm	Sensitive
	Ciprofloxacin	CIP	5mcg	22mm	Sensitive
	Bacitracin	B	8mcg	-	Resistant
E. coli	Chloramphenicol	C	10mcg	-	Resistant
	Gentamicin	GEN	10mcg	18mm	Sensitive
	Nitrofurantoin	NIT	100mcg	-	Resistant
	Ceftriaxone	CTR	30mcg	-	Resistant
	Nalidixic acid	NA	30mcg	-	Resistant
	Erythromycin	E	10mcg	-	Resistant
K. pneumoniae	Chloramphenicol	C	10mcg	13mm	Intermediate
	Gentamicin	GEN	10mcg	23mm	Sensitive
	Nitrofurantoin	NIT	100mcg	18mm	Sensitive
	Ceftriaxone	CTR	30mcg	-	Resistant
	Nalidixic acid	NA	30mcg	22mm	Sensitive
	Amoxyclav	AC	30mcg	8mm	Resistant
P.aeruginosa	Chloramphenicol	C	10mcg	-	Resistant
	Gentamicin	GEN	10mcg	32mm	Sensitive
	Amoxyclav	AC	30mcg	-	Resistant
	Ciprofloxacin	CIP	5mcg	35mm	Sensitive
	Nalidixic acid	NA	30mcg	-	Resistant

Antimicrobial activity was evaluated by two ways viz measuring zone of inhibition and quantitative determination of spices extract for MIC and MBC. Among four pathogens, spices extracts were found most effective against *S. aureus* and *K. pneumoniae* only. Chili extract proved to be most effective against *S. aureus* with zone of inhibition of 26mm. However, garlic and onion extract were found least effective.

Table 3: Antimicrobial activity of selected crude extract of spices against microorganisms

Spice extract	Diameter of zone of inhibition (mm) against microorganisms			
	S. aureus	*E. coli*	*K. pneumonia*	*P. aeruginosa*
Ginger	16mm	-	12mm	-
Garlic	15mm	-	14mm	-
Turmeric	16mm	-	14mm	-
Chili	26mm	-	14mm	-
Onion	15mm	-	14mm	-
DW	-	-	-	-
DMSO (10%)	-	-	-	-

The minimal inhibitory concentrations and minimum bactericidal concentrations for the spices extracts against examined bacterial strains are presented in table no (4). The lowest MIC and MBC which could inhibit microbial which could inhibit microbial growth was recorded for Turmeric. From the microbial sensitivity side of view, *K. pneumoniae* was the most sensitive bacteria to the examined spices with MIC of 31.25mg/ml and MBC of 62.5mg/ml.

Table 4: Minimum inhibitory concentration (mg/ml) and minimum bactericidal concentration of various spice extracts

spices extract	Minimum Inhibitory Concentration		Minimum Bactericidal Concentration	
	S. aureus	*K. pneumoniae*	*S. aureus*	*K. pneumoniae*
Ginger	500mg/ml	500mg/ml	1000mg/ml	1000mg/ml
Garlic	500mg/ml	500mg/ml	1000mg/ml	1000mg/ml
Turmeric	62.5mg/ml	31.25mg/ml	125mg/ml	62.5mg/ml
Chili	500mg/ml	250mg/ml	1000mg/ml	500mg/ml
Onion	500mg/ml	500mg/ml	1000mg/ml	1000mg/ml

Regarding the combined effect of spices, mixture of ginger and garlic showed highest effectiveness among all mixture with zone of inhibition of 26mm against *S. aureus*. Similarly, combined mixture of ginger and garlic was found least effective with zone of inhibition of 12mm against *K. pneumoniae*. Garlic and onion was found ineffective against both organisms.

Table 5: Antimicrobial activity of their combination against microorganism

Mixture of spices	Diameter of zone of inhibition(mm) against organisms			
	S. aureus	*E. coli*	*K. pneumoniae*	*P. aeruginosa*
Gi+Ga	26mm	-	12mm	-
Tu+Ch	21mm	-	16mm	-
Tu+Gi	24mm	-	14mm	-
Ch+Gi	24mm	-	14mm	-
Ga+On	-	-	-	-

Note: Gi+Ga=Ginger and Garlic, Tu+Ch= Turmeric and chili,Tu+Gi= Turmeric and Ginger, Ch+Gi= Chili+Ginger, Ga+On= Garlic and Onion

Combined extract of Turmeric and Chili, Turmeric and Ginger, Chili and Ginger were found to have synergistic effect against *S. aureus* with FIC value of 0.28, 0.28,0.25 respectively whereas the extract of ginger and garlic was found to have additive effect (FIC value = 1). Similarly, these all combined extract were found to have additive effect against *K. pneumoniae.* Combined extract of Garlic and Onion was found to have antagonistic effect against both isolates.

Table 6: Minimum inhibitory concentration (mg/ml) of various spice extracts

Combined extract	*S. aureus*			*K. pneumonia*		
	MIC Value	FIC Value	Inference	MIC Value	FIC Value	Inference
Gi+Ga	250mg/ml	1	Add	250mg/ml	1	Add
Tu+Ch	15.63mg/ml	0.28	Syn	15.63mg/ml	0.56	Add
Tu+Gi	15.63mg/ml	0.28	Syn	13.63mg/ml	0.53	Add
Ch+Gi	62.5mg/ml	0.25	Syn	125mg/ml	0.75	Add
Ga+On	500mg/ml	2	Anta	500mg/ml	2	Anta

Keys: Add: Additive effect, Syn: Synergistic effect, Anta: Antagonistic

DISCUSSION

During study, it was found that most of extract was affective against *S. aureus* and *K. pneumoniae* whereas, *E. coli* and *P. aeruginosa* were resistant against extract. In case of *S. aureus*, the ginger extract produces ZOI of 16mm, whereas in case of *K. pneumoniae*, it produced ZOI of 12mm. The main constituents of ginger are sesquiterpenoids with zingiberene. Other compounds include β-sesquiphellandrene, bisabolene and farnesene, which are sesquiterpenoids and trace monoterpenoid fraction (Malu et al. 2008). In similar fashion ethanolic extract of turmeric produced ZOI of 16mm in case of *S. aureus* whereas only 14mm ZOI against *K. pneumoniae*. The antimicrobial activity of turmeric is reported to be due to the presence of essential oil, curcumins, curcuminoids, turmeric oil, turmerol and valeric acid (Gul et al. 2015). Ethanolic extract of chili produced highest zone of inhibition against *S. aureus* (ZOI = 26mm). Capsaicin, a well-studied chemical component of the capsicum species and one of the pungent capsainoids found in chili peppers, has already demonstrated a high degree of biological activity affecting nervous, cardiovascular and digestive system. The ethanolic extract of garlic and onion extract produced least ZOI of 15mm. In previous studies, it has been demonstrated that allicin is the main component of garlic that exhibits antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein synthesis are also partially inhibited (Yadav et al. 2015).

In this study, ethanol was used as a solvent although it itself has antimicrobial properties. The study is justified as the ethanol was evaporated when heated 40°C for 24 hrs. On other hand, it is also classified as polar solvent. This means the solvent is miscible in water and it will extract mostly ionic compounds from spices. It has better dissolving capabilities compared to water because it has a slightly low dipole and is dielectric (Ramli et al. 2017). Moreover, according to Marriott (2010), ethanol, ethyl acetate and acetone are the solvents permitted for use in the preparation of food ingredients.

Overall the effectiveness of ethanolic extract of spices is higher in Gram positive than Gram negative. Grace et al. (2017) found similar result i.e. ethanolic extract of ginger is best effective against *S. aureus* when compared to other Gram negative. Generally, in Gram negative bacteria, their outer membrane serves as permeability barrier which allows only small hydrophilic molecules to pass through into all, restricting their route of penetration for certain antimicrobial compounds and excluding larger molecules. Besides these, they also possess multidrug resistant pumps which exclude some of antibacterial compounds across barrier (Marriott 2010).

Similarly, effect of combination of spices extracts have proven to be feature of antimicrobial and antioxidant treatment due to number of important considerations viz (i) they increase activity through use of compounds with synergistic or additive activity, (ii) they thwart drug resistance (ii) they decrease required doses, reducing both cost and adverse/toxic side effects and (iv) they increase the spectrum of activity (Baljeet et al. 2015).

From the foregoing findings, combination of selected spices produced zone of inhibition larger than

individual use. The combined extract of ginger and garlic showed highest ZOI of 26mm against *S. aureus* whereas turmeric and chili mixture produced highest ZOI of 16 mm against *K. pneumoniae.* Both the isolates were found resistant to the combined garlic and onion extract. But Aliyu et al. (2015) reported that ethanolic extract of garlic produced zone of inhibition ranging from 3.59-15.80mm against *S. aureus* whereas the ginger extract produced zone of inhibition ranging from 13-28mm. Their combined extract of garlic and ginger extract produced zone of inhibition of 33.60mm.

MIC value was calculated for only two organisms i.e. *S. aureus* and *K. pneumoniae* as these two organism were found to be most sensitive to ethanolic extracts whereas other two isolates were found to be insensitive to the extract as there was no any visible zone of inhibition. Although various spices extracts showed different inhibitory effect against tested microorganisms, similar value of MIC ranging from 500-1000µg/ml was observed in case of each individual spices extract against both isolates except for turmeric extract. This showed that the same concentration of various individual extract was effective to different extents in inhibiting the growth of tested microorganism. MIC value for turmeric was found to be 62.5mg/ml while MBC was found to be 125mg/ml. similarly, in case of *K. pneumoniae*, MIC value for turmeric was found to be 31.25mg/ml and MBC was found to be 62.5mg/ml.

The MIC of combined extracts however fluctuated from 15.63mg/ml to 500mg/ml and the most sensitive microbial species in relation to the MIC of combined extract was *S. aureus.* The MIC of most of combined extract was reduced to 25% of the MIC of individual extracts. The FIC values of combined extracts showed that the combination of ethanolic extract of chili and ginger (FIC index=0.25), turmeric and chili (FIC index= 0.28) & turmeric and ginger (FIC index = 0.28) displayed synergistic effect (FIC index < 0.5) against *S. aureus* while combined extract of ginger and garlic showed additive effect (FIC index = 1). On other hand all the combined extracts showed additive effect against *K. pneumoniae* except garlic and onion.

All the bacteria employed for the study were also subjected to antibiotic susceptibility test. The resistivity and sensitivity of antibiotics against the organism was determined based on CSLI (2014) guideline. The highest ZOI of inhibition was given by Ciprofloxacin against *P. aeruginosa*. Most of antibiotic was found resistant against *E. coli*.

Hence the synergistic and additive effect of these spices against tested spices against test microorganisms supports the use of these spices in combinations. The results of study revealed that combined ethanolic extracts of spices were more effective as antimicrobials, as antimicrobial properties of spices depend not only on chemical composition but also on the lipophilic properties and water solubilities. Combination of various compounds may have contributed to the observed synergistic and additive effects. The multiple mode of action may include degradation of cell wall, disruption of cytoplasmic membrane, leakage of cellular components, alteration of fatty acid and phospholipids constituents, changes in synthesis of DNA and RNA and destruction of protein translocation (Baljeet et al. 2015). Hence it is possible that combining spice extracts could lead to synergistic or additive inhibitory potential against both food spoilage and pathogenic microorganisms. Most studies attributed additive and synergistic effects to phenolic and alcoholic compounds.

Although the research is conducted by systematic protocol, the variables used in research are too small to draw strong and specific conclusion. The study only focuses on pathogenic organism by using four bacterial isolates and limited number of spices. From beginning to the end of research, tests were performed using crude extract without conforming its purity.

CONCLUSION

Spices extract seems more effective against *Staphylococcus aureus* than *Klebsiella pneumoniae* with MIC value of 62.5µg/ml and 31.25µg/ml and MBC value of 125µg/ml 62.5µg/ml respectively The MIC of most of combined extract was reduced 25% of the MIC of individual extracts. The FIC values of combined extracts showed that the combination of ethanolic extract of chili and ginger (FIC index=0.25), turmeric and chili (FIC index= 0.28) & turmeric and ginger (FIC index = 0.28) displayed synergistic effect (FIC index < 0.5) against *Staphylococcus aureus* while combined extract of ginger and garlic showed additive effect (FIC index = 1). On other hand all the combined extracts showed additive effect against *Klebsiella pneumoniae* except garlic and onion. Therefore, combinations of extracts can provide additive as well as synergistic

effects making them more effective antimicrobial agents. Consequently, the selection of spices, for use in preventing food-borne bacterial infection, is both interesting and worthwhile for food safety.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aktug SE and Karapinar M (1986). Sensitivity of some common food-poisoning bacteria to thyme, mint and bay leaves. *Int J Food Microbiol* **3**: 349-354.

Aligu AM, Saleman SS and Aligu MV (2015). Synergistic effect of *Allium sativum*(garlic) and *Zingiberofficinale* (ginger) against *E. coli* and *Staphylococcus aureus*. *Int J Sci and Eng Res* **6**: 1350-1356.

Al-Mohammod MA (2009). Efficacy of Crude Extracts of Garlic (*Allium sativum*) Against Nosocomial *E. coli, Staphylococcus aureus, Streptococci pneumonia* and *Pseudomonas aeruginosa*. *J Med plants Res* **3**: 179-185.

Baljeet SY, Simmy G, Ritika Y and Roshanlal Y (2015). Antimicrobial activity of individual and combined extracts of selected spices against some pathogenic and food spoilage microorganisms. *Int Food Res J* **22**: 2594-2600.

Brito-Argaez L, Moguel-Salazar F, Zamudia F, Gonzalez-Estrada T and Islas-Flores (2009). Characterization of a Capsicum Chinese seed peptide Fraction with Broad Antibacterial Activity. *Asian J of Biochem* **4**: 77-87.

Britto AJD, Gracelin DHS, Benjamin P and Kumar JR (2012). Antimicrobial potency and Synergistic effects of a few South Indian spices against antibiotic resistant bacteria. *Int J of Natural products and Resources* **3**: 557-562.

Byadgi SA and Vastrad JV (2018). Phytochemical Screening and Antimicrobial Activity of Hibiscus rosa-sinesis Leaf Extracts. *Int J Curr Microbiol Appl Sci* **7**: 3329-3334.

Chandarana H, Baluja S and Chanda S (2005). Comparison of antibacterial activities of selected species of Zingerberaceacae family and some synthetic compounds. *Turk J Bio* **29**: 83-97.

CLSI (2014). Performance standards for antimicrobial susceptibility testing; twenty-second Informational supplement 33. CLSI Document M100–S20. Wayne, PA.

Dingle J, Red WW and Solomons Gl (2012). The Enzymatic Degradation of pectin and polysaccharides II Application of the Cup Assay Method to The Estimation of Enzyme. *J Sci Food Agric* **4**: 149-153.

Foster S (2011). Ginger (*Zingiberoffi cinale*) - your food is your medicine. Available at

Grace US, Sankari M and Gopi (2017). Antimicrobial activity of ethanolic extract of Zingiber Officinal-An invitro study. *J Pharm Sci & Re* **9**: 1417-1419.

Gul P and Bakht J (2015). Antimicrobial activity of turmeric extract and its potential use in food industry. *J Food sci Technol* **52**: 2272-2279.

Juven BJ, Konner J, Sched F and Weisslowiez H (1994). Factors that interact with the antibacterial of thyme essential oil and its active constituents. *J of App Microbiol* **76**: 626-631.

Maharjan D, Singh A, Lekhak B, Basnyat S and Gautam LS (2012). Study on antimicrobial activity of common spices. *Nepal Journal of Science and Technology* **12**: 312-313

Malu SP, Obochigo, Tawo EN and Nyong BE (2016). Antibacterial activity and medicinal properties of ginger (*Zingiberofficinale*). *Global J of pure and Appl Sci* **15**: 365-366.

Marriott RJ (2010). Grenner chemistry preparation of traditional flavor extracts and molecules. *Agro Food Industry Hi-Tech* **21**: 46-48.

Panpatil VV, Tattari S, Kota N, Nimgulkar C and Polasa K (2013). In vitro evaluation of antioxidant and antimicrobial activity of spice extracts of ginger, turmeric and garlic. *J Pharmacogn Phytochem* **3**: 143-148.

Rajendhran J, Arun Mani M and Navaneethan K (2008). Antimicrobial activity of some selected medicinal

plants. *Geobios (Jodhpur)* **25:** 208-282.

Sagdic O, Karahan AG, Ozean M and Ozean G (2003). Effect of some spices extracts on bacterial inhibition. *Food Sci and Tech Int* **9**: 5353-5359.

Sandur SK, Manoj KP, Bokyung S, Pornngarm K, Vladimir B and Bharar BA (2007) Curcumin, dimethoxycurcumin and turmerones differentially regulate anti-inflammatory and antiproliferative responses through ROS-independent mechanism **28**: 1765-1773.

Singh BR and Mohd B (2015). Method of determinig fractional inhibitory concentration (FIC). Division of Epidemiology Indian Veterinary Research Institute: 201-207.

Tankukar R and Maharjan S (2017). Screening and evaluation of antimicrobial activity of medicinal plants. A project work of B.Sc. presented to Department of Microbiology, Pinnacle College, Tribhuvan University. Lalitpur, Nepal: 20-28.

Tyler VE (2002). The honest herbal, a sensible guide to the use of herbs and related remedies, New york: Pharmaceutical Products Press pp. 300-375.

Yadav S, Trivedi NA and Bhatt TD (2015). Antimicrobial activity of fresh garlic juice: An invitro study. *Ayu* **36**: 203-207.

Effect of *Psidium guajava* L on Biofilm Forming Multidrug Resistant Extended Spectrum Beta Lactamase (ESBL) Producing *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: In order to investigate the relative efficacy of *Psidium guajava* L (guava) leave, in vitro antibacterial effect of ethanolic extract and leaf tea of guava against pathogenic *Pseudomonas aeruginosa* was carried out.

Methods: This study was carried out at microbiology laboratory of KIST medical college and teaching hospital, Lalitpur. *Pseudomonas aeruginosa* was isolated from different clinical samples. Antibiotic susceptibility of the isolates was performed by Kirby-Bauer disk diffusion method. Biofilm formation was detected by microtitre culture plate method and ESBL production by combine disk methods. Antimicrobial activity of guava leaf tea and ethanol extract of leaf were determined by agar well diffusion method.

Results: Ethanol extract of fresh guava leaves exhibited higher antibacterial activity than dry and fresh leaves tea, but significantly less than the standard antibiotics. In this study, 7% of the total bacterial isolates were *P. aeruginosa* with 65.30% of these isolates being MDR. Similary, significant number of these MDR strains ie. 83.67% of these *P. aeruginosa* isolates produced biofilm. While as 6.12% of the isolates were ESBL producer.

Conclusion: The leaves extract of guava have shown effective result against *P. aeruginosa* and could serve as good source of antibacterial agents. Guava leaves extract can be an economic alternative to antibiotics. However, active compound of this extract need to be purified and pharmacologically tested before its application.

Key words: Guava, *Pseudomonas aeruginosa*, antibacterial activity, AST

INTRODUCTION

Infectious diseases are the major cause of death in the developing countries. Although the global burden of infectious disease was reduced by using antimicrobial agents the spread of resistant pathogen has diminished the effectiveness of the antibiotics (Bisht et al. 2016). An inheritent resistant bacteria, *Pseudomonas aeruginosa,* belonging to Gram-negative aerobic bacilli of Pseudomonadaceae family, are posing challenge in treatment of various infections it cause such as: urinary tract infections, respiratory infections, otitis media, skin and soft tissue infections, bone and joint infections, and bacteremia. Besides, it can also cause serious systemic infections particularly in people with compromised immune systems including patients of burn suffer, cystic fibrosis, cancer and AIDS (Neopane et al. 2017). It has emerged as one of the leading causes of nosocomial infections. *P. aeruginosa* is the sixth most frequently isolated nosocomial pathogen, causing 7.3% of all hospital acquired infection in the US (Weiner et al. 2016). Infections due to *P. aeruginosa* are difficult to eradicate due to their intrinsic resistance as well as their ability to acquire resistance to different antibiotics. The resistance is due to over expression of efflux pump, acquisition of extended spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs), target site or outer membrane modification, porin mutations and plasmid enzymatic modification (Heydari and Eftekhar 2015). *P. aeruginosa* can form biofilms, which exponentially increase antibiotic

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resistance. The three exo-polysaccharides that mainly contribute to the biofilm formation in this bacteria are; alginate, Psl (Polysaccharides Synthesis Locus), and Pel (Pellicle). Alginate confers additional protection against antimicrobials and the immune system while Psl and Pel contribute to aggregation and adherence (Nithyalakshmi et al. 2015). Hence, dealing with multi-drug resistant strain of these bacteria is challenging.

Opting for an alternative natural plant product to inhibit this bacteria we have selected Guava leave (*Psidium gaujava* L). Guava leave (*Psidium gaujava* L), a phyto-therapeutic plant used in folk medicine, and is believed to have active antimicrobial compound which can be used to treat and cure various diseases (Mailoa et al. 2014). Different parts of the plant have been used in traditional medicine against ailments like malaria, gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, controlling of diabetes, hypertension, and obesity (Biswas et al. 2013).

Study in Nepal has shown the association of nosocomial infections with multidrug resistant (MDR) *P. aeruginosa*. The occurrence of biofilm and MDR bacteria in a hospital setting possess a therapeutic problem, as well as a serious concern for infection control management. Therefore, minimizing the use of antibiotics and possibly substituting with antimicrobial compound from natural source can be an alternative to subside the growing antibiotic resistant problem. This study was therefore undertaken to evaluate the antimicrobial activity of guava leaf tea and extract on *P. aeruginosa* isolated from various clinical samples.

MATERIALS AND METHODS

P. aeruginosa was isolated from different clinical samples such as pus/wound, blood, sputum and urine at KIST medical college and teaching hospital, Lalitpur, a tertiary care hospital from 15th June 2017 to 15th Dec 2017. They were identified by standard microbiological techniques. Antibiotic susceptibility of the isolates was performed by Kirby-Bauer disk diffusion method on Mueller Hinton agar as per CLSI guidelines (CLSI 2015). The isolates resistant to ≥ 1 agent in ≥ 3 antimicrobial categories were considered multidrug resistant (MDR). ESBL production was screened using two disks, ceftazidime (30µg) and cefotaxime (30 µg) according to the CLSI guidelines. An inhibition zone of ≤22 mm for ceftazidime and ≤ 27 mm for cefotaxime indicated a probable ESBL producing strain which was further confirmed by combination disc method.

Detection of biofilm production

Biofilm production was detected by microtitre culture plate method (TCP), a quantitative test as described by Christensen et al. (1995). Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37˚C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms used was *P. aeruginosa* ATCC 27853. Negative control wells contained inoculated sterile broth without inoculum. The plates were incubated at 37˚C for 24 hr. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA auto reader at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. Interpretation of biofilm production was done according to the criteria of Stepanovic et al. (2000).

Preparation of guava tea and ethanol extract

Guava leaf samples were randomly collected from guava tree in Kathmandu Valley. The leaves were washed with sterile distilled water. Extract first (fresh leaf tea) was prepared by boiling ten leaves in conical flask containing 200 ml of distilled water. Extract second (dry leaf tea) was prepared by boiling freeze dried leaves of the same weight and numbers. Extract third and fourth (ethanol extract of fresh and dried leaves respectively) were prepared by using absolute ethanol as a solvent. The leaves pieces were added to solvent in sterile flask and wrapped in aluminum foil to avoid evaporation and exposed to light. The flasks were placed on a platform shaker at 70 rpm for 3 days. The mixtures were then transferred to tubes and centrifuged for 10 min at 4,000 rpm. The supernatant was collected and stored at 4°C until use.

Determination of antimicrobial activity of guava leave tea against *P. aeruginosa*

Antimicrobial activity of tea and extract were done by agar diffusion method in triplicate on Mueller Hinton Agar (MHA) plates according to the guidelines of the National Committee for Clinical Laboratory Standards. Prior to streaking the plates with bacteria, 5mm diameter wells were punched into the medium using a sterile borer. The surface of the agar plate was swabbed with 0.5 McFarland standarized broth culture of isolated *P. aeruginosa* and allowed to dry for 5min. Each 100µl aliquots of extract and leaves tea were dispensed into each well of MHA plates swabbed with bacteria. For positive control tobramicin was placed at the centre of the plate. Ethanol was used as negative control. *P. aeruginosa* ATCC 27853 was used as positive control organism. The plates were incubated for 24 hours and zone of inhibition was noted.

RESULTS

Out of total 3000 specimen processed, bacterial growth was observed in 32% samples and *P. aeruginosa* was isolated from 7% of the total sample. Bacterial isolates other than *P. aeruginosa* accounted for 25% of the total (Figure 1).

Antibiotic susceptibility pattern and prevalence of MDR *P. aeruginosa*

Most of the isolates were sensitive towards colistin and resistant towards cefepime followed by imipenem and cefotaxime (Table 1). Among the total *P. aeruginosa* isolated in this study 65.3% (*n*=32) were MDR (Figure 2).

Biofilm production detection in *P. aeruginosa*

Out of 49 isolates of *P. aeruginosa* 35 (71.40%) were strong biofilm producer, while 6 (12.24%) were weak producers and 8 (16.32%) were non-biofilm producers (Figure 3).

ESBL production in *P. aeruginosa*

Among the total *P. aeruginosa* isolates, 3 (6.12%) were found to be ESBL producer. Significant numbers of biofilm producers were MDR isolates ($p<0.05$) (Table 2).

Antimicrobial activity of guava leave

Guava leaf tea (fresh and dry leaves tea) showed antimicrobial activity against *P. aeruginosa* isolates. However, compared to the antimicrobial activity of standard antibiotic tobramicin, the zone of inhibition shown by guava leaves was less (Figure 4). The inhibition zone given by fresh leaves for both guava leaf tea and ethanol extract were higher compared to the dry leaves (Figures 4 and 5).

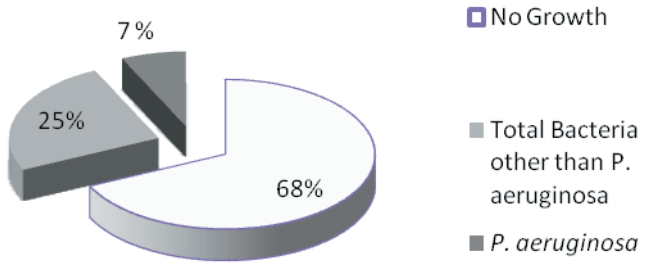

Figure 1: Growth pattern of bacterial isolates in clinical samples

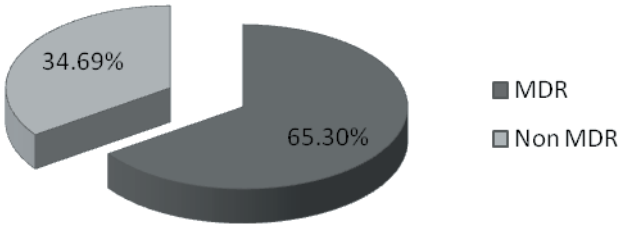

Figure 2: Percentage of MDR strain from the total isolates of *P. aeruginosa*

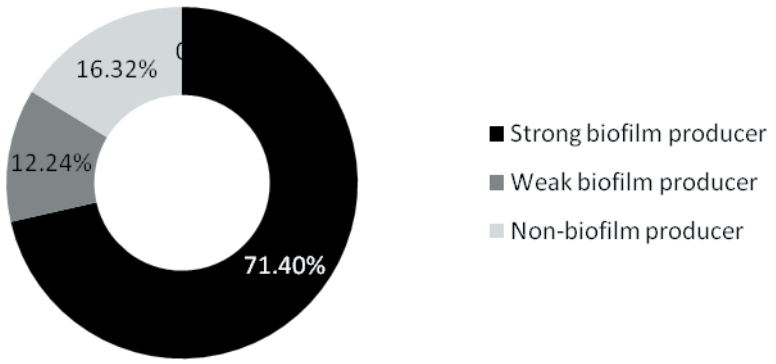

Figure 3: Percentage of biofilm producing *P. aeruginosa*

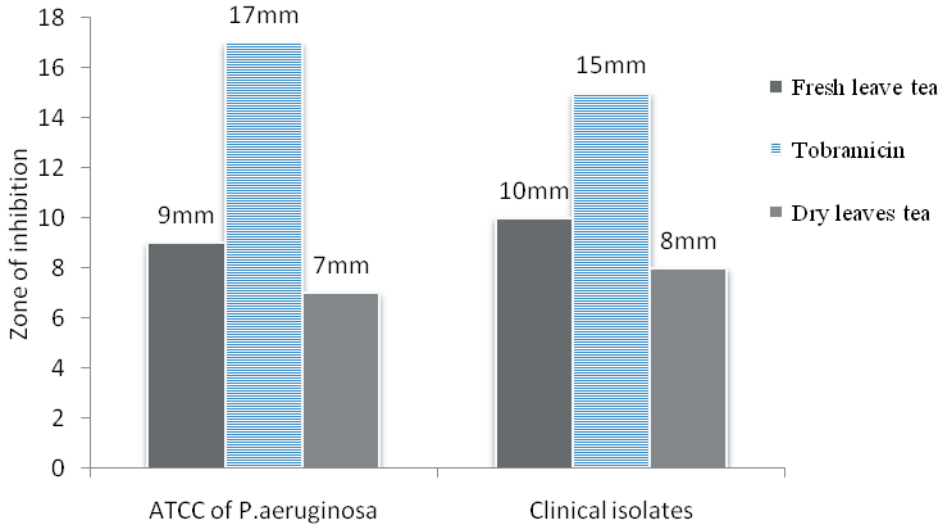

Figure 4: Antimicrobial activity of guava leaves tea and tobramicin against *P. aeruginosa*

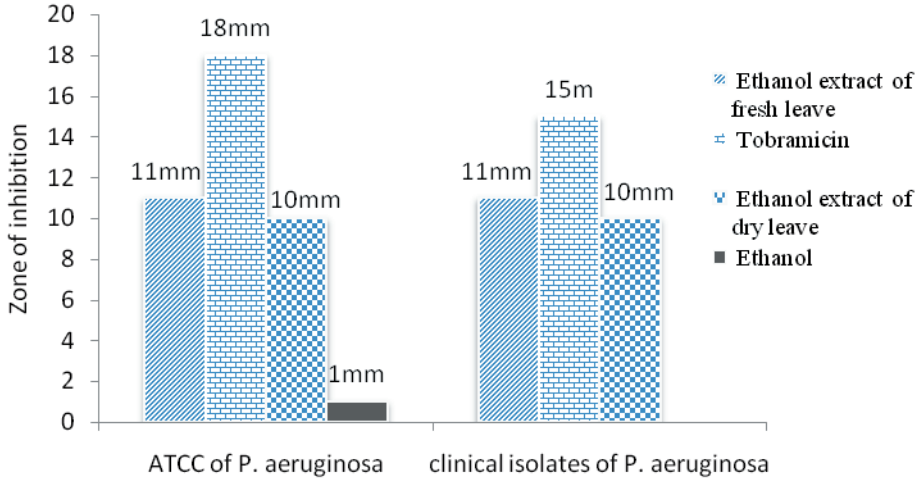

Figure 5: Antimicrobial activity of ethanol extract of guava leaves and tobramicin against *P. aeruginosa*

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

Antibiotic	Sensitive %	Intermediate %	Resistant %
Piperacillin	31(63.3)	-	18 (36.7)
Piperacillin/Tazobactam	27 (55.1)	3 (6.1)	19 (38.8)
Ceftriaxone	20 (40.8)	7 (14.3)	22 (46.9)
Ceftazidime	22 (44.9)	4 (8.2)	23 (46.9)
Cefepime	16 (32.7)	-	33 (67.3)
Aztreonam	29 (59.2)	6 (12.2)	14 (28.6)
Gentamicin	29 (59.2)	-	20 (40.8)
Amikacin	24 (49.0)	1 (2.0)	24 (49.0)
Imipenem	19 (38.8)	1 (2.0)	29 (59.2)
Meropenem	24 (49.0)	2 (4.1)	23 (46.9)
Ciprofloxacin	30 (61.0)	-	19 (38.8)
Colistin	46 (93.9)	-	3 (6.1)
Polymyxin-B	32 (65.3)	5 (10.2)	12 (24.5)
Cefotaxime	20 (40.8)	3 (6.1)	26 (53.1)
Tobramycin	29 (59.2)	1 (2.0)	19 (38.8)

Table 2: Comparison between MDR and biofilm production

MDR	Biofilm producer		Total (%)	p-value
	Positive (%)	Negative (%)		
Positive	31 (96.8)	1 (3.2)	32 (100.0)	0.02
Negative	10 (58.82)	7 (41.18)	17 (100.0)	
Total	**41 (83.67)**	**8 (16.33)**	**49 (100.0)**	

DISCUSSION

P. aeruginosa is an opportunistic nosocomial pathogen of great important due to its resistance to multiple antibiotics (Goel et al. 2013). In this study, prevalence of *P. aeruginosa* from different clinical samples was found to be 27.22% which is less compared to Ali et al. (2015) and Goel et al. (2013) who reported that 39% and 37.7% of *P. aeruginosa* were isolated from ICU patients in Pakistan and India, respectively.

High prevalence of MDR *P. aeruginosa* (65.3%) was reported in this study which is similar to the finding of Fatima et al. (2012) where MDR isolates accounted for 73.9%. This finding is also corroborated with MDR *P. aeruginosa* in Southeast Asia, where 71% reported during 2007-2009 (Suwantarat and Carroll 2016). A high prevalence of MDR *P. aeruginosa* seen in hospital acquired infections was due to selective pressure exerted by over usage of broad spectrum antibiotics. However, the emergence of MDR is related to the empirical use of antibiotics rather than the rational use of broad-spectrum antibiotics before the sample collection.

Prevalence of ESBL producing *P. aeruginosa* was 6.12%. Among the ESBL producer 66.67% were MDR and 6.25% MDR were ESBL producer, which is similar to a study by Stepanovic et al. (2000). Shaikh et al. (2015) reported 25.13% isolates of *P. aeruginosa* were ESBL positives. The ESBL producing *P. aeruginosa* isolates exhibited co-resistance against most of the antibiotics tested.

In this study, 83.67% isolates of *P. aeruginosa* were biofilm producer. Maita and Boonbumrung (2014) reported 79.4% biofilm producer in Thailand. Similar to this study (89.3%) was reported by Sharma and Chaudhary (2015) but lower (48.8%) by Tamaraiselvi et al. (2015). The biofilm production was independent of antibiotic susceptibility profile of the bacteria. When the degree of adhesion of the biofilm is high, the penetration of the antimicrobial compound into its structure is reduced resulting in the increased resistance of the bacteria.

Antibacterial activity of ethanol extract of fresh leaves showed higher activity (11mm) followed by ethanol extract of dry leaves and least activity by dry guava tea solution (7mm). Guava leaf extract and leaf tea exhibited some antibacterial activity against *P. aeruginosa* but less compared to the standard antibiotic tobramycin. Biswas et al. (2013) reported that *P. guajava* has antibacterial effect against both Gram-negative and Gram-positive bacteria. It was due to the presence of alkaloids, flavonoids, tannins, saponins,

glycosides and terpenoids in the leaves extracts of *P. guajava* (Savoia 2012). These phytochemicals have in vitro inhibitory activity against some clinical bacterial isolates. In Brazil, Sanches et al. (2005) reported that the aqueous extracts of *P. guajava* leaves, roots and stem bark were active against the Gram positive bacteria but not against Gram negative species. This can be due to the outer membrane of Gram negative bacteria which act as barrier for penetration of numerous antibiotic molecules. Besides, the enzymes present in the periplasmic space have ability to break down foreign molecules.

CONCLUSION

P. aeruginosa is a pathogen of interest in most of the hospital acquired infection. Increase in the drug resistant *P. aeruginosa* is a great challenge in treatment of infections caused by it. Since crude ethanol extract of guava leave and its tea was able to inhibit *P. aeruginosa*, the compound present in guava leaves extract and tea contain some antimicrobial compounds which can effectively control pathogenic bacteria. It has potential for use in therapy against infections caused by pathogens. Thus we recommend additional test from pure extracts along with further pharmacological evaluation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

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**: 10-21.

Bisht R, Chanyal S and Agrawal PK (2016). Antimicrobial and phytochemical analysis of leaf extract of medicinal plants. *Asian J Pharm Clinic Res* **9**: 131-136.

Biswas B, Rogers K, McLaughlin F, Daniels D and Yadav A (2013). Antimicrobial activities of leaf extracts of guava (*Psidium guajava* L.) on two Gram-negative and Gram-positive bacteria. *Int J of Microbiol* **7**: 122-129.

Centers for Disease Control and Prevention (2015). Health care associated Infections. CDC, Atlanta, USA **4**: 1-14.

Christensen GD, Simpson WA and Younger JA (1995). Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices. *J Clin Microbiol* **22**: 996-1006.

Fatima A, Naqvi SB, Kkali SA, Perveen S and Jabeen S (2012). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* isolated from patients of lower respiratory tract infections. *Springer Plus* **1**: 70.

Goel V, Sumati A, Hogade SA and SG Karadesai (2013). Prevalence of extended spectrum β-latamases, AmpC beta lactamase, and metallo- β - lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter baumanni* in an intensive care unit in a tertiary care hospital. J Sci Sco 40: 28-31.

Heydari S and Eftekhar F (2015). Biofilm formation and β-Lactamase production in burn isolates of *Pseudomonas aeruginosa*. *Jundishapur J Microbio* **4:** 111-123.

Mailoa MN, Mahendradatta M, Laga M and Djide N (2014). Antimicrobial activities of tannins extract from guava leaves (*Psidium gaujava* L) on pathogens microbial. *Int J of Sci and Tech Res* **1**: 236-241.

Maita P and Boonbumrung K (2014). Association between biofilm formation of *Pseudomonas aeruginosa* clinical isolates versus antibiotics resistant and genes involved with biofilm. *J Chem and Pharma Res* **6**: 1022-1028.

Neopane P, Nepal HP, Gautam R, Paudel R, Ansari S and Shrestha S (2017). Is there correlation of biofilm formation with multidrug resistance and esbl production in *Pseudomonas aeruginosa*. *European J of Biomed* **4** : 366-372.

Nithyalakshmi J, Akila K, Mohanakrishnan K and Sumathi G (2015). Evaluation of resistance profile of *Pseudomonas aeruginosa* with reference to biofilm production – An emerging challenge. *J of Den and Med Sci* **14**: 13-18.

Sanches RN, Garcia AD, Schiavini SM, Nakamura CV and Filho DB (2005). An evaluation of antibacterial activities of *P. guajava* (L). *An Int J* **48:** 429-436.

Savoia D (2012). Plant-derived antimicrobial

compounds. *Future Microbio* **7**: 979-90.

Shaikh S, Fatima J, Shakil S, Mohd S, Rizyi D and Amijakamal M (2015). Prevalence of multidrug resistant and extended spectrum beta-lactamase producing *Pseudomonas aeruginosa* in a tertiary care hospital. *Saudi J Bio Sci* **22**: 62-64.

Sharma I and Chaudhary D (2015). Detection of Pel A gene in *P. aeruginosa* from clinical samples using polymerase chain reaction with reference to biofilm production in N E India. *Ind J Res* **4:** 119-121.

Stepanovic S, Vukovi D and Hola V (2000). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. *APMIS* **115**: 891-9.

Suwantarat N and Carroll KC (2016). Epidemiology and molecular characterization of multi-drug resistant Gram negative bacteria in Southeast Asia. *Antimicrob Res and Infect Cont* **5**: 1-8.

Tamaraiselvi S, Swarma SR, Madhavan R and Gomathi S (2015). Biofilm production and multi drug resistant among the isolates from acute and chronic wound infection. *Int J Pharma Bio Sci* **6**: 1197-1203.

Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, Edwards JR and Sievert DM (2016). Antimicrobial resistant pathogens associated with healthcare-associated infections: summary of data reported to the national healthcare safety network at the Centres for Disease Control and Prevention, 2011-2014. *Infect Cont and Hosp Epid* **4**: 1-14.

Extended Spectrum Beta-lactamase Producing Gram Negative Bacterial Isolates from Urine of Patients Visiting Everest Hospital, Kathmandu, Nepal

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ABSTRACT

Objectives: The study was aimed to determine the prevalence of Extended Spectrum Beta Lactamase (ESBL) producing Gram negative pathogens from urine samples along with their antimicrobial resistance.

Methods: This cross-sectional study was conducted from December 2015 to May 2016 at Everest Hospital, Kathmandu. Mid-stream urine samples were collected and processed for culture by standard loop streak method. Identified bacterial isolates were tested for Antibiotic Susceptibility by modified Kirby Bauer disc diffusion method and, were subjected to ESBL screening by using 30µg cefotaxime and ceftazidime. ESBL production was confirmed by combination disc method.

Results: Of the three hundred urine samples, 22.7% (67/300) showed significant growth. Four different bacterial species were identified. Among the isolates, *E. coli* was the most common pathogen (71.64%) followed by *Klebsiella pneumoniae* (14.92%), *Pseudomonas* spp (8.95%) and *Acinetobacter* spp (4.48%). Altogether 92.54% (n=62) isolates were sensitive to gentamicin, 89.55% (n=60) to amikacin, and 79.10% (n=53) to nitrofurantoin. 70.10% (n=47) isolates were resistant to antibiotic ampicillin while 62.68% (n=42) were found as multi-drug resistant (MDR) and 29.8% (n=20) were ESBL producers.

Conclusion: The overall prevalence of MDR and ESBL among uropathogens is low in comparison to other studies though it is essential to have a regular monitoring of ESBL producing clinical isolates in laboratory practice.

Key words: Uropathogens, Mid-stream urine, Antimicrobial resistance, ESBL, MDR

INTRODUCTION

Urinary tract infection (UTI) is a common bacterial infection prevailing in developing countries like Nepal. UTI is defined as a condition in which the urinary tract is infected with a pathogen causing inflammation. The emergence and occurrence of UTI is increasing day by day. The major Gram-negative bacteria involved in causing UTIs are *E. coli*, *Klebsiella* spp, *Proteus* spp, *Pseudomonas* spp, *Citrobacter* spp, *Acinetobacter spp* with most leading uropathogens *E. coli* and *Klebsiella pneumoniae* that belongs to Enterobacteriaceae family (Dromigney et al. 2005).

Clinical experience has indicated the presence of numerous cases of antibiotic resistance to common antibiotics by uropathogens in both developed and developing countries (Gupta 2002). The resistivity has posed challenges in choosing empiric regimens. The cause for resistivity against most prescribed broad-spectrum beta-lactam antibiotic for treatment against

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Gram negative bacterial infection is the production of extended spectrum beta lactamases. These ESBLs enable these bacilli highly efficient in inactivating third generation cephalosporins, monobactams and penicillins (Hawkey 2008) but cannot inactivate cephamycins or carbapenems and are inhibited by clavulanic acid (Bradford 2001; Bush 2001). Several risk factors for ESBL producing Gram negative bacterial infections have been described for the most frequent antimicrobial exposure mostly to third generation cephalosporins resulting in increased morbidity, mortality and costs of health care (Chakraborty et al. 2016).

Prevalence of ESBL producing uropathogens varies widely even in closely related regions. Various studies have reported ESBL producing bacteria in Nepal. Failure in the treatment of infection especially caused by ESBL producing organisms need to be under controlled monitoring in developing countries to avoid widespread distribution of multidrug resistant uropathogens (Chakrawarty et al. 2015). Therefore, this study seeks to evaluate the prevalence of ESBL producing Gram negative bacterial isolates and their existing antibiotic susceptibility pattern.

MATERIALS AND METHODS

This cross-sectional hospital based prospective study was conducted from December 2015-May 2016 in microbiological laboratory of Everest Hospital. Patients (inpatients and outpatients) clinically suspected of UTI of different ages and sexes were selected for the study. A total of 300 mid-stream urine samples were collected and processed according to standard operating protocols during the study period.

Suspected samples were cultured onto Cystine Lactose Electrolyte Deficient (CLED) agar (Hi-Media Pvt. Ltd., India) and Gram-negative bacteria were isolated. The isolates with significant bacteriuria of 10^5 colonies/ml were identified based upon the standard laboratory procedures involving morphological characteristics, Gram's staining, rapid tests(catalase and oxidase) and biochemical tests IMViC (Indole, Methyl red, Voges Proskauer, Citrate), triple sugar iron agar test, oxidation-fermentation test and urease test (Harley and Prescott 2002). Each identified isolate was subjected to invitro antibiotic susceptibility test by modified Kirby-Bauer disc diffusion method as recommended by CLSI guidelines on Muller Hinton Agar (CLSI 2006). Commercially available antibiotic tested were ampicillin, amikacin, cotrimoxazole, ofloxacin, nitrofurantoin, nalidixic acid, ceftriaxone, gentamicin and imipenem. MDR isolates were detected based on their resistance to two or more antibiotics (Cheesbrough 2006; CLSI 2014).

The isolates exhibiting reduced susceptibility to cefotaxime (30µg) and ceftazidime (30µg) were considered as potential ESBL producers. The ESBL production was phenotypically confirmed by combination disc method (CLSI 2014). The disc used were cefotaxime and ceftazidime alone and cefotaxime and ceftazidime in combination with clavulanic acid. A ≥ 5mm increase in growth inhibition zone for any antimicrobial associated with clavulanic acid in comparison with the inhibition zone of antibiotic tested alone confirmed ESBL production.

The collected data analysis was done by SPSS version 20 software and chi-square test was done as a test of significance.

RESULTS

Out of 300 mid-stream urine samples collected, 22.3% (n=67) showed the significant growth among 12 inpatients and 288 outpatients of different age groups and of both the sexes. Four different Gram negative bacteria were isolated; *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter* spp.

From the samples collected between 0-80 age groups, the maximum growth was observed in age group 20-30 years i.e. 22 (32.8%) followed by age group 30-40 years 9 (13.4%) and 70-80 years 9 (13.4%). Among 119 male, 21 (17.64%) showed significant growth and among female 46 (25.4%) showed significant growth. Among males, maximum growth was observed in 20-30 years (23.8%) whereas in females maximum number of growth was found in 20-30 years (37%) followed by age group 10-20 years (15.21%). The prevalence of UTI was found higher in female than male (Table 1).

Table 1: Age and gender wise distribution of patients with isolates

Age group	Male		Female		Total	
	No.	%	No.	%	No.	%
0-10	3	14.28	3	6.5	6	8.96
10-20	1	4.76	7	15.21	8	11.94
20-30	5	23.8	17	36.95	22	32.83
30-40	4	19.04	5	10.86	9	13.43
40-50	1	4.76	5	10.86	6	8.96
50-60	1	4.76	3	6.5	4	5.97
60-70	1	4.76	2	4.34	3	4.48
70-80	5	23.8	4	8.69	9	13.43
Total	21		46		67	

Similarly, from 300 mid-stream urine samples collected, only four different Gram negative bacteria were isolated. Among them, *Escherichia coli* was found predominant with significant growth of 71.64% followed by *Klebsiella pneumoniae* (14.93%), *Pseudomonas aeruginosa* (8.95%) and *Acinetobacter* (4.5%) (Table 2)

Table 2: Microbiological profile of urine isolates

Organism isolated	Total no. of isolates	%
Escherichia coli	48	71.64
Klebsiella pneumoniae	10	14.93
Pseudomonas aeruginosa	6	8.95
Acinetobacter spp	3	4.48
Total	**67**	**100**

The antibiotic susceptibility test profile of the identified isolates was determined by modified Kirby Bauer disc diffusion method. The Gram-negative bacteria showed highest sensitivity to gentamicin (92.54%), amikacin (89.55%) and nitrofurantoin (79.10%) respectively. Similarly high resistant rate was found against ampicillin (70.15%), nalidixic acid (46.26%) followed by cotrimoxazole and ceftriaxone (32.83%). (Table 3)

Table 3: Antibiotic susceptibility profile of Gram-negative isolates

Antibiotics	Sensitive		Intermediate		Resistant		Total
	No.	%	No.	%	No.	%	
Ampicillin	18	26.8	2	2.985	47	70.15	67
Ofloxacin	47	70.15	4	5.97	16	23.88	67
Nalidixic acid	30	44.77	6	8.95	31	46.26	67
Nitrofurantoin	53	79.10	6	8.95	8	11.94	67
Cotrimoxazole	36	53.73	9	8.95	22	32.83	67
Ceftriaxone	38	56.72	7	10.45	22	32.83	67
Amikacin	60	89.55	4	5.97	3	4.48	67
Imipenem	37	55.22	4	5.97	26	38.80	67
Gentamicin	62	92.54	1	1.49	4	5.97	67

About 62.68% of total Gram-negative isolates were MDR. Higher rate of MDR was observed in *E. coli* (66.7%). Similarly, out of 67 isolates, 20 were confirmed as ESBL producer. Prevalence of ESBL was found high in *E. coli* 31.25% (15/48). Total prevalence of ESBL producing Gram negative bacteria was 29.8%. There was no significant association between Gram negative isolates and ESBL production ($p \leq 0.05$) (Table 4).

Table 4: Profile of ESBL producing bacterial isolates

Organism isolated	Total no. of isolates	No. of MDR (%)	ESBL producers (%)
Escherichia coli	48	32(66.7)	15(31.25)
Klebsiella spp	10	4(40)	3(30)
Pseudomonas spp	6	4(66.7)	1(16.7)
Acinetobacter	3	2(66.7)	1(33.3)
Total	**67**	**42**	**20(29.8)**

DISCUSSION

Increasing number of recent reports on bacterial resistance to beta-lactam antibiotics is of serious concern today as these drugs are used for treatment of most bacterial infections. Failure of empirical therapy is increasing proportionally to increasing rate of ESBL producing pathogens. So, detection of ESBL producing bacteria is highly important and this study was carried out with the same motive.

In this study, out of 300 midstream urine samples processed, 22.7% (n=67) showed the significant growth. This growth rate is similar to the other study done in Nepal which have shown 16.88% and 16% growth rate (Poudyal et al. 2011; Tiwari 2014). This growth rate was found higher than the study done by Chander and Shrestha, 2013 with 9.34% whereas lower than the study of Karki 2010 with 58.8%. The study showed higher prevalence of UTI among females 68.66% (n=46) than in males 31.43% (n=21). This rate is similar to study done by Yadav and Satyam 2017; Chaudhary et al. 2016. The reason for higher rate of UTI in female is due to their shorter length of urethra and complex physiology. In female higher growth rate was found in the age group of 20-30 years of age 36.95% which may be due to their sexual activity during this period--a potential factor for UTI. Also, at the age group of 10-20 years, growth was found higher. This might be due to their poor sanitary practices and hormonal changes during the phase.

In this study, out of 67 bacteria isolated, maximum number of *E. coli* was isolated with 71.64% (n=48) followed by *Klebsiella pneumoniae* 14.93% (n=10), *Pseudomonas aeruginosa* 8.95% (n=6) and *Acinetobacter* 4.48% (n=3) respectively. Similar predominance of *E. coli* was found in the recent study which showed 84% growth of *E. coli* and 16% of *Klebsiella* (Yadav et al. 2015). Likewise, similar results were seen in other studies done by Baral et al. 2012; Das et al. 2000; Sharma et al. 2000. The reason for the higher isolation of *E. coli* is due to their commensalism property with ability to bind to the glycoconjugate receptor of epithelial cells of human urinary tract. *Klebsiella* is another major uropathogens isolated from urine samples. These bacteria have several defense mechanisms enabling them to spread infection faster.

In this study, gentamicin and amikacin with the susceptibility rate of 92.54% and 89.55% respectively were found to be the most active drug against Gram negative isolates followed by nitrofurantoin with 79.10% susceptibility. Ampicillin was found as the most resistant drug with 70.10% followed by other antibiotics: ofloxacin, nalidixic acid, cotrimoxazole and ceftriaxone.

In this study, 62.68% of the total Gram negative isolates were found as MDR. This result was in consistent with the study reported from National Public Health Laboratory (Poudyal et al. 2011). In our study, ESBL production rate was 33.3%, 31.25%, 30% and 16.7% of *Acinetobacter*, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively. The overall prevalence of ESBL production was 29.8%. This rate is slightly lower in compare to other studies from Nepal. Though previous studies have shown similar prevalence of 24%, 25%, 25.8%, 26.8%, 31.3% and 33.2% (Ansari et al. 2015, Khanal et al. 2013, Neupane et al. 2016; Pant et al. 2016; Pokharel et al. 2014; Yadav et al. 2015) whereas higher than the study of Chander and Shrestha 2013 which have reported 13.5%. The cause for lower prevalence rate of ESBL producing uropathogens may be due to low sample collection and low patient flow in the hospital. ESBL prevalence is increasing day by day due to self-medication, suboptimal quality of antimicrobial drugs and poor community and personal hygiene (Walson et al. 2001).

CONCLUSION

E. coli was yet again the predominant bacteria isolated from urine sample. Gram negative isolates were highly sensitive to gentamicin, amikacin followed by nitrofurantoin and highly resistant to ampicillin.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Ansari S, Nepal HP, Gautam R, Shrestha S, Neupane P, Gurung G and Chapagain ML (2015). Community acquired multi-drug resistant clinical isolates *E. coli* in a tertiary care center of Nepal. *Antimicrob Resist and Infect Control* **4**: 15.

Baral P, Neupane S, Marasini BP, Ghimire KR, Lekhak B and Shrestha B (2012). High prevalence of multidrug resistance in bacterial pathogens from Kathmandu, Nepal. *BMC Research Notes* **5**: 38.

Bradford PA (2001). Extended spectrum beta-lactamases in the 21st century: Characterization, epidemiology and detection of this important resistance threat. *Clin Microbiol Rev* **14**(4): 933-951.

Bush K (2001). New beta-lactamases in Gram negative bacteria: diversity and impact on the selection of antimicrobial therapy. *Clin Infect Dis* **3**(7): 1085-1089.

Chakraborty S, Mahsna K, Sarker PK, Alam MDZ, Karin MIA and Sayem SMA (2016). Prevalence, antibiotic susceptibility profiles and ESBL production in *Klebsiella pneumoniae* and *Klebsiella oxytoca* among hospitalized patients. *Peridicum Biologorum* **118** (1): 53-58.

Chakrawarty A, Dongol P, Khanal H, Subba P and Banerjee JJ (2015). Extended spectrum beta-lactamases detection and multiple antibiotic existance indexing of *E. coli* from urine samples of patients from a referral hospital of Eastern Nepal. *Int J Appl Sci Biotechnol* **3**(3): 423-426.

Chander and Shrestha (2013). Prevalence of extended spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates in a tertiary care hospital in Kathmandu, Nepal. *BMC Research Notes* **6**: 487.

Chaudhary P, Bhandari D, Thapa K, Thapa P, Shrestha D, Chaudhary HK, Shrestha A, Parajuli H and Gupta BP (2016). Prevalence of Extended spectrum beta-lactamase producing *Klebsiella pneumoniae* isolated from urinary tract infected patients. *J Nepal Health Res Counc* **14**(33): 111-115.

Cheesbrough M (2006). District laboratory Practice in Tropical Countries Part-2, Cambridge University Press.

Clinical and Laboratory Standards Institute/ NCCLS (2006). Performance Standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M100-S15: Clinical and Laboratory Standards Institute, Wayne PA.

Clinical and Laboratory Standards Institute/NCCLS (2014). Performance Standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M100-S15. Clinical and Laboratory Standards Institute.

Dromigney JA, Nabeth P, Juergens-Behr A and Perrier Ggros-Claude JD (2005). Risk factors for antibiotic resistant Escherichia coli isolated from community acquired urinary tract infections in Dakar, Senegal. *J Antimicrob Chemother* **56**: 236-239.

Hawkey PM (2008). Prevalence and clonality of extended spectrum beta-lactamases in Asia. *Clin Microbiol Infect* **14** (Suppl 1): 159-65.

Karki M (2010). Prevalence of microorganisms in the intensive care unit (ICU) patients and their association with indoor environment. MSc dissertation submitted to the Central Department of Microbiology, Tribhuvan University. pp: 7-88

Kateregga JK, Kantume R, Atuhaire C, Lubowa MN and Ndukui JG (2015). Phenotypic expression and prevalence of ESBL producing Enterobacteriaceae in samples collected from patients in various wards of Mulago Hospital, Uganda. *BMC Pharmacology and Toxicology* **16**:14.

Khanal S, Joshi DR, Bhatta DR, Devkota U and Pokhrel BM (2013). Beta-lactamase producing multidrug resistant bacterial pathogens from tracheal aspirates of intensive care unit patients at National Institute of Neurological and Allied Sciences, Nepal. *ISRN Microbiol* 2013: 847569.

Koirala J, Pokhrel BM, Dahal RK, Mishra SK, Khadka

PK and Tuladhar NR (2006). Multi-drug resistant and extended spectrum beta lactamases (ESBL) producing *Salmonella enterica* (Serotypes Typhi and Paratyphi A) from blood isolates in Nepal: Surveillance of resistance and a search for newer alternatives. *Int J Infect Dis* **10**: 434-438.

Neupane S, Pant ND, Khatiwada S, Chaudhary R and Banjara MR (2016). Correlation between biofilm formation and resistance towards different commonly used antibiotics along with ESBL production in uropathogenic Escherichia coli isolated from the patients suspected of urinary tract infection visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. *Antimicrob Resist Infect Control* **5** :5.

Panta K, Ghimire P, Rai SK, Mukhiya RK, Singh RN and Rai G (2013). Antibiogram typing of Gram negative isolates in different clinical samples of a tertiary hospital. *Asian J Pharm Clin Res* **6**: 153-156.

Pant ND, Bhandari R, Poudel A and Sharma M (2016). Assessment of the effectiveness of three different cephalosporins/clavulanate combinations for the phenotypic confirmation of extended spectrum beta-lactamases producer bacterial isolates from urine samples at National Public Health Laboratory, Kathmandu, Nepal. *BMC Research Notes* **9**: 390.

Pfaller MA and Segretti J (2006). Overview of the epidemiological profile and laboratory detection of extended spectrum beta-lactamases. *Clin Infect Dis* **15**(42): 153-163.

Poudyal S, Bhatta DR, Shakya G, Upadhyaya B, Dumre SB and Buda G (2011). Extended spectrum beta-lactamases producing multidrug resistant clinical bacterial isolates at National Public Health Laboratory, Nepal. *Nepal Med Coll J* **13**(1): 34-38.

Pokhrel RH, Thapa B, Kafle R, Shah PK and Tribuddharat C (2014). Co-existence of beta-lactamases in clinical isolates of *Escherichia coli* from Kathmandu, Nepal. *BMC Research Notes* **7**:694

Walson JL, Marshall B, Pokhrel BM, Kafle KK and Levy SB (2001). Carriage of antibiotic-resistant faecal bacteria in Nepal reflects proximity to Kathmandu. *J Infect Dis* **184**: 1163-1169.

Yadav KK, Adhikari N, Khadka R, Pant AD and Shah B (2015). Multidrug resistant Enterobacteriaceae and extended spectrum beta-lactamase producing *Escherichia coli*: a cross-sectional study in National Kidney Center, Nepal. *Antimicrob Resist Infect Control* 4:42.

Prevalence of Asymptomatic Bacteriuria during Pregnancy at a Tertiary Care Hospital of Province No. 2, Nepal

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ABSTRACT

Objectives: The objective of this study was to determine the prevalence of asymptomatic bacteriuria in pregnant women, identify the causative agent responsible for urinary tract infection (UTI) and its antibiotic susceptibility.

Methods: The mid-stream urine sample was streaked on the MacConkey agar (MA) and Blood agar (BA) medium by the semi-quantitative culture technique. Identification of significant isolates was done by standard microbiological techniques. Antibiotic susceptibility test of the isolated organisms was done by modified Kirby Bauer disc diffusion method.

Results: The prevalence rate of asymptomatic urinary tract infection (AUTI) among pregnant women was found to be 42%. The highest number of UTI cases found during pregnancy was in between age 21-25 years (52.22%), in second gravida (51.59%), during 3rd trimester of pregnancy (49.68%) and in winter with 52.22%. *E. coli* was principal organism to cause AUTI (35.48%) during pregnancy. Amikacin, imipenem and nalidixic acid were effective towards Gram negative bacilli whereas vancomycin, tetracycline and amoxyclav were effective towards Gram positive cocci.

Conclusion: All pregnant women visited for antenatal checkups should be advised for the culture and sensitivity test of their urine specimens which will reduce the maternal and child health complications. Different screening test and awareness programme should be conducted at regular interval of time for prevention of AUTI during pregnancy.

Key words: Asymptomatic bacteriuria, *E. coli*, Pregnancy, Pyelonephritis

INTRODUCTION

Asymptomatic bacteriuria (ASB) is the presence of bacteria more than 10^5 per ml in a midstream of urine sample (Yadav et al. 2014) in significant number of a person without symptoms of urinary tract infection (UTI) or bacteriuria. It is an important global health problem prevailing in all age groups. UTI can be classified based on association with complications as, complicated or uncomplicated UTI (Yadav and Prakash, 2016b) and also based on the site involved as upper urinary tract leads to symptomatic bacteriuria or lower urinary tract leads to asymptomatic bacteriuria (Emamghorashi et al. 2012).

The normal physiological changes during pregnancy with reduction in immunity, increased plasma, decreased urine volume and gestational induced glycosuria, pregnant women are more prone to UTI (Lucas and Cunningham, 1993). In addition, increased age, number of childbirths, number of intercourses per week, diabetes, recessive sickle cell anemia, previous history of UTI, immunodeficiency and urinary tract abnormalities can increase the risk of UTI in pregnant women (Giraldo et al. 2012; Raza et al. 2011). As per recent reports, around 25% to 30% women develop symptomatic UTI whereas 2% to 10% women develop ASB during pregnancy (Schnarr and Smaill 2008).

UTI aetiology are diverse showing the geographical variability of causative agents of Gram-negative bacteria

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such as *Escherichia coli*, *Klebsiella* species, *Enterobacter* species, *Citrobacter* species, *Pseudomonas* species, *Proteus* species and Gram-positive bacteria like *Enterococcus* species, Streptococci, *Candida albicans* and *Staphylococcus saprophyticus* (Yadav et al. 2014). *E. coli* is the most common organism causing both community as well as hospital acquired UTI. Considering the importance of UTI in pregnant women which is responsible for several complications, its diagnosis and treatment are essential to maintain the health of mother and baby.

In Nepal, most of health care centers do not carry out routine urine culture test for every pregnant woman during her antenatal checkups presumably due to poor health education, high cost and time duration of culture result usually 2-3 days. Although, the clinicians and healthcare personnel look for the presence of glucose and protein analysis in urine by strip urinalysis method to diagnose UTI that poorly quantify the extent and grade of infection in pregnant women which do not give a clear picture of AUTI or UTI. Very few or negligible studies have been implicated in the prevalence and therapeutic consideration of AUTI at Southern Terai of Nepal. So, it is troublesome to know how frequent this disease is common in this reason among pregnant women. During pregnancy, the development of the AUTI to the symptomatic UTI can be prevented based on proper diagnosis. Therefore, this study was focused to identify the prime importance of causative agent of asymptomatic UTI and prevalence of this disease among at a tertiary care hospital of Dhanusha District at Province No. 2, Nepal which may be helpful to disseminate knowledge regarding asymptomatic UTI as many are unaware of it in this region.

MATERIALS AND METHODS

Study design: A cross-sectional descriptive study was conducted among the pregnant women attending for antenatal care check up at Department of Obstetrics and Gynecology and all the laboratory procedures were carried out in Microbiology Department of Clinical Pathology and Laboratory Medicine at Ram Janaki Hospital, Janakpurdham, Nepal from March 2017 to August 2018. This study included 378 women of reproductive age group who were pregnant.

Ethical consideration: Ethical approval was taken from Ram Janaki Technical Institute and Ram Janaki Hospital, Janakpurdham, Nepal. Informed written verbal consent was also obtained from the participants prior to the study

Inclusion and exclusion criteria of participants: Pregnant women on attending for antenatal check up at the hospital were included while members of staff of the hospital, patients with previous history of preterm labor or spontaneous abortion and those who did not give their consent were excluded.

Specimen collection and processing: The Mid Stream Urine (MSU) samples were collected in the clean, sterile, dry, wide-necked leak-proof container. The distinctive instruction was followed by the patient for the sample collection. When immediate processing was not possible, the specimen was refrigerated at 4-6°C, and when a delay of more than 2 hours, boric acid (1.8 % w/v) was added as preservative to the urine.

Culture of specimen: Media were prepared as instructed by the manufacturer company (Himedia). The urine sample was streaked on the MacConkey agar (MA) and Blood agar (BA) medium by the semi-quantitative culture technique using a standard loop. After mixing the urine sample in the container thoroughly, a loopful of sample was touched to the centre of the plate, from which the inoculum was spread in a line across the diameter of the plate. Without flaming the loop was drawn across the entire plate, crossing the first inoculum streak numerous times to produce isolated colonies. The plates were incubated aerobically at 37°C overnight.

The number of colonies was counted and the number of bacteria i.e. Colony Forming Unit (CFU) per ml urine estimated in accordance to the volume of urine inoculated and reported as:- Less than 10^4 /ml organisms - Not significant, 10^4 -10^5 /ml organisms - Doubtful significance (suggest repeat specimen) and More than 10^5 /ml organisms - Significant bacteriuria

Identification of the isolates: Identification of significant isolates was done by microbiological techniques as described in the Bergey's Manual of Systematic Bacteriology (2nd Edition) which involves morphological appearance of the colonies, staining reactions and biochemical properties. Gram positive organisms were tested by catalase test, oxidase test, coagulase test and their specific biochemical tests. Gram negative isolates were identified on the basis of various biochemical tests such as catalase test, oxidase test, O/F test, MR/VP test, SIM test, citrate test, urease test, TSI test.

Antibiotic susceptibility testing: Antibiotic susceptibility test of the isolated organisms was done

by modified Kirby Bauer disc diffusion method. Bacterial inoculum was prepared by suspending the freshly grown bacteria in 2 ml of sterile nutrient broth and incubated at 37 °C for 3-4 hours. The turbidity of tube was matched with 0.5 Mc Farland turbidity standards. The inoculum was then streaked on entire Muller-Hinton agar (MHA) plate. Antibiotic discs were placed around the outer edge of the plate and incubated overnight at 37 °C. Diameter of zone of inhibition was measured and zone diameter criterion was used to interpret the level of susceptibility to each antibiotic (CLSI, 2013).

Statistical analysis: The data were analyzed using SPSS 18.0 version and Microsoft excels 2007. The Chi-square test was used to test for the positive cases of UTI during pregnancy in relation to age, gravidity, seasons and trimester. The p-value ($p<0.05$) was considered as statistically significant.

RESULTS

Prevalence rate of AUTI among pregnant women: Out of total 378 pregnant women, the prevalence rate of AUTI among pregnant women was found to be 42% (Figure 1).

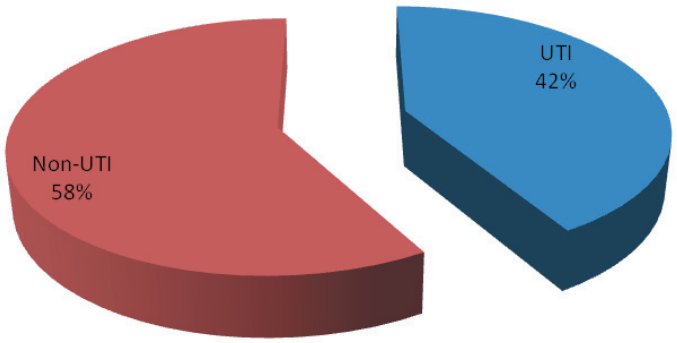

Figure 1: Prevalence rate of AUTI among pregnant women

Age-wise distribution of prevalence of AUTI in pregnant women: The highest number of AUTI cases found during pregnancy was in between age 21 to 25 years (52.22%) followed by age 26 to 30 years (19.74) (Table 1).

Table 1: Age-wise distribution of prevalence of AUTI in pregnant women

Age groups (years)	No. of pregnant women (%)	No. of positive cases (%)	p-value
≤ 20	78 (20.63)	26 (16.56)	
21-25	149 (39.41)	82 (52.22)	
26-30	105 (27.77)	31 (19.74)	0.09
>30	46 (12.16)	18 (11.46)	
Total	**378**	**157 (41.53)**	

Prevalence of AUTI in pregnant women in relation to gravidity: The more number of positive cases of AUTI during pregnancy was found in second gravida (51.59%) followed by prime gravida (29.93%). (Table 2).

Table 2: Prevalence of AUTI in pregnant women in relation to gravidity

Gravidity	No. of pregnant women (%)	No. of positive cases (%)	p-value
Prime gravida	133 (35.18)	47 (29.93)	
Second gravida	168 (44.44)	81 (51.59)	0.50
Multi gravid	77 (20.37)	29 (18.47)	
Total	**378**	**157**	

Trimester pattern of prevalence of AUTI in pregnant women: Most of the study participant were attacked by uropathogens during 3rd trimester of pregnancy (55.31%) and was statistically significant (p=0.0001) (Table 3).

Table 3: Prevalence of AUTI in pregnant women in relation to trimester

Trimester	No. of positive cases		Total	p-value
	Present (%)	Absent (%)		
1st trimester (1st 12 weeks)	26 (26.53)	72 (73.46)	98	
2nd trimester (13-28 weeks)	53 (38.12)	86 (61.87)	139	0.0001
3rd trimester (29-40 weeks)	78 (55.31)	63 (44.68)	141	

Bacteriological profile of uropathogens causing AUTI in pregnancy: *E. coli* was found to be predominant organism to cause urinary tract infection (35.48%) followed by *K. pneumoniae* (18.27%) during pregnancy (Figure 2).

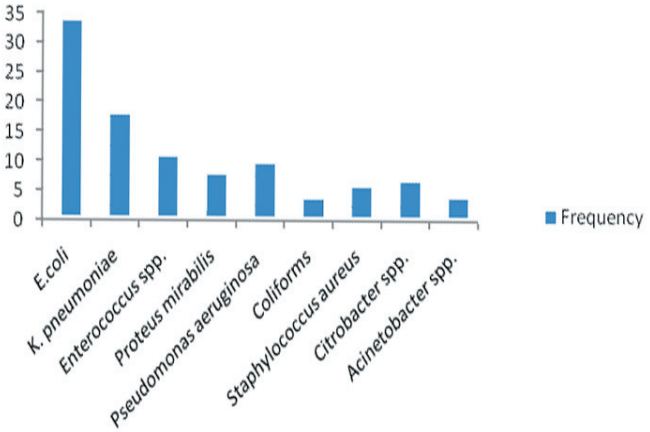

Figure 2: Profile of uropathogens causing AUTI in pregnancy

Antibiogram of Gram negative bacilli: More Gram negative bacilli were sensitive towards amikacin (69.89%) followed by imipenem (65.59%) and nalidixic acid (61.29%) while least sensitive towards cotrimoxazole (31.18%) (Table 4).

Table 4: Antibiotic susceptibility pattern of Gram negative bacilli

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin	65 (69.89)	4 (4.30)	24 (25.80)
Gentamycin	41 (44.08)	15 (16.12)	37 (39.78)
Ciprofloxacin	49 (52.68)	10 (10.75)	34 (36.55)
Cotrimoxazole	29 (31.18)	2 (2.15)	62 (66.660
Norfloxacin	36 (38.70)	8 (8.60)	49 (52.68)
Nitrofurantoin	52 (55.91)	14 (15.05)	27 (29.03)
Nalidixic acid	57 (61.29)	3 (3.22)	33 (35.48)
Imipenem	61 (65.59)	7 (7.52)	25 (26.88)

Antibiogram of Gram positive cocci

More Gram positive cocci were sensitive towards vancomycin (77.41%) followed by tetracycline (73.11%) and amoxyclav (70.96%) while least sensitive towards erythromycin (49.46%) (Table 5).

Table 5: Antibiotic susceptibility pattern of Gram positive cocci

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Amoxyclav	66 (70.96)	7 (7.52)	20 (21.50)
Ciprofloxacin	53 (56.98)	11 (11.82)	29 (31.18)
Erythromycin	46 (49.46)	9 (9.67)	38 (40.86)
Linezoid	60 (64.51)	2 (2.15)	31 (33.33)
Tetracycline	68 (73.11)	5 (5.37)	20 (21.50)
Teicoplanin	54 (58.06)	3 (3.22)	36 (38.70)
Vancomycin	72 (77.41)	3 (3.22)	18 (19.35)

DISCUSSION

The prevalence of significant asymptomatic bacteriuria (bacterial count >1x10^{5}cfu/ml) called the asymptomatic UTI in pregnancies. This study reveals the prevalence rate of AUTI among pregnant women was 42% which is almost in accordance with a study conducted by Valentina and Srirangaraj in 2016, the prevalence of UTI in pregnant women was 45%. Yadav and Yadav in 2018 from Chitwan Medical College reported AUTI of 51.83%. Similarly, Adabara et al. in 2012, Little et al. in 2016, Mokube et al. in 2013 and August et al. in 2012, accounted the prevalence of UTI as 75%, 66%, 23.5% and 21.15% respectively. Whereas, Mobasheri et al. in 2002, Hernandez et al. in 2007, Tadesse in 2007, Obirikorang et al. in 2012, Turpin et al. in 2007, Demilie et al. in 2012 and Thakre et al. in 2012, reported the low prevalence of UTI as 7- 10%.

The reason behind for higher prevalence could be attributed to the low income status of the patients, anemia, sexual activity during pregnancy, lack of proper personal, environmental hygiene, population susceptibility, poor housing, ventilation, sanitation and drainage systems (Dutta, 2008; Kolawole et al. 2009). The other established fact is that the urethra in females is shorter, wider and close to the anus contributes to the higher prevalence of UTI in women. Being in close proximity, the bacteria from the rectum can easily go up the urethra increasing the rate of infections (Kolawole et al. 2009; Ebie et al. 2009). Moreover, biochemical, hormonal and immunological normal physiological changes in pregnancy to reduce ureteric muscular tone in ureter and urethra, and increase in mechanical pressure from the gravid uterus, leading to urinary stasis, which act as good culture media, favoring the bacterial growth and multiplication in urine (Obiogbolu et al. 2001)

Age group in pregnancies revealed a significant difference in the prevalence of asymptomatic bacteriuria ($p<0.05$). The highest number of UTI cases found during pregnancy was in between 21 and 25 years. Similar finding was also obtained by Adeyeba et al. in 2002, Amadi et al. in 2007, Akinleye et al. in 2014, Valentina and Srirangaraj in 2016 and Yadav and Yadav in 2018. This could be recognized that the subjects with this age group are sexually more active which could favor the incidence of UTIs (Dutta 2008; Adeyeba et al. 2002).

Majority of the studies show a higher prevalence of UTI in multigravida and stress on the fact that the prevalence of UTI in pregnancy increases with parity (Emamghorashi et al. 2011). The result of this study demonstrates 29.93%, 51.59% and 18.47% of pregnant women suffered from asymptomatic UTI with respect to prime, second and multi gravida respectively. More number of positive cases of UTI during pregnancy was found in second gravida of 51.59% followed by prime gravida of pregnancy with 29.93% and was found to be statistically insignificant ($p=0.50$). This may be due to glycosuria, which is present in 70% of pregnant women, increases the urinary level of estrogen and progesterone, and decreases the patient's ability to fight invasive bacteria (Rizvi et al. 2011). In contrast to this study, Lavanya et al. in 2002, Marahatta et al. in 2011 and Valentina and Srirangaraj in 2016 found the higher prevalence of UTI associated with pregnancy among the primigravida.

Increased parity, age and gestational age increases the risk of UTI in pregnant women. Most of the study participants were infected by uropathogens during 3rd trimester of pregnancy. An article published by researcher of Chitwan Medical College, Nepal accounted the highest prevalence (45.33%) of asymptomatic bacteriuria was observed in the second trimester of pregnancy (Yadav and Yadav, 2018). A similar finding was also reported by Paty in 2018 with high percentage of asymptomatic bacteriuria in the second trimester of pregnancy which is in accordance with this study.

E. coli was found to be predominant organism to cause urinary tract infection. A similar result was also found by Valentina and Srirangaraj, 2016; Paty, 2018. This could be the reason for Gram negative bacteria being the dominant etiologic agent of UTI due to poor or unhygienic genital practices by pregnant women who may find it difficult to clean their anus properly after defecating or clean their genital after passing urine leading to infection by faecal contamination. Another reason behind for *E. coli* proliferation may be due to the increased levels of amino acids and lactose in pregnancy (Obiogbolu et al. 2009).

More Gram negative bacilli were sensitive towards amikacin followed by imipenem and nalidixic acid while least sensitive towards cotrimoxazole. More Gram positive cocci were sensitive towards vancomycin

followed by tetracycline and amoxyclav while least sensitive towards erythromycin. Valentina and Srirangaraj in 2016 reported among the Gram negative bacilli, higher degree of sensitivity was observed with Nitrofurantoin, Amikacin and Imipenem and among the Gram positive cocci, higher degree of sensitivity was observed with Vancomycin, Amoxyclav and Tetracycline. These findings are in line with the findings by Yadav and Yadav in 2018.

The diverse pattern of antimicrobial sensitivity and resistance among different communities and hospitals is due to indiscriminate use of antibiotics causing resistant strains (Yadav and Prakash, 2016a). The increase in antibiotic resistant pattern could be due to antibiotic abuse due to lack of knowledge to health practitioner and consumer. Also, the low cost and availability of antibiotics without prescriptions in this area could be another contributing factor for antibiotic abuse, and thus the resistance (Yadav and Yadav, in 2018)

CONCLUSION

This study highlights the moderate prevalence of UTI among pregnant women regardless to women's age, parity and gestational age. Routine screening and urine culture at least once in each trimester of pregnancy could be useful to investigate asymptomatic or symptomatic UTI to minimize maternal and neonatal morbidity and mortality.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Adabara NU, Momoh JA, Bala JD, Abdulrahman AA and Abubakar MB (2012). The prevalence of bacterial urinary tract infections (UTI) among women attending antenatal clinic in the general hospital, Minna in Niger state. *IJBR* **3**(3)**:** 171-173.

Adeyeba OA, Adekoya JA, Lowed AO and Adesiji YO (2002). Urinary Tract Infections amongst Patients attending Sexually Transmitted Disease Clinic in Ibadan, Nigeria. *J Sci Eng* **9**(4)**:** 4552-4560.

Akinleye OM, Olaniyan JAT, Folorunso JB, Ipaye TO, Abdulraheem LD and Ayodele FA (2014). Asymptomatic Bacteriuria among antenatal Women Attending private Hospital in Lagos, Nigeria. Sch. *J App Med Sci* **2**(6D)**:** 3076-3080.

Amadi ES, Enemuo OB, Uneke CJ, Nwosu OK, Onyeagba RA and Ugbogu OC (2007). Asymptomatic bacteriuria among pregnant women in Abakaliki, Ebonyi State, Nigeria. *J Med Sci* **7**(4)**:** 698-700.

August SL and De Rosa MJ (2012). Evaluation of the prevalence of urinary tract infection in rural Panamanian women. *PloS ONE* **7**(10)**:** 0047752.

CLSI (2013). Performance Standards for Antimicrobial Disk Susceptibility Testing; Twenty third informational supplements. Clinical and Laboratory Standards Institute 950 West Valley Road, Suite 2500 Wayne, PA 19087 USA pp. 116-122.

Demilie T, Beyene G, Melaku S and Tsegaye W (2012). Urinary bacterial profile and antibiotic susceptibility pattern among pregnant women in northwest Ethiopia. *Ethiop J Health Sci* **22**(2)**:** 121-128.

Dutta DC (2008). DC Dutta's textbook of Obstetrics and Gynecology. 6thed: New central book agency.

Ebie MY, Kandakai-Olukemi YT, Ayanbadejo J and Tanyigna KB (2001). Urinary Tract Infections in a Nigerian Military Hospital. *Nigerian J Microbiol* **5**(1)**:** 31-73.

Emamghorashi F, Mahmoodi N, Tagarod Z and Heydari ST (2012). Maternal urinary tract infection as a risk factor for neonatal urinary tract infection. *Iran J Kidney Dis* **6**(3)**:** 178–180.

Giraldo PC, Araújo ED, Junior JE, Amaral RLGD, Passos MRL, Gonçalves AK (2012). The Prevalence of Urogenital Infections in Pregnant Women Experiencing Preterm and Full-Term Labor. *Infect Dis Obstetrics Gynecol* 1–4.

Hernandez BF, Lopez Carmona JM, Rodriguez Moctezuma JR, PeraltaPedrero ML, Rodriguez Gutierrez RS and Ortiz Aguirre AR (2007). Asymptomatic bacteruiria frequency in pregnant women and uro pathogen in vitro antimicrobial sensitivity. *Ginecol Obstet Mex* **75:** 325-331.

Kolawole AS, Kolawole OM, Kandaki-Olukemi YT, Babatunde SK, Durowade KA and Kolawole CF (2009). Prevalence of urinary tract infections (UTI)

among patients attending Dalhatu Araf Specialist Hospital, Lafia, Nasarawa State, Nigeria. *Int J Medic Med Sci* **1**(5)**:** 163-167.

Lavanya SV and Jogalakshmi D (2002). Asymptomatic bacteriuria in antenatal women. *Indian J Med Microbiol* **20:** 105-106.

Little P, Turner S, Rumsby K, Jones R, Warner G, Moore M, Lowes JA, Smith H, Hawke C, Leydon G and Mullee M (2016). Validating the prediction of lower urinary tract infection in primary care: sensitivity and specificity of urinary. *Int J Curr Microbiol App Sci* **5**(1)**:** 452-460.

Lucas MJ and Cunningham FG (1993). Urinary tract infection in pregnancy. *Clinical obstet Gyeacol* **36**:555-68.

Marahatta R, Dhungel BA, Pradhan P, Rai SK and Choudhury DR (2011). Asymptomatic Bacteriuria among pregnant women visiting Nepal medical college teaching hospital, Kathmandu, Nepal. *Nepal Med Coll J* **13**(2)**:** 107-110.

Mobasheri E, Tabbaraei A, Ghaemi E, Mojerloo M, Vakili M and Dastforooshan M (2002). Distribution of asymptomatic bacteriuria in pregnant women referring to Dezyani Hospital in Gorgan. *J Gor Univer Med Sci* **4:** 42-6.

Mokube MN, Atashili J, Halle-Ekane GE, Ikomey GM and Ndumbe PM (2013). Bacteriuria amongst pregnant women in the Buea hospital district, Cameroon: Prevalence, predictors, antibiotic susceptibility patterns and diagnosis. *PLoS ONE* **8**(8)**:** e71086.

Obiogbolu CH, Okonko IO, Anyamere CO, Adedeji AO, Akanbi AO and Ogun AA, et al. (2009) Incidence of urinary tract infections (UTIs) among pregnant women in Akwa metropolis, South eastern Nigeria. *Sci Res Essay* **4**: 820-824.

Obirikorang O, Quaye L, Bio FY, Amidu N, Acheampong I and Addo K (2012). Asymptomatic bacteriuria among pregnant women attending antenatal clinic at the University Hospital, Kumasi, *Ghana J Med Biomed Sci* **1**(1)**:** 38-44.

Paty PB (2018). Prevalence of Urinary Tract Infection in Pregnant Women in a Tertiary Care Hospital of Odisha. *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)* **17**(5)**:** 47-50.

Raza S, Pandey S and Bhatt CP (2011). Microbiological analysis of isolates in Kathmandu Medical College Teaching Hospital, Kathmandu, Nepal. *Kathmandu Univ Med J* **9**(36):

Rizvi M, Khan F, Shukla I and Malik A (2011) Rising prevalence of antimicrobial resistance in urinary tract infections during pregnancy: necessity for exploring newer treatment options. *J Lab Physicians* **3**(2)**:** 98–103.

Schnarr J and Smaill F (2008). Asymptomatic bacteriuria and symptomatic urinary tract infections in pregnancy. *Eur J Clin Invest* **38**(S2)**:** 50-57.

Tadesse A, Negash M and Ketema LS (2007). Asymptomatic bacteriuria inpregnancy: assessment of prevalence, microbial agents and their antimicrobial sensitivity pattern in Gondar Teaching Hospital, North West Ethiopia. *Ethiop Med J* **45:** 143-149.

Thakre SS, Dhakne SS, Thakre SB, Thakre AD, Ughade SM and Kale P (2012). Can the Griess nitrite test and urinary pus cell count of 5 cells per microlitre of urine in pregnant women be used for screening or the early detection of urinary tract infections in rural India. *J Clin Diagn Res* **6**(9)**:** 1518-1522.

Turpin CA, Minkah B, Danso KA and Frimpong EH (2007). Asymptomatic bacteriuria in pregnant women attending antenatal clinic at Komfo Anokye teaching hospital, Kumasi,Ghana. *Ghana Med J* **41:** 26-9.

Valentina Y and Srirangaraj S (2016). Pregnancy associated Urinary Tract Infection: Prevalence and Screening. *Int J Curr Microbiol App Sci* **5**(1)**:** 452-460.

Yadav K and Prakash S (2016a). Antimicrobial Resistance (AMR): A Global Problem. *Global Journal of Public Health and Epidemiology* **3**(1)**:** 120-138

Yadav K and Prakash S (2016b). Antimicrobial Resistance Pattern of Uropathogens Causing Urinary Tract Infection (UTI) Among Diabetics. *Biomedical Research Int* **1:** 07-15

Yadav K, Prakash S, Serayi RC, Shilpkar T and Shrestha S (2014). Antimicrobial susceptibility test of pathogens isolated from urinary tract infection suspected cases *Janaki Medical College Journal of Medical Sci* **2**(1)**:** 28-34

Yadav LK and Yadav RL (2018). Asymptomatic UTI in pregnancy attending at tertiary care of Nepal. *Int J Res Med Sci* **6**(4)**:** 1119-1128

Comparative Microbiological Assessment of Drinking Water Collected from Different Areas of Kathmandu Valley

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ABSTRACT

Objectives: This study was aimed to evaluate different water samples in terms of total bacterial load and total coliform load in comparison with different parameters such as pH, type of samples, chlorination status, turbidity, temperature and collection areas.

Methods: Altogether 250 water samples were collected; 110(44%) were ground water samples, 60(24%) were public tap water samples and 80(32%) were bottled water samples. Total Coliform load was evaluated using Membrane Filtration Technique and Total Bacterial Load was evaluated using Aerobic Plate Count Technique. pH was measured using pH meter, turbidity was measured using nephelometer, temperature was measured using temperature probe and bottled water were considered chlorinated.

Results: Among these 250 samples, the highest mean bacterial load was seen in public tap water samples (137×10^3 cfu/ml) and least mean bacterial load was seen on bottled water sample (28×10^3). Similarly, highest mean coliform load was seen on ground water samples (81 cfu/ml) and least mean coliform load was seen on bottled water samples (6 cfu/ml).

Conclusion: This study has concluded that ground water samples contain large number of coliforms which suggest it might be fecally contaminated. Also the result had shown even bottled water contained coliforms. Therefore, it is recommended to treat ground water before using. Proper care must be taken during manufacture of bottled water.

Key words: Water samples, total bacterial load, total coliform load.

INTRODUCTION

Water covers 70% of the earth surface and is also present in varying amount in the atmosphere. It is an essential component of all cells and a requirement for life. Consequently, 250 million people are exposed to water-borne disease resulting in 10-20 million deaths every year (Pironcheva 2004). It is well known that 88% of the global diarrheal diseases are water-borne infections caused by drinking unsafe and dirty water (Gundry et al. 2004). Nepal faces a serious crisis of potable drinking water in both urban cities and rural areas.

In Nepal, every summer, water borne epidemics (of diarrhea, cholera, typhoid, etc.) hit different parts of the country including Kathmandu valley and cause a heavy death toll. Most of the water sources in Kathmandu valley do not comply with the guidelines provided by WHO (Bottino 1991).

This study was conducted with an objective to assess the microbiological quality of water in terms of heterotrophic bacteriological load and coliform load. This research would contribute to evaluate the safety of drinking water and provide information on further treatment necessary for drinking water. The results of this research can be an open source for academic and be put to scrutiny for any and all.

MATERIALS AND METHODS

The study was conducted on water sample collected

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from different places of Kathmandu valley. The study was conducted in Microbiology Laboratory of Kathmandu College of Science & Technology, Kamalpokhari, Kathmandu, Nepal. The water samples were collected in pre-sterilized glass BOD bottles (15lbs at 121°C for 15 minutes) of 300ml capacity and transported in an ice box containing freezer ice packs. These samples were processed as soon as arrival in the laboratory. pH was measured on site by using automatic digital pH meter (Hanna instruments) and temperature by dipping bulb of standard temperature probe (Hanna instruments). Turbidity was determined by using nephelometer (Hanna instruments). Jar water and bottled water sample was considered chlorinated. A total of 100ml each water sample was filtered through 0.45µm membrane filter (Pall Corporation). The membrane filter was aseptically transferred to Eosin Methylene Blue agar (EMB, HiMedia Laboratories) and incubated at 37°C for 24hrs. Coliforms were enumerated by observing pink colonies with or without metallic sheen grown in EMB agar. Bacterial load was determined using plate count agar (PCA) technique. Enumeration was done after incubation at required temperature and time. Comparative distribution of bacterial and coliform load was done with various parameters.

RESULTS

A total of 250 samples were collected from various sources such as ground 110(44%), public tap water 60(24%) and bottled water 80(32%) as shown in fig.1

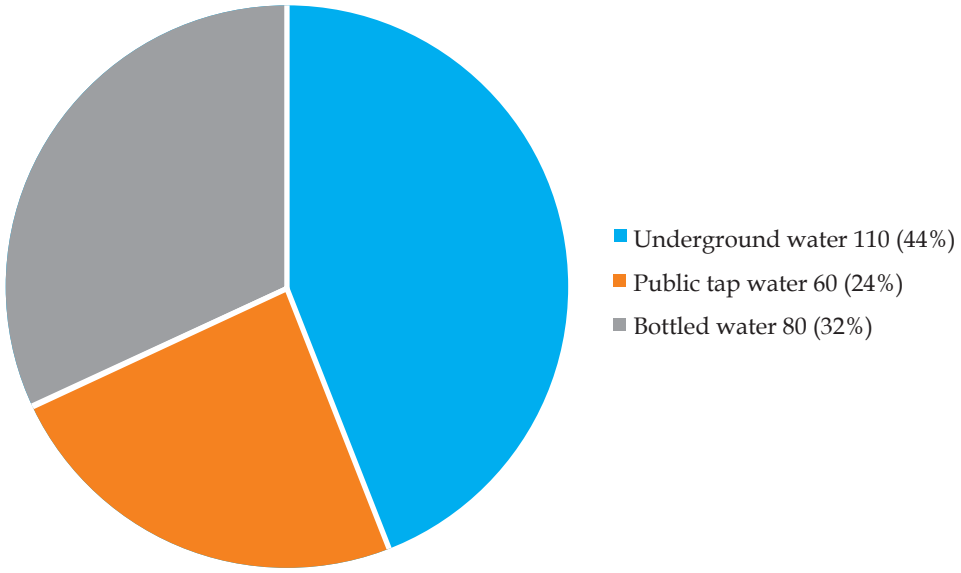

Figure 1. Distribution of water from various sources

These collected samples were designated as Area 1, Area 2, Area 3 & Area 4. Area 1 consist of places such as Kamalpokhari, Kamaladi, Teku, Tripureshwor, Kirtipur, Dhobidhara, Bagbajar, Dillibajar, Nagpokhari, Gairidhara and Panipokhari. Area 2 - Kapan, Bafal, Sundarijal, Boudha, Bansbari, Baluwatar, Balaju. Area 3- Kuleshwor, Anamnagar, Sitapaila, Patan, Sanepa, Bhaktapur. Area 4 - Tahachal. These area wise distribution of water samples are shown in fig 2.

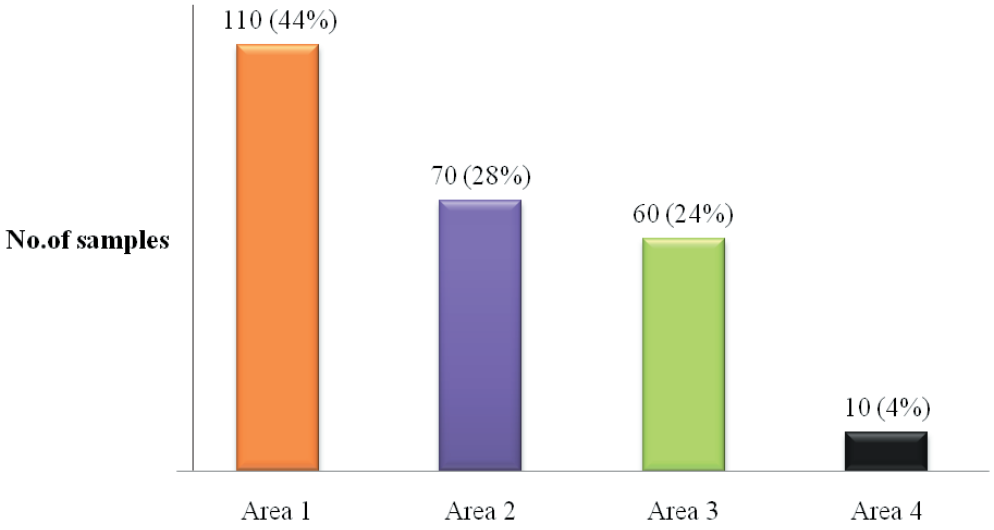

Figure 2. Area wise distribution of water samples

The highest bacterial load was observed on public tap water (137×10^3) cfu/ml and least bacterial load was observed on bottled water samples (28×10^3) cfu/ml as shown in table 1.

Table 1: Distribution of bacterial load in different water samples.

Type of samples	Total no. of samples	Mean bacterial load (cfu/ml)
Ground water	110	130×10^3
Public tap water	60	137×10^3
Bottled water	80	28×10^3

The highest mean coliform load was seen on ground water samples (81 cfu/100ml) and least mean coliform load was seen on bottled water samples (6cfu/100ml) (Table 2).

Table 2: Distribution of coliform load in different water samples.

Type of samples	Total no. of samples	Mean coliform load (cfu/ 100ml)
Ground water	110	81
Public tap water	60	30
Bottled water	80	6

The highest mean coliform load with respect to different Areas was observed on area 2 (55cfu/100ml) & least mean coliform load was seen on Area 4 (3 cfu/100ml) (table 3).

Table 3: Distribution of coliforms in water samples of different areas.

Area	Total no. of samples	Mean coliform load (cfu/100ml)
Area 1	110	45
Area 2	70	55
Area 3	60	32
Area 4	10	3

The highest bacterial and coliform load with respect to different temperature range was observed on temperature range (26°C to 27°C) i.e. 123×10^3 cfu/ml and 61 cfu/100ml respectively while the least bacterial and coliform load was observed in temperature range (24°C to 25°C) i.e. 41×10^3 cfu/ml and 4cfu/100ml (Table4).

Table 4: Distribution of bacterial and coliform load in water samples in different temperature range

Temperature	Total No. of sample	Mean bacterial load (cfu/ml)	Mean coliform load (cfu/ 100ml)
24 - 25°C	10	41×10^3	4
25-26°C	50	71×10^3	6
26-27°C	60	123×10^3	61
27-28°C	130	89×10^3	56

The highest mean bacterial and coliform load in different water samples with respect to turbidity was observed on turbid water (85×10^3 cfu/ml and 74 cfu/100ml) while least bacterial and coliform load was observed in non-turbid water (68×10^3cfu/ml and 30 cfu/100ml) (table 5)

Table 5: Distribution of bacterial and coliform load in water samples with respect to turbidity

Turbidity	Total No. of samples	No. of bacterial load (cfu/ml)	No. of coliform load (cfu/100ml)
Turbid	70	85×10^3	74
Non-turbid	18	68×10^3	30

The highest mean bacterial and coliform load in different water samples with respect to water treatment was seen on non-treated water (106×10^3 cfu/ml & 83 cfu/100ml) while the least mean bacterial & coliform load was observed in treated water (28×10^3 cfu/ml & 23 cfu/100ml) (table 6).

Table 6: Distribution of bacterial and coliform load in water samples with respect to water treatment.

Chlorination	Total No. of samples	Total bacterial load (cfu/ml)	Total coliform load (cfu/100ml)
Chlorinated	80	28×10^3	23
Non-chlorinated	170	106×10^3	83

The highest mean bacterial and coliform load in different water samples with respect to pH range was seen between 5-6 & 6-7 (94×10^3 cfu/ml and 50 cfu/100ml) while the least mean bacterial & coliform load was observed in pH range between 6-7 and 5-6 (67×10^3 cfu/ml and 46 cfu/100ml) (table 7).

Table 7: Distribution of bacterial and coliform load in water samples with respect to pH

pH	Total No. of sample	Mean bacterial load (cfu/ml)	Mean coliform load (cfu/100ml)
5-6	130	94×10^3	46
6-7	120	67×10^3	50

DISCUSSION

This study assesses the physiochemical and microbiological quality of bottled water, ground water and public tap water collected around different areas of Kathmandu valley. The highest number of sample was underground water (110) followed by bottled water (80) and public tap water (60).

The result was found to be consistent with various other studies conducted, which found 82.6% and 92.4%, drinking water samples were not consistent with the WHO guideline of drinking water (Prasai 2007). In similar study, total coliform positive rate of 80% was reported for mineral or euroguard treated water (Rai et al. 2012). In present study, it showed ground water samples contain highest bacterial & coliform loads. Similar study done in Kathmandu valley, Nepal enumerated 267 cfu/ml coliform load in ground water samples (Pant 2011). Following ground water, mean coliform load was high in public tap water i.e. 30cfu/100 ml. A similar study found 91.18% of contamination by coliforms (Aryal et al. 2012). This may be due to contamination in pipeline system, drainage system and discontinuity in water supply pattern or carelessness. Contamination in bottles may be due to improper handling, improper disinfections, infiltration of contaminated water, leakage etc.

On evaluating data of distribution of coliform in water samples of different areas, Area 2 showed highest number of coliform distribution. This may be due to the fact that most of the samples from area 2 were ground water samples. Similarly, coliform distribution was higher in turbid water sample and temperature range of 27 °C to 28 °C. In this study, the highest distribution of coliform was observed at pH 6-7. This may be due to fact that most of coliforms are neutrophillic and optimally grow in neutral pH.

CONCLUSION

This study concludes that ground water, public water and even bottled water consist of high bacterial load

& coliform contamination. Ground water was found to be unsafe for drinking purpose as it contained highest coliform contamination. Bottled water was seen much safer than ground and public tap water as it contained comparatively less bacterial load and coliform contamination. Ground water must be treated properly before using for drinking purpose. Public tap water must be properly disinfected before using and bottled water must be monitored properly during manufacturing.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Aryal J, Gautam B and Sapkota N (2012). Drinking Water quality assessment. *J Nepal Health Res Council* **10**(22): 192-196.

Bottino A, Thapa A, Scatoline A, Ferino B, Sharma S and Pradhananga MT (1991). Pollution in Water Supply System of Kathmandu City. *Journal of Nepal Chemical Society* **10**: 33-44.

Gundry S, Wright J and Conroy R. (2004). A systematic review of the health outcomes related to household water quality in developing countries. *J Water Health* **2:** 1–13.

Pant BR (2011). Ground water quality in the Kathmandu Valley of Nepal, *Environmental Monitoring and Assessment* **178** (1-4): 477-85

Pironcheva (2004). Water Management Practises in Rural and Urban Homes; a case of of Bangladesh on Ingestion of Polluted water. *J. Public Health* **112**: 317-321.

Prasai T, Lekhak B, Joshi DR and Baral MP (2007). Microbiological Analysis of Drinking Water of Kathmandu Valley. *Scientific World* **5**(5): 112-114.

Rai SK, Ono K, Yanagida J-I, Ishiyama- Imura Kurokawa M and Rai CK (2012). A large-scale study of bacterial contamination of drinking water and its public health impact in Nepal. *Nepal Med Coll J* **14**(3): 234-240.

Beta-Lactamases Production in Multi-drug Resistant *Acinetobacter* species Isolated from Different Clinical Specimens

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ABSTRACT

Objectives: To determine the prevalence of *Acinetobacter* spp. from different clinical specimens and detect different types of β-lactamase enzymes.

Methods: Different clinical samples were collected and 125 *Acinetobacter* spp. were isolated. Various biochemical tests were carried out to speciate the *Acinetobacter* spp. The antibiotic susceptibility pattern and β-lactamase enzymes like Extended spectrum β-lactamase (ESBL), Metallo β-lactamase (MBL) and AmpC β-lactamase were determined.

Results: Of the total 125 isolates, the most predominant species was *Acinetobacter calcoaceticus-A. baumannii* (Acb) complex (80%). Highest rate of isolation of *Acinetobacter* species were from in-patients (neonates' blood sample). Among all, 44.8% isolates were found to be MDR with the majority being resistant to aminoglycosides, carbapenems and fluoroquinolones but not to colistin. ESBL, MBL and AmpC beta-lactamase was detected in 43.2%, 15.2% and 1.6% of the isolates respectively.

Conclusion: *Acinetobacter calcoaceticus-A. baumannii* complex should be considered for detection in hospitalized patients. The analysis of antibiotic susceptibility pattern and β-lactamases would be helpful to establish network surveillance in order to maintain and control the spread of these resistant strains.

Key words: *Acinetobacter* species, Acb complex, ESBL, MBL, AmpC beta-lactamase.

INTRODUCTION

Gram-negative bacteria cause different infections, which are becoming increasingly prevalent and constitute a serious threat to public health worldwide. Systemic infections from these organisms are difficult to treat and carry unacceptably high mortality, as high as 50% because of lack of efficacious treatment regimens (Kaye and Pogue 2015).

Genus *Acinetobacter* comprises more than 50 validly named species. The most significant among them is *A. baumannii* (Kolk et al. 2019) that commonly infects immuno-compromised patients (Park et al. 2017). They are ubiquitous organisms and prevail in natural environments (Kolk et al. 2019). They also represent the normal flora in humans (Almasaudi 2016).

Acinetobacter have emerged as one of the most troublesome classes of pathogen in health care-associated infections (Silveira et al. 2019). They cause various infections like hospital-acquired pneumonia, community-acquired pneumonia, bacteremia, trauma and wound infection, urinary tract infection, meningitis and other manifestations like endocarditis, peritonitis, opthalmitis or keratitis associated with contact lens use following eye surgery (Almasaudi 2016).

Acinetobacter species are well suited for genetic exchange and have the remarkable capacity for acquisition of foreign genetic material, which helps in obtaining resistance to the antibiotics (Kolk et al. 2019). *AbaR1* resistance cluster, which is an 86-kb region, have been identified in *Acinetobacter* spp. that contains 45

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resistance genes in MDR isolates (Perez et al. 2007).

Bacterial resistance against β-lactam antibiotics is now becoming threat in the interventions of antibiotics due to the production of β-lactamase enzymes. Khanal et al. (2013) reported the prevalence of ESBL, MBL and AmpC β-lactamase producing *Acinetobacter* to be 9.09%, 10.90% and 46.80% respectively. In another study conducted by Bhandari et al. (2015), 12.5% ESBL, 63.8% MBL and 31.37% AmpC β-lactamase producing *Acinetobacter* were reported. OXA-51 of *A. baumannii* is involved in cephalosporin resistance (AmpC) (Gordon and Wareham 2009).

The spread of multi drug resistant *Acinetobacter* infection has been increasing and is creating a problem in the treatment. The early detection of MDR isolates and their ability to produce β-lactamase enzyme is necessary to neutralize the serious threat. So this study was conducted with the objective to identify different *Acinetobacter* species and to detect various types of β-lactamases (ESBL, MBL and AmpC β-lactamase) produced by it that could be helpful for the treatment and analysis of resistance mechanism of this bacterium and to search the alternative therapeutic options.

MATERIALS AND METHODS

Sample size and study population

The study was conducted in Paropakar Maternity and Women's Hospital, Kathmandu, Nepal over a period of six months from May to November 2018. *Acinetobacter* spp. were isolated from various clinical specimens received in Microbiology laboratory of the hospital. A total of 10,265 samples were investigated which included blood sample, ear swab, Eustachian tube, high vaginal swab (HVS), pus and urine. The samples were collected from females and neonates.

Ethical issues

The informed consent was taken from adult females. In case of neonates, the informed consent was taken from their guardians. Permission to conduct the study was obtained from the participating hospital.

Isolation and identification of *Acinetobacter* species

The specimens were directly inoculated on Blood agar and MacConkey agar plates and incubated at 37°C for 24 hours. The species of *Acinetobacter* was identified phenotypically in the laboratory by series of biochemical tests (Table 1) (Gupta et al. 2015).

Table 1: Phenotypic characteristics of *Acinetobacter* spp.

Name of test	Acinetobacter species				
	Acb. complex	*A. lwoffii*	*A. Haemolyticus*	*A. junnii*	*A. radioresistens*
Gram staining	Gram negative cocci or coccobacilli				
Catalase	+	+	+	+	+
Oxidase	–	–	–	–	–
Motility	–	–	–	–	–
Urease	V	V	–	–	–
Citrate	+	–	+	+	–
OF glucose	+	–	V	–	–
Nitrate reduction test	–	–	–	–	–
Hemolysis	–	–	+	–	–
Gelatin Hydrolysis	–	–	+	–	–
Growth at 42	+	–	–	–	–
Chloramphenicol sensitivity	R	S	R	R	R
Arginine hydrolysis	+	–	+	+	+

+: Positive, - : Negative, V: Variable, S: Sensitivity, R: Resistant, OF: Oxidative-fermentative.

Antibiotic susceptibility testing

The antibiotic susceptibility tests were performed on Muller-Hinton agar (MHA) via modified Kirby-Bauer method of disk diffusion following guidelines of CLSI (2018). In this study the antibiotics used were Amikacin (30 µg), Ampicillin (10 µg), Azithromycin (15µg), Cefixime (5µg), Cefotaxime (30 µg), Cefalexin (30 µg), Ciprofloxacin (5µg), Colistin (10 µg), Gentmicin (10 µg), Meropenem (10 µg), Nitrofurantoin (300 µg), Norfloxacin (10 µg), Piperacillin (100 µg), Piperacillin-tazobactam (100/10 µg), Tetracycline (30 µg), and Trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Criterion for multidrug resistance

The defining criterion for an isolate to be multidrug resistant (MDR) was set as resistance to three or more drugs belonging to different structural classes (Magiorakos et al. 2012).

Tests for ESBL

The ESBL production in bacterial isolates was screened by using Cefotaxime disc (30µg) and Ceftazidime disc (30 µg). If the zone of inhibition was less than or equal to 25 mm for Cefotaxime and if it was less than or equal to 22mm for Ceftazidime, the isolate was considered as potential ESBL producer on the basis of guidelines of CLSI (2018). The screened isolates were further confirmed by combined disc method. Cefotaxime (30 µg), Cefotaxime-clavulanate (30/10µg) and Ceftazidime (30 µg), Ceftazidime-clavulanate (30/10 µg) were used for confirmation of ESBL producing strains. After overnight incubation at 37°C, greater than or equal to 5 mm increase in a zone of diameter for either Cefotaxime/clavulanate (30/10 µg) or Ceftazidime/clavulanate (30/10 µg) vs the zone diameter of Cefotxime (30µg) or Ceftazidime (30µg) was interpreted as ESBL producer as recommended by CLSI (2018).

Tests for MBL

The screening test for the MBL production was performed by using Imipenem disc (10µg). If the zone of inhibition was less than or equal to 18 mm for Imipenem, the isolate was considered as potential MBL producer as stated by CLSI (2018). The screened isolates were further confirmed by combined disc method using Imipenem (10 µg) alone and in combination with EDTA. After overnight incubation at 37°C, if the increase in inhibition zone with Imipenem-EDTA disc was greater than or equal to 7 mm than the Imipenem (10 µg) alone, it was interpreted as MBL producer as stated by Anwar et al. (2016) and Sujatha and Goyal (2017).

Tests for AmpC β-lactamase

AmpC-lactamase production was screened by using Cefoxitin (30 µg) disc. If the zone of inhibition was less than or equal to 14 mm for Cefoxitin, the isolate was considered as potential AmpC β-lactamase producer (Saad et al. 2016). The screened isolates were further confirmed by disc approximation test. Imipenem (10µg), Ceftazidime (30 µg), Cefoxitin (30 µg) and Amoxicillin-clavulanate (20/10µg) were used for the confirmation of AmpC β-lactamase producing strains. After overnight incubation at 37°C, the plate was examined for any blunting or flattening of the zone of inhibition between the ceftazidime disc and the imipenem, cefoxitin and amoxicillin-clavulanate discs. The presence of any blunting or flattening of the zone was interpreted as AmpC β-lactamase producer (Saad et al. 2016).

RESULTS

Out of 10,265 clinical specimens, 807 (7.86%) were found to be culture positive and the occurrence of *Acinetobacter* was found to be 125 (15.48%). 113 (23.01%) of *Acinetobacter* species were isolated from in-patients and 12 (3.78%) from out-patients (Table 2).

Table 2: Status of bacterial infections in suspected patients

Category	Culture		Total N (%)	Acinetobacter spp. N (%)
	Positive N (%)	Negative N (%)		
In-patients	491 (13.96)	3025 (86.04)	3516 (34.25)	113 (23.01)
Out-patients	316 (4.68)	6433 (95.32)	6749 (65.74)	12 (3.78)
Total	**807 (7.86)**	**9458 (92.16)**	**10265**	**125 (15.48)**

Amidst the *Acinetobacter* isolates, 65 (52%) were obtained from neonates' blood whereas only 1 (0.8%) was obtained from ear swab (Figure 1).

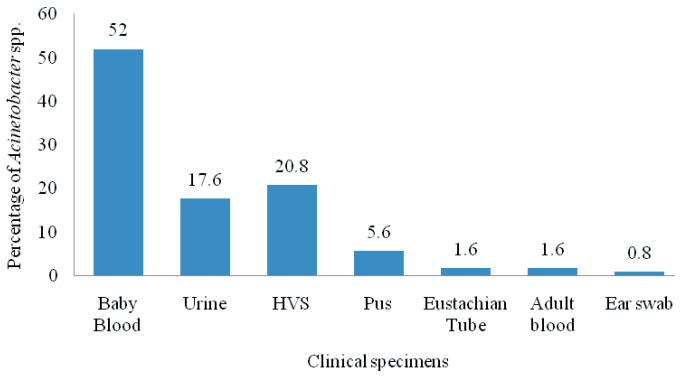

Figure 1: Distribution of *Acinetobacter* spp. in clinical specimens

Out of 125 *Acinetobacter* spp., the most predominant species was *Acinetobacter calcoaceticus-baumannii (Acb* complex*)* (80%) followed by *A. lowffii* (10.4%) (Figure 2).

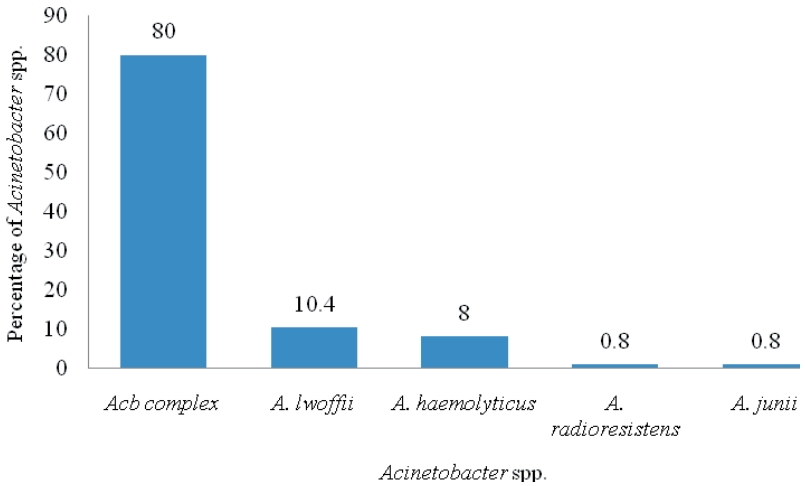

Figure 2: Distribution of various species of *Acinetobacter*

Amongst total 125 *Acinetobacter* spp., 67 (53.6%) of the isolates were resistant towards Cefotaxime and 55 (44%) towards Piperacillin while all the isolates were sensitive towards Colistin followed by Tetracycline (85.6%) (Table 3).

Table 3: Antibiotic susceptibility profile of *Acinetobacter* spp. (n=125).

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	93	74.4	32	25.6
Cefotaxime	58	46.4	67	53.6
Ciprofloxacin	104	83.2	21	16.8
Colistin	125	100	0	0
Gentamicin	92	73.6	33	26.4
Meropenem	90	72	35	28
Piperacillin	70	56	55	44
Piperacillin-Tazobactam	100	80	25	20
Tetracycline	107	85.6	18	14.4
Trimethoprim-Sulfamethoxazole	79	63.2	46	36.8

Of the total *Acinetobacter* spp. 56 (44.8%) were MDR, 32 (57.14%) were ESBL producer, 18 (32.14%) were MBL producer and 2 (3.57%) were AmpC β-lactamase producer. The ESBL production and MBL production in MDR isolates were found to be statistically significant while the AmpC β-lactamase production in MDR isolates was found to be statistically insignificant (Table 4).

Table 4: Profile of β-lactamase producing *Acinetobacter* species

Tests	ESBL		MBL		AmpC ß-lactamase	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
MDR	32 (57.14)	24 (42.86)	18 (32.14)	38 (67.86)	2 (3.57)	54 (96.43)
Non MDR	22 (31.88)	47 (68.12)	1 (1.45)	68 (98.55)	0 (0)	69 (100)
Total	54	71	19	106	2	123
p-value	**0.005**		**0.001**		**0.114**	

Among 125 *Acinetobacter* spp. 57.14% of the isolates were MDR and ESBL producer, 32.14% were MDR and MBL producer, 3.57% of the isolates were MDR and AmpC producer, 17.85% of the isolates were MDR and both ESBL as well as MBL producer while 1.78% of the isolates produced all the three beta lactamase enzymes along with being MDR isolate (Table 5).

Table 5: Relationship between MDR, ESBL, MBL and AmpC β-lactamase production in *Acinetobacter* spp.

Characteristics	*Acinetobacter spp.*	
	No.	%
MDR + ESBL	32	57.14
MDR + MBL	18	32.14
MDR + AmpC	2	3.57
MDR + ESBL + MBL	10	17.85
MDR + ESBL + AmpC	1	1.78
MDR + MBL + AmpC	1	1.78
MDR + ESBL + MBL + AmpC	1	1.78

DISCUSSION

Acinetobacter species are ubiquitous organisms and prevail in natural environments (Kolk et al. 2019). They also represent the normal flora in humans (Almasaudi 2016). Transmission of isolate is usually through the hands of staff, contaminated equipment or overall hospital environment. Moreover, the virulence factors of *Acinetobacter* spp. are porins, surface structures such as capsular polysaccaharide and lipopolysaccaharide (LPS), phospholipases, iron acquisition systems, outer membrane vesicles, protein secretion systems, regulatory proteins, biofilm associated proteins, different types of binding proteins. They are also well suited for genetic exchange and have the remarkable capacity for acquisition of foreign genetic material, which helps in obtaining resistance to the antibiotics (Kolk et al. 2019).

The incidence of *Acinetobacter* spp. from in-patients was found to be 90.4%, which is in accordance with previous studies carried out by Gupta et al. (2015) and Joshi et al. (2017). The incidence of *Acinetobacter* infection was highest in in-patients and highest number of bacteria was isolated from neonates' blood as also stated by Gupta et al. (2015). It is because *Acinetobacter* spp. is low virulence organism responsible for opportunistic infections in immuno-compromised patients, which increases the incidence of nosocomial infections. One of the reasons for the increased nosocomial infections by *Acinetobacter* spp. might be their endurance in dry conditions for long period of time and survival in a hospital environment and on the surface of healthcare worker hands (Park et al. 2017). The immune system of neonates is immature when they are born and it takes time to fully develop this immunity and thus they are easily attacked by various bacterial pathogens (Park et al. 2017).

The predominantly isolated species was *Acinetobacter calcoaceticus baumanii (Acb complex)* as also reported by Raina et al. (2015) and Gupta et al. (2015). Almost half of the isolates were multi-drug resistant which is consistent with previous reports by Pathak et al. (2017) and Shrestha et al. (2015). The development of resistance in *Acinetobacter* spp. may be due to the presence of wide array β-lactamases that hydrolyze and confer resistance to penicillins, cephalosporins and carbapenems, presence of efflux pumps and loss of porin proteins. Also the inappropriate use of antibiotics and lack of hygiene practices are also the factors that help in the spread of antibiotic resistant bacteria (Awad et al. 2016; Khanal et al. 2013).

Of the total *Acinetobacter* spp., 54 (43.2%) were ESBL producer, 19 (15.2%) were MBL producer and 2 (1.6%) were AmpC β-lactamase producer while 1.78% of the isolates (*Acinetobacter calcoaceticus baumanii*) produced all the three beta lactamase enzymes along with being MDR isolate. ESBL production and MBL production in MDR *Acinetobacter* spp. was found to be statistically significant while AmpC production in MDR *Acinetobacter* spp. was found to be statistically insignificant.

ESBL production might be due to the presence of ESBL producing genes like bla_{OXA-23} and antibiotic genes that can be transferred to other bacteria horizontally through conjugation and due to excessive use of broad spectrum antibiotics (Joshi et al. 2017; Shrestha et al. 2017). The acquisition of MBL-encoding genes such as *vim1, vim2, imp1* and *imp2* is one of the ways to acquire resistance to carbapenems like imipenem, meropenem and ertapenem (Davoodi et al. 2015). Phenotypic detection of AmpC β-lactamase enzyme is generally considered inappropriate because there are no standardized screening methods and also there are no CLSI recommended guidelines (Saad et al. 2016). Molecular methods are the most reliable and appropriate methods for the detection of AmpC β-lactamase enzyme (Delgado et al. 2016).

Acinetobacter spp. are becoming the troublesome pathogen with multiple antibiotic resistance mechanisms, especially in hospital settings. Thus, infection prevention and control measures are required to minimize or prevent the transmission of infections and antibiotic stewardship programs can be implemented effectively in hospitals for optimizing the treatment of infections and reducing adverse events associated with antibiotic use.

CONCLUSION

Acinetobacter calcoaceticus-baumannii was the most common bacterial isolate which was mostly recovered from neonates' blood. All isolates were sensitive to Colistin while more than half of the isolates were resistant towards Cefotaxime and Piperacillin. Significant proportions of ESBL, MBL and AmpC beta lactamase producers were MDR. This suggests for regular monitoring of these resistant pathogens for their control.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Almasaudi SB (2016). *Acinetobacter* spp. as nosocomial pathogens: Epidemiology and resistance features. *Saudi J Biol Sci* **25**(3): 586-596.

Anwar M, Ejaz H, Zafar A and Hamid H (2016). Phenotypic Detection of Metallo-Beta-Lactamases in Carbapenem Resistant *Acinetobacter baumannii* Isolated from Pediatric Patients in Pakistan. *J Pathog* **6**: 7-12.

Awad HA, Mohamed MH, Badran NF, Mohsen M and Abd-Elrhman AS (2016). Multidrug-resistant organisms in neonatal sepsis in two tertiary neonatal ICUs, Egypt. *J Egypt Pub Heal Assoc* **91**(1): 31-38.

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* **1**: 1-6.

Clinical and Laboratory Standard Institute (CLSI 2018). Performance standards for antimicrobials susceptibility testing: 24th informational supplement (M100-S23). CLSI, Wayne PA, USA.

Davoodi S, Ali Baroumand M, Sepehriseresht S and Pourgholi L (2015). Detection of VIM- and IMP-type Metallo-Beta-Lactamase Genes in *Acinetobacter baumannii* Isolates from Patients in Two Hospitals in Tehran. *Iran J Biotechnol* **13**(1): 63-67.

Delgado DY, Barrigas ZP, Astutillo SG, Jaramillo AP and Ausili A (2016). Detection and molecular characterization of β-lactamase genes in clinical isolates of Gram-negative bacteria in Southern Ecuador. *Braz J Infect Dis* **20**(6): 627-630.

Gordon NC and Wareham DW (2009). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents* **35(3)**: 219-226.

Gupta N, Gandhan N, Jadhav S and Mishra R (2015). Isolation and identification of *Acinetobacter* species with special reference to antibiotic resistance. *J Nat Sci Biol Med* **6**(1): 159-162.

Joshi PR, Acharya M, Kakshapati T, Leungtongkam U, Thummeepak R and Sitthisak S (2017). Co-existence of bla_{OXA-23} and bla_{NDM-1} genes of *Acinetobacter baumannii* isolated from Nepal: antimicrobial resistance and clinical significance. *Antimicrob Resist Infect Control* **6**: 21-27.

Kaye K and Pogue J (2015). Infections Caused by Resistant Gram Negative Bacteria: Epidemiology and Management. *Pharmacotherapy* **35**(10): 949-962.

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. *ISRN Microbiol* **2013**: 1-5.

Kolk J, Endimiani A, Granubner C, Gerber V and Perreten V (2019). *Acinetobacter* in veterinary medicine, with an emphasis on *Acinetobacter baumannii*.*J Glob Antimicrob Resist* **16**: 59-71.

Magiorakos AP, Srinivasan A and Carey RB (2012). Multi drug resistant, extensively drug-resistant and pandrug-resistant bacteria: an international

expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**(3): 268–281.

Park G, Kang C, Cha M, Cho S, Seok H, Lee J, Kim J, Ha Y, Chung D, Peck K, Lee N and Song J (2017). Bloodstream infections caused by *Acinetobacter* species with reduced susceptibility to tigecycline: clinical features and risk factors. *Int J Infect Dis* **62**: 26-31.

Pathak P, Jaishi N, Yadav BK and Shah PK (2017). Prevalence of Extended Spectrum Beta Lactamases (ESBL) and Metallo Beta Lactamases (MBL) Mediated Resistance in Gram Negative Bacterial Pathogens. *Tribhuvan University Journal of Microbiology* **4**: 49-54.

Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, and Bonomo RA (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii. Antimicrob Agents Chemother* **51**(10): 3471-3484.

Peymani A, Nahaei MR, Farajnia S, Hasani A, Mirsalehian A, Sohrabi N and Abbasi L (2011). High prevalence of Metallo-beta-lactamase-producing *Acinetobacter baumannii* in a teaching hospital in Tabriz, Iran. *Jpn J Infect Dis* **64**(1): 69-71.

Raina D, Sharma N, Mahawal BS, Khanduri A and Pandit A (2015). Speciation and antibiotic resistance pattern of *Acinetobacter* species in a tertiary care hospital in Uttarakhand. *Int J Medi Resear Heal Sci* **5**(4): 89-96.

SaadN, Munir T, Ansari M, Gilani M, Latif M and Haroon A (2016). Evaluation of phenotypic tests for detection of Amp C beta-lactamases in clinical isolates from a tertiary care hospital of Rawalpindi, Pakistan.*J Pak Med Assoc* **66**(6): 658-661.

Shrestha S, Tada T, Akiyama T, Ohara H, Shimada K, Satou K, Teruya K, Nakano K, Shiroma A, Sherchand JB, Rijal BP, Hirano T, Kirikae T and Pokhrel BM (2015). Molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* isolates in a university hospital in Nepal reveals the emergence of a novel epidemic clonal lineage. *Int J Antimicrob Agents* **46**(5): 526-531.

Silveira F, Nedel WL, Cassol R, Pereira PR, Deutschendorf C and Lisboa T (2019). *Acinetobacter* etiology respiratory tract infections associated with mechanical ventilation: what impacts on the prognosis? A retrospective cohort study. *J Crit Care* **49**: 124-128.

Soudeiha M, Sokhn E, Daoud Z, Sarkis D (2018) Molecular epidemiology and clonality of *Acinetobacter* spp. in a Lebanese hospital over a period of one year. *J Infect Dev Ctries* **12:** 17-22.

Sujatha R and Goyal R (2017). A Study of Phenotypic & Genotypic Assays For Detection of Metallo β- Lactamase Producing *Pseudomonas aeruginosa* With Special Reference to New Delhi Metallo β-Lactamase. *J Den Med Sci* **16**(7): 90-96.

Antibacterial Property of Extract of *Erveniastrum nepalense* (Edible Lichen) Collected from Hilly Regions of Eastern Nepal

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ABSTRACT

Objectives: To evaluate the antibacterial property of *Everniastrum nepalense*(edible lichen)collected from eastern part of Nepal and determine Minimum Inhibitory Concentration (MIC) of lichen extract.

Methods: *Everniastrum nepalense* was collected from local Rai and Limbu community in a plastic bag from four hilly districts (Pachthar, Taplejung, Dhankuta and Bhojpur) of Eastern, NepaMoisture content in lichen samples were removed by air drying and grinded to powdery form. The methanolic extracts of lichens of different places were prepared by solvent extraction process using Soxhlet apparatus and tested against human pathogenic bacteria by disc diffusion method. Then, Minimum Inhibitory Concentration (MIC) of lichen extracts was determined using well diffusion method.

Results: The lichens extract against the test bacterial isolates were performed by agar well diffusion method. Overall, it was observed that all these lichen extracts from Eastern Nepal had inhibitory effect on both Gram-positive and Gram-negative bacteria. Out of seven bacterial samples used, all bacterial samples were inhibited by the lichen extracts except *Escherichia coli*. The effect of lichen extract collected from Bhojpur district had maximum zone of inhibition against *Staphylococcus aureus*, *Salmonella* Typhi and *Klebsiella* spp were found to be 13 mm, 14 mm and 14 mm respectively. Similarly, zone of inhibition against *Bacillus subtilis* was found to be 17 mm from samples of Dhankuta, which was highest value than other 3 districts viz; Bhojpur, Taplejung and Pachthar District. Likewise, *Pseudomonas aeruginosa* showed highest inhibition value i.e. 18 mm on Pachthar sample whereas, *Shigella* showed 15 mm on Dhankuta sample. This study revealed that inhibitory capacity of edible lichen (*Everniastrum nepalense*) against test bacteria was not the same. It varied from place to place.

Conclusion: This study has concluded that *Everniastrum nepalense*has potential antibacterial property against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella* spp, *Shigella* spp, *Klebsiella* spp and *Pseudomonas aeruginosa* except *E. coli* along with nutritional value and can be used as safe alternative and economic herbal medicine to treat infectious diseases.

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

INTRODUCTION

Lichens are being used in traditional foods and medicines since millenia and also play vital roles in ecosystem function and human welfare (Crawford 2015). According to WHO, approximately 80% of present world's people depend on traditional medicine for primary health cares. Indigenous traditional medicine has considerable economic benefits as well as it can be used in the treatment of various diseases (Azaizeh et al. 2003).

According to Crockett et al. (2003), Lichens are the symbiotic association between two organisms (Fungi and its photosynthetic partner algae) which live together intimately.The fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites in all lichens (Ahmadijan 1993**)**. Lichens, frequently called 'Jhyau' or 'Tare' in Nepal, are classified as a cluster of lower life-forms of fungi (Shah 2014). Although about 8% of the terrestrial ecosystem consists of lichens and more than 20,000 lichen species

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are distributed throughout the world, their biological activities and biologically active compounds remain unexplored to a great extent (Toma et al. 2001).

Lichens are considered as valuable plant resources which are used as medicines, food, fodder, dyes perfume, spice, and for miscellaneous purposes (Hegnauer 1962**).** Lichens are also used for monitoring the air pollution because they are very sensitive to various air pollutants (Jezierski et al. 1999).

The use of lichens in medicine is based on the fact that they contain unique and varied biologically active substances, mainly with antimicrobial actions. Because of marked antimicrobial activity of secondary metabolites, lichens, macrofungi, and vascular plants attract great attention of investigators as new significant sources of bioactive substances (Karaman et al. 2003). The intensive use of antibiotics has selected for antibiotic resistance factors and facilitated the spread of multiply resistant microorganisms.

Lichen metabolites constitute a wide variety of biological function including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects (Shukla et al. 2010; Manojlovic et al. 2010).

Various lichens and its extracts having usnic acid have been used for medicinal, perfumery, cosmetic as well as ecological purposes. In some commercial products such as creams, toothpaste, mouthwash, deodorants and sunscreen products, usnic acid has been formulated as a pure substance as well as in some cases used as an active principle, in others as a preservative. In addition to the antimicrobial activity against human and plant pathogens, usnic acid has been shown to exhibit antiviral, antiprotozoal, antiproliferative, antiinflammatory and analgesic activities (Ingolfsdottir 2002). Slow growth and long duration (maximum lifetime spans to several thousand years) of existence in the specific and even extreme conditions are consistent with their abundance in protective metabolites against different physical and biological influences (Denton and Karlen 1973). Ecological effects, such as antigrowth, antiherbivore and anti-insect properties of lichens have also been reported (Ingolfsdottir 2002). Edible lichen (*Everniastrum cirrhatum, E. nepalense* and *Parmotrema cetratum)* is also supposed to be effective in healing various diseases. Several lichen species have been used in folk medicine for treatment of stomach diseases, diabetes, whooping, cough, pulmonary tuberculosis, cancer, skin diseases. Lichen extracts can inhibit *Bacillus subtilis* which mainly causes nausea and diarrhea. So, it is good treatment for such a problem. Recently, many plants have received attention as sources of antibiotics (Basile et al. 2000).

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 (Rudrappa and Bais 2008).

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 (Thapa et al., 2018)

The potential bioactive compound of natural plant extracts that are economical. Among seven extracts examined *Snyzygium aromaticum, Pisidium guajava and Elaeocarpus ganitrus sh*owed the best antibacterial activity against *S. aureus* (Sakha et al. 2018).

In this regard, edible lichen from Eastern part of Nepal is of a significant interest. Therefore, in this study, antibacterial activity and MIC of methanolic extracts of *Erveniastrum nepalense* were evaluated against human pathogenic bacteria. This study was designed to contribute scientific proof for utilization of locally available edible lichen as herbal medicine in comparison to the commercial antibiotics having various side effects.

MATERIALS AND METHODS

Collection of lichen samples

Everniastrum nepalense was collected in a plastic bag from various areas of Pachthar, Taplejung, Dhankuta and Bhojpur districts of Eastern Nepal in May 2018 and this study was carried out up to July 2018 months in the microbiology laboratory of Central Campus of Technology, Hattisar, Dharan.

Test bacteria such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella* spp, *Shigella* spp, *Klebsiella* spp and *Pseudomonas aeruginosa* obtained from the Department of Microbiology, Central Campus of Technology, Dharan, were used for study of antimicrobial activity of lichen extracts.

Preparation of extracts

Ten gram (10 g) of collected sample of *Everniastrum nepalense* was weighed and placed in individual thimble and enclosed in it. After enclosing the sample,

Methanol was taken as a solvent. Each thimble with sample was placed on individual Soxhlet apparatus for solvent extraction. The solvents were left to siphoned single time. Then again, the solvent was added, so as to cover thimble. The temperature was adjusted at around 55-60°C, where the boiling point of methanol is 64.7°C. The process of siphoning was started and done until the extraction becomes transparent. The extracts were poured on a beaker and concentrated to dryness using rotary evaporator. This process was repeated when more lichen extract was required. The extracts were then ready for testing antimicrobial activity (Jasuja et al. 2013).

Preparation of standard inoculums of test bacteria

The antibacterial activity *of Everniastrum nepalense* extract was tested against test bacteria such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella* spp., *Shigella* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa.*

For this, 0.5 McFarland standard inoculums from pure culture of test bacteria were prepared in a separate test tube containing nutrient broth at 37° C and were maintained.

Evaluation of antibacterial activity

The antibacterial activity was screened by both agar well diffusion (Basri and Nor 2014) on Mueller-Hinton agar (MHA) against test bacteria. The 20ml of sterilized Muller Hinton Agar was poured into sterile petri plates, after solidification, 100µl of fresh culture of Test bacteria (0.5 Mc-Farland standards) were uniformly swabbed on the respective plates. The wells of 6 mm diameter were bored in the inoculated plates using sterile cork borer and the lichen extracts of 100 ml were loaded into respective well and incubated at 37°C for 24 hours. After incubation the diameter of inhibitory zones formed around the well were measured.

Minimum Inhibitory Concentration

MIC was determined using well diffusion method. The prepared MHA plates were inoculated with respective test bacteria, (*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Shigella* spp., *Klebsiella* spp. and *Salmonella* Typhi*).*

Standardization of bacterial suspension: McFarland standard was used as a reference to adjust turbidity of bacterial sample. Bacterial suspension was grown in Nutrient broth for 18-24h, followed by the matching of bacterial suspension to the turbidity equivalent to 0.5 McFarland solutions.

Determination of MIC: The prepared MHA plates were inoculated with respective test organisms, i.e. *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli, Shigella* spp., *Klebsiella* spp. and *Salmonella* Typhi.

Seven wells of 6 mm diameter were made at least 1.5 mm edge of the plate. Each well was labeled for the amount of extract to keep on. Various lichen extracts of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL and 3.125 mg/mL of extract were respectively poured in six wells and dimethyl sulphoxide (DMSO) solution as a control was poured in remaining one well. Whole system were kept undisturbed and allowed to dry for few minutes. The plates were inoculated at 37°C for 24 hours for the determination of minimum inhibitory concentration. The concentrations were made by mixing DMSO with crude solid extracts. The MIC was interpreted as the lowest concentration of the extract that showed visible zone of inhibition when compared to control plate well that contained only the DMSO.

RESULTS

In this study, methanolic extracts of edible lichen collected from four different places namely Bhojpur, Dhankuta, Panchthar and Taplejung were evaluated for their antibacterial activity against human pathogenic bacteria such as ATCC strain of *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella* spp., *Shigella* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa* by agar well assay method.

Overall, it was observed that all these lichen extracts from Eastern part of Nepal had inhibitory effect on both Gram-positive and Gram-negative bacteria. Out of seven test bacteria, all were inhibited by the lichen extracts except Gram-negative *Escherichia coli* (Table 1).

The effect of extract of lichen collected from Bhojpur district showed that the maximum zone of inhibition against *Staphylococcus aureus*, *Salmonella* Typhi and *Klebsiella* spp., were found to be 13 mm, 14 mm and 14 mm respectively. Similarly, that of Dhankuta district against *Bacillus subtilis* was found to be 17 mm, which was highest value than other 3 districts viz; Bhojpur, Taplejung and Pachthar districts. Similarly, extract of Dhankuta sample showed highest inhibition values (15 mm) against *Shigella* spp., whereas extract of Pachthar sampleshowed highest inhibition values (18 mm) against *Pseudomonas aeruginosa*. Hence, all the

bacterial species except *Escherichia coli* were inhibited by different lichen extracts.

Table 1: Effect of extracts on test bacteria on well diffusion

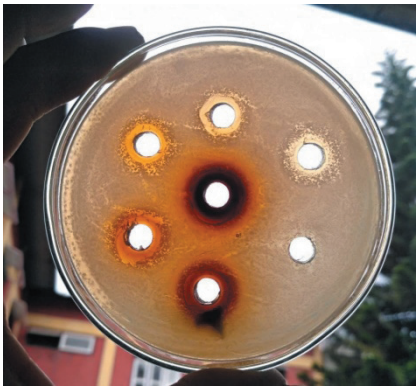
Organisms	Zone of inhibition (mm) using lichen extract of			
	Bhojpur	Dhankuta	Panchthar	Taplejung
E. coli	-	-	-	-
S. aureus	13	12	12	12
S. Typhi	14	13	11	12
B. subtilis	14	17	16	12
P. aeruginosa	10	14	18	15
Klebsiella spp	14	10	10	13
Shigella spp	12	15	12	11

Table 2: Zone of inhibition of test bacteria for MIC determination

Organisms	MIC (mg/mL) value of lichen collected from			
	Bhojpur	Dhankuta	Panchthar	Taplejung
E.coli	-	-	-	-
S. aureus	12.5	25	6.25	25
S. Typhi	25	6.25	6.25	50
B. subtilis	6.25	6.25	6.25	6.25
P. aeruginosa	25	6.25	6.25	6.25
Klebsiella spp	6.25	25	50	50
Shigella spp	25	6.25	50	50

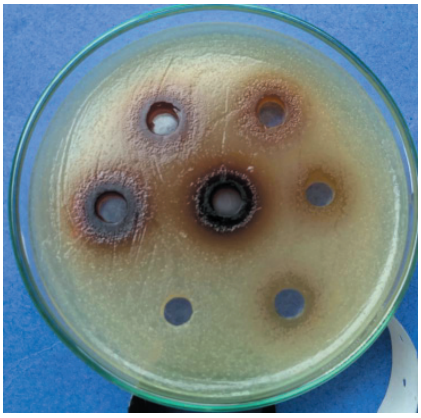
All Lichen extracts showed no any antibacterial activity against *Escherichia coli*. Hence, it is concluded that there was no effect on growth of *E. coli*. MIC values of the lichen extracts of Taplejung and Dhankuta against *Staphylococcus aureus* were same (25 mg/mL) whereas that of Bhojpur and Pachthar was found to be 12.5 mg/mL and 6.25 mg/mL respectively (Table 2).

MIC values of the lichen extracts of Bhojpur, Dhankuta, Pachthar and Taplejung against *Salmonella* Typhi were 25 mg/mL, 6.25 mg/mL, 6.25 mg/mL and 50 mg/mL respectively. Similarly, MIC value of the lichen extracts of all four districts against *Bacillus subtilis* were same i.e. 6.25 mg/mL. Hence, *B. subtilis* was equally affected by lichens extracts. Likewise, MIC value of the lichen extracts of Bhojpur against *Pseudomonas aeruginosa* was found to be 25 mg/mL whereas 6.25 mg/mL on all remaining three lichens extracts from remaining three districts. On other hand, *Klebsiella* and *Shigella* have same value of MIC with Pachthar and Taplejung lichen extracts i.e. 50 mg/mL whereas, their value differs within Bhojpur and Dhankuta lichen extracts viz; 6.25 mg/mL, 25 mg/mL and 25 mg/mL, 6.25 mg/mL on Bhojpur and Dhankuta samples respectively.

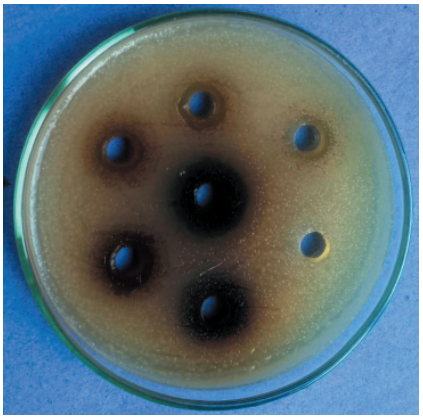


Photograph 1: Zone of inhibition against *Bacillus subtilis* by Bhojpur sample

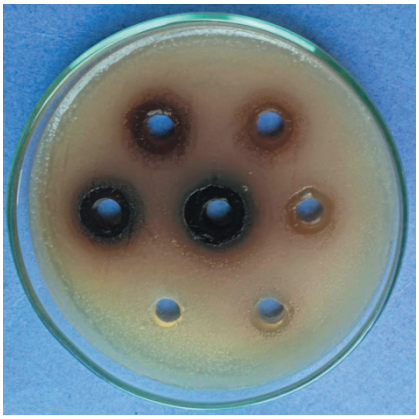
Photograph 2: Zone of inhibition against Shigella spp by Taplejung sample



Photograph 3: Zone of inhibition against Staphylococcus aureus by Pachthar sample



Photograph 4: Zone of inhibition against *Pseudomonas aeruginosa* by Dhankuta sample



Photograph 5: Zone of inhibition against *Salmonella* Typhi by Dhankuta sample

So, it was concluded that lichen sample from Pachthar was more effective against *Staphylococcus aureus*. Samples from Dhankuta and Pachthar were equally effective against *S*. Typhi than other two samples.So, it is concluded that *P. aeruginosa* was less affected by Bhojpur sample while both *Shigella* spp and *Klebsiella* spp showed most resistance against the lichen extracts of Pachthar and Taplejung samples. Therefore, MIC value of the lichen extracts of Bhojpur against *Klebsiella* spp. was found to be effective and that of lichen extracts of Dhankuta was effective against *Shigella* spp. Overall it is concluded that the lichen extracts from lichens of Dhankuta was the most effective against test bacteria being used except *Escherichia coli*. DMSO solution is used as a control for each system.

DISCUSSION

Plant product drugs and herbal remedies have been employed since prehistoric times to treat human and animal diseases and several countries still rely on plants and herbs as the main sources of drugs (Ogbonnia et al. 2008).

Lichens, the symbiotic organisms of fungi and algae, are slow-growing organisms (Fiedler et al., 1986). Their secondary metabolites as the "lichen substances," are amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers. They have been used by humans for centuries as food, dye and therapeutic traditional medicine. Their efficacy is due to the synthesis of unique secondary compounds, a number of which have important biological roles (Perry et al. 1999).

For this study, edible lichen (*Everniastrum nepalense*) was collected from four different districts of eastern Nepal viz. Bhojpur, Dhankuta, Panchthar and Taplejung.

Lichens are used as medicinal plants as well as food commodities by the some ethnic groups especially Rai and Limbu from Kirat community of Eastern Nepal. (Limbu 2013).

The edible lichens such as *Everniastrum cirrhatum, E. nepalense* and *Parmotrema cetratum* are are famous for its delicacy and high nutritional values and hence are widely eaten along with cooked blood, intestinal parts of pigs with spices and seasonings by the Limbu and Rai communities (Limbu and Rai 2012; Limbu 2013).

Extracts were evaluated for their antibacterial activity against human pathogenic bacteria such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella* spp., *Shigella* spp., *Klebsiella* spp. and *Pseudomonas aeruginosa* by agar well assay method.

The extracts of all edible lichens showed inhibitory effects against all test bacteria except *Escherichia coli*. This study is similar to the result obtained by Dulger et al. (1998).The species of the edible lichens including *Everniastrum cirrhatum, E. nepalense* and *Parmotrema cetratum* shown to exhibit inhibitory effect against bacteria including clinical isolates (Limbu and Rai 2012**).** The lichen extracts containing bio-active compounds showed inhibitory activity against wide variety of human pathogens including clinical strains (Kosanić et al. 2014).

Inhibitory Zone shown by methanol extracts of Lichen collected from four different districts varied according to bacteria used. Range of zone of inhibition obtained as results was 10 to 18 mm against test bacteria. The intensity of the antimicrobial effect depended on the type of extract, its concentration, and the tested microorganisms (Srivastava et al. 2013).Acetone and methanol extract showed almost equal activity against *S. aureus* whereas ethanol extract was found to be more effective against *B. cereus* and *P. aeruginosa*. The reason for different sensitivity of bacteria can be found in different transparency of the cell wall (Yang and Anderson 1999).

From the result obtained, it is concluded that lichen extracts showed less antibacterial activity on Gram-negative bacteria as compared to Gram-positive bacteria which is matched with that of Lawrey (1986). This can be justified with reference to Lodhia et al. (2009) and Nalubega et al. (2011) because the lesser inhibitory activity of solvent extracts against the Gram negative bacterium could be described to the presence of an outer membrane that possess hydrophilic polysaccharides chains and forms an additional barrier for extracts as well as antibiotics whereas the cell wall of the Gram-positive bacteria consists of peptidoglycan (mureins) and teichoic acids.

Behera et al. (2005) reported that the acetone, methanol, and light petroleum extracts of lichen were effective against *Bacillus licheniformis*, *B. megaterium*, and *S. aureus*. Karagoz et al. (2009) reported antibacterial activity of aqueous and ethanolic extracts lichens like *Lecanora muralis*, *Peltigerapolydactyla*, *Ramalina farinacea*, and *Xanthoria elegans*.

Everniastrum nepalense showed that MIC values were varying between 6.5 mg/mLand 50 mg/mL. Minimum inhibitory concentrations (MICs) was used to characterize the biological activity of various lichen solvent extracts.

In this study it was obtained that Dhankuta sample was the most effective against all test bacteria except *Escherichia coli*. Similarly, Panchhar and Bhojpur samples were ranked as second and third antimicrobial effective samples respectively. Taplejung sample was considered as a sample having least antimicrobial activity because their MIC values for all the isolates except *Escherichia coli* were greater. Less the MIC value is, more effective towards bacterial sample to inhibit. For each test sample, DMSO was used a control. *Usnea ghattensis* showed that MIC values were varying between 25 and 3.125g/mL (Madamombe and Afolayan 2003).

As lichen extracts of four districts have showed good antibacterial activity against the test bacterial isolates namely *Staphylococcus aureus, Salmonella* Typhi, *Pseudomonas aeruginosa, Bacillus subtilis, Klebsiella* spp a*nd Shigella* spp. Hence, it is concluded that lichens are a good medicinal plant which is also supported by Karagoz et al. (2009).

On the basis of this finding, the methanolic extract of edible lichen (*Everniastrum nepalense*) possess a good candidate in the search for a natural antimicrobial agent against infections or diseases caused by the test bacteria except *E. coli*. The extracts of lichens should be further analyzed to isolate the specific antibacterial properties in them. Clinical trials should be carried out to explore the potential of the extracts in the treatment of the infectious diseases.

CONCLUSION

This study revealed that the lichen extract showed highest inhibitory effect on Gram-positive bacteria such as *Staphylococcus aureus, Bacillus subtilis* and mild inhibitory effect against Gram-negative bacteria such as *Pseudomonas aeruginosa, Salmonella* Typhi, *Shigella* spp, *Klebsiella* spp comparatively. Gram-negative *Escherichia coli* showed resistance to the lichen extracts from all four districts viz, Bhojpur, Taplejung, Dhankuta and Pachthar.

Hence, lichens are much effective against Gram-positive bacteria than that of Gram-negative bacteria. Therefore, it is assumed that lichens are ethno-botanically important which are still in use as medicinal as well as food commodities by local ethnic community and can be used for the treatment of various diseases. This study also revealed that lichens might be useful as an antimicrobial agent following further extensive investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ahmadijan V (1993). The Lichen Symbiosis. New York: John Wiley & Sons.

Azaizeh H, Fulder S, Khalilk and Said O (2003). Ethnomedicinal knowledge of local Arab practicioners in the Middle East Region. *Fitoterapia* **74**: 98-108.

Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Montesano D, Cobianchi RC, Vuotto ML and Ferrara L (2000). Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia* **71**: 110-116.

Basri DF and Nor NHM (2014). Phytoconstituent Screening and Antibacterial Activity of the Leaf Extracts from *Canarium odontophyllum* Miq. *American Journal of Plant Sciences* **5**: 2878-2886.

Behera BC, Verma N, Sonone A and Makhija U (2005). Antioxidantand antibacterial activities of lichen *Usnea ghattensis in-vitro*. *Biotechnology Letters* **27**(14): 991–995.

Crawford SD (2015). Lichens used in traditional medicine. In: Rankovie B, editor. Lichen secondary metabolites. *Switzerland: Springer International Publishing:* 27–80.

Crockett M, Kageyama S, Homen D, Lewis C, Osborn J and Sander L (2003). Antimicrobial properties of four Pacific Northwest lichens. *Oregon State University Press, Coruallis* 386.

Denton GH and Karlen W (1973). Lichenometry: its application to Holocene moraine studies in Southern Alaska and Swedish Lapland. *Arctic and Alpine Research* **5**: 47–372.

Dulger B, Gucin F and Aslan A (1998). *Cetraria islandica* (L) Ach. Likenin Antimikrobial . *Tr J. Biol* **22**: 111-118.

Fiedler P, Gambaro V, Garbarino JA and Quihot W (1986). Epiphorellic acids 1 and 2, two diaryl ethers from the lichen *Cornicularia epiphorella*. *Phytochemistry* **25**: 461-465.

Hegnauer R (1962). Chemotaxonomie der Pflanzen; eine Dbersicht iiber die Verbreitung und die Systematische Bedeutung der Pflanzenstoffe. Birkhauser, Basel.

Ingolfsdottir K (2002). Usnic acid. *Phytochemistry* **61**: 729-736.

Jasuja ND, Sharma SK, Saxena R, Choudhary J, Sharma R and Joshi SC (2013). Antibacterial, antioxidant and phytochemical investigation of *Thuja orientalis* leaves. *Journal of Medicinal Plants Research* **7**(25): 1886-1893.

Jezierski A, Bylinska E, and Seaward MRD (1999). Electron paramagnetic resonance (EPR) investigations of lichens—1: effects of air pollution. *Atmospheric Environment* **33**(28): 4629–4635.

Karagoz A, Dogruoz N, Zeybeck Z and Aslan A (2009). Antibacterial Activity of some lichen extract. *Journal of Medicinal Plant Research* **3**(12): 1034-1039.

Karaman I, Sanin F, Gulluce M, Ogutcu H, Sengul M and Adiguzel A (2003). Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *Journal of Ethnopharmacology* **85**: 231–235.

Kosanic M, Seklic D, Markovic S and Rankovic B (2014). Evaluation of antioxidant, antimicrobial and anticancer properties of selected lichens from Serbia. *Digest Journal of Nanomaterials and Biostructures* **9**(1): 273-287.

Kumar PV, Chauhan SN, Padh H and Rajani M (2006). Search for antibacterial and antifungal agents from selected Indian medicinal plants. *J. Ethnopharmacol*. **107**: 182-188

Lawrey JD (1986). Biological role of lichen substances. *The Bryologist* **89**: 111-112.

Limbu DK and Rai BK (2012) Ethnomedicinal practices among the limbu community in Limbuwan Eastern Nepal. *Glob J Hum Soc Sci H* **8**(2): 7-29.

Limbu RK (2013). Exploring indigenous knowledge system and Limbu cultural identity in Nepal. *J Univ Grants Comm* **2**: 122-137.

Lodhia MH, Bhatt KR and Thaker US (2009). Antibacterial activity of essential oils from Palmarosa, Evening primerose, Lavender and Tuberose. *Indian Journal of Pharmaceutical Sciences* **71**(2): 134-136.

Madamombe IT and Afolayan AJ (2003). Evaluation of antimicrobial activity of extracts from South African *Usnea barbata*. *Pharmaceutical Biology*

41(3): 199–202.

Manojlovic NT, Vasiljevic P, Juskovic M, Najman S, Jankovic S and Milenkovic-Andjelkovic A (2010). HPLC analysis and cytotoxic potential of extracts from the lichen, *Thamnolia vermicularisvar. subuliformis*. *Journal of Medicinal Plant Research* **4 (**9):817–823.

Manojlovic NT, Vasiljevic PJ and Markovic ZS (2010). Antimicrobial activity of extracts and various fractions of chloroform extract from the lichen *Laurera benguelensis*. *Journal of Biological Research* **13**: 27–34.

Nalubega R, Kabasa JD, Olila D and Kateregga J (2011). Evaluation of antibacterial activity of selected ethnomedicinal plants for poultry in Masaka district, Uganda. *Research Journal of Pharmacology* **5**(2):18-21.

Ogbonnia SO, Enwuru NV, Onyemenem EU, Oyedele GA and Enwuru CA (2008). Phytochemical evaluation and antibacterial profile of *Treculia africana* Decne bark extract on gastrointestinal bacterial pathogens. *Afr. J. Biotechnol*. **7**: 1385-1389.

Perry NB, Benn MH, Brennan NJ, Burgess EJ, Ellis G. Galloway D J, Lorimer SD and Tangney S (1999). Antimicrobial, antiviral and cytotoxic activity of New Zeland lichens. *Lichenologist* **31**: 627-636.

Rowe JG, Saenz MT and Garcia MD (1989). Contribution a a'letudedel' activiteantibacterienne de queques lichens du suddel'Espagne. *Pharmaceutical Francaise* 47:89–94.

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. *JAgric Food Chem* **56**: 1955-1962.

Sakha H, Hora R, Shrestha S, Acharya S, Dhakal D, Thapaliya S and Prajapati K (2018). Antimicrobial Activity of Ethanolic Extract of Medicinal Plants against Human Pathogenic Bacteria. *TUJM* **5**(1): 1-6

Shah NC (2014). Lichens of commercial importance in India. *Scitech J.* **1**:32-36.

Shukla V, Joshu GP and Rawat MSM (2010). Lichens as a potential natural source of bioactive compounds. *Phytochemistry Review* **9**: 303-314.

Srivastava P, Upreti DK, Dhole TN, Srivastava AK and Nayak MT (2013). Antimicrobial Property of Extracts of Indian Lichen against Human Pathogenic Bacteria, Interdisciplinary Perspectives on Infectious Diseases Volume 2013, Article ID 709348, 6 pages http://dx.doi.org/10.1155/2013/709348

Thapa B, Singh A and Tuladhar R (2018). In vitro Antibacterial effect of medicinal plants against multidrug resistant Gram negative bacteria. *TUJM* **5**(1): 25-31

Toma N, Ghetea L, Nitu R and Corol DI (2001) Progress and perspectives in the biotechnology of lichens. *Romanian Biotechnological Letters* **6**:1–15.

Yang Y and Anderson EJ (1999). Antimicrobial activity of a porcine myeloperoxidase against plant pathogenic bacteria and fungi. *Journal of Applied Microbiology* **86** (2): 211–220.

Detection of Methicillin Resistant *Staphylococcus aureus* in Dairy Products and Anterior Nares of Dairy Workers

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ABSTRACT

Objectives: To isolate methicillin resistant *Staphylococcus aureus* (MRSA) from anterior nares of dairy workers and dairy products and assess the antibiotic susceptibility pattern of the isolates.

Methods: Swab samples collected from anterior nares of dairy workers and dairy product (butter) were inoculated into mannitol salt agar and incubated at 37°C for 24 hours. Identification was done based on colony characteristics, Gram's staining, catalase, oxidase and coagulase test. Antibiotic susceptibility testing was done by modified Kirby Bauer disc diffusion method. MRSA was confirmed by using cefoxitin disc.

Results: A total of 109 *S. aureus* (98 from dairy workers and 11 from butter samples) were isolated. Out of them 32 MRSA were isolated from dairy workers and 4 from butter samples. The association between age group and MRSA was found insignificant (p = 0.115). The association of MRSA between male and female workers was found significant (>0.05). About 86% of the MRSA isolates were susceptible to Gentamicin (86.11%) followed by Ciprofloxacin (77.78%).

Conclusion: Detection of MRSA among dairy workers and dairy products warrants proper handling and adequate control measures to prevent transmission of MRSA from dairy industry.

Key words: MRSA, dairy products, dairy workers, carriage

INTRODUCTION

Staphylococcus aureus is considered to be one of the leading causes of food borne illness. Milk, dairy products and meats are often contaminated with this bacterium. Foodstuff contamination may occur directly from infected food producing animals or may result from poor hygiene during production processes or the retail and storage of foods, since humans may carry the microorganisms (Hennekinne et al. 2010). *S. aureus* colonizes skin and mucosa of humans and animals, with nasal carriage rates between 30% and 50% among the adult human population (Sakr et al. 2018). While colonization of the anterior nares is usually asymptomatic, it serves as a reservoir for the spread of the organism. Carries are at increased risk to develop bacteremia which in 80% of cases is caused by the strain colonizing their nares (Hassoun et al. 2017). The rapid emergence of antibiotic resistance among *S. aureus* is also known to play a crucial role in the epidemiology of staphylococcal infections (Wertheim et al. 2005).

Methicillin resistant *S. aureus* (MRSA) has become an increasingly important and serious public health concern due to the morbidity, mortality and cost that is associated with these infections annually. Once exposed to MRSA, animals may become colonized, and serve as reservoirs to transmit the infection to other animals or humans. Data has shown that with human to animal transmission, there is a possibility that until the animal is free of infection, re-transmission from the animal and subsequent human to human transmission can occur (American Veterinary Medical Association 2009). Therefore, this study aims to isolate MRSA from anterior nares of dairy workers and dairy products and assess the antibiotic susceptibility pattern of the isolates.

MATERIALS AND METHODS

This descriptive cross-sectional study was carried out

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in the quality control laboratory of Dairy Development Corporation, Balaju, Kathmandu, Nepal. A total of 109 samples from dairy workers and dairy products were investigated.

Sample collection and processing

Samples were collected aseptically from nasal swabs of both noses from dairy workers of different working units. The samples were also collected from butter samples by means of sterile cotton swab in separate sterile screw-capped test-tube. All the samples were labeled properly and processed in the laboratory as soon as possible. In case of delay, samples were stored at refrigerator (2-8^{o}C).

Each sample was inoculated into mannitol salt agar plates and incubated at 37^{o}C for 24 hours. Identification of *S.aureus* was done based on colony characteristics, Gram's staining, catalase, oxidase and coagulase test (Chakraborty 2011). All identified *S. aureus* isolates were processed for antibiotic susceptibility testing by modified Kirby Bauer disc diffusion method as recommended by Clinical Laboratory Standards Institution (CLSI, 2013). Antibiotic discs (Hi Media Laboratories, Pvt. Limited, India) such as ciprofloxacin (5 µg), clindamycin (2 µg), gentamicin (10 µg), penicillin G (10 U), teicoplanin (10 µg) were used for antibiotic susceptibility tests.

The isolates showing zone of inhibition of ≤ 21 mm around cefoxitin disc (30 µg) were considered as MRSA (CDC 2019). Data were analyzed by IBM SPSS statistics 21 software. Frequency and percentage for descriptive and Chi Square test with cross tab for inferential statistics were used.

RESULTS

Altogether 109 *S. aureus* were isolated of which 98 (89.90%) were from dairy workers and 11 (10.10%) from dairy products. Out of 98 isolates recovered, 63.26% (n= 62) were from male and 36.34% (n= 36) were from female workers. Similarly, 7 (63.63%) of *S. aureus* were isolated from plain butter (exported) and 4 strains (36.36%) were from table butter.

The maximum numbers of MRSA were isolated from dairy workers of age group 40-49 years followed by 30-39 years. However, the number of MRSA and age was not statistically significant (Table 1).

Table 1: Distribution of MRSA among different age groups of dairy workers

Age in years	No. of *S. aureus*	MRSA N (%)	p- value*
20-29	8	2 (25.00)	0.115
30-39	28	11 (39.28)	
40-49	47	15 (31.91)	
50-59	15	4 (26.67)	
Total	**98**	**32**	

*p-value calculated using chi-square test

MRSA was isolated more from male workers as compared to female workers (Table 2).

Table 2: Distribution of MRSA among different gender of dairy workers

Gender of workers	No. of *S. aureus*	MRSA N (%)	p- value*
Male	56	20 (35.71)	0.001
Female	42	12 (28.57)	
Total	98	32	

*p-value calculated using chi-square test

Antibiotic susceptibility pattern showed that most of the MRSA isolates were susceptible to Gentamicin followed by Ciprofloxacin (Table 3).

Table 3: Antibiotic susceptibility pattern of MRSA isolates

Antibiotics used	Susceptible N (%)	Resistant N (%)
Ciprofloxacin	28 (77.78)	8 (22.22)
Clindamycin	26 (72.22)	10 (27.78)
Gentamicin	31 (86.11)	5 (13.89)
Penicillin G	24 (66.67)	12 (33.33)
Teicoplanin	27 (75.00)	9 (25.00)

DISCUSSION

Isolation of 109 strains of *S. aureus* from dairy workers and dairy sample i.e. table butter and plain butter (exported) in our study is similar to that conducted by Crago et al. (2014). Andre et al. (2008) found that 75% (3/4) of the investigated persons were colonized with *S. aureus*. Huber et al. (2010) found that a nasal carriage rate was approximately 27% in healthy adult populations. Two studies with pre-clinical medical students showed that 35.2% and 29% were *S. aureus* nasal carriers (Bischoff et al. 2005). Our result is consistent with these findings.

Similarly, 11 strains of *S. aureus* were detected from butter sample which was 10.10% of total isolates. Among them 7 strains (63.63%) were isolated from plain (exported) butter whereas 4 strains (36.36%) were from table butter. Due to predominantly manual handling during butter packaging, contamination with *S. aureus* might occur. Highest number of MRSA in our study were found at the age of range of range 40-49 years. However, the association between age groups and MRSA was found statistically insignificant which signifies that presence of MRSA does not depend on the age group. The detection of MRSA in workers and butter suggests that healthy human could play a role in the spread of MRSA among animals, workers, and the food environment (Crago et al. 2014).

Livestock derived food product that could lead to MRSA food intoxication or serve as vehicle for MRSA transmission is raw milk, when contaminated raw milk is used for the production of cheese. This was reported in Italy, where two MRSA strains of unknown origin were found in dairy cheese products (Normanno et al. 2007). As these strains were found to harbor genes for expression of common staphylococcal enterotoxins, they had the potential to cause food poisoning (Normanno et al. 2007).

The major limitation of our study is that this we have not correlated the strains isolated from dairy workers and dairy product. Further studies should be done to correlate the strains upto sequence level.

CONCLUSION

Detection of MRSA among dairy workers and dairy products warrants proper handling and adequate control measures to prevent transmission of MRSA from dairy industry.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Andre MC, Campos MR, Borges LJ, Kipnis A, Pimenta FC and Serafini AB (2008). Comparison of *Staphylococcus aureus* Isolates from Food Handlers, Raw Bovine Milk and Minas Frescal Cheese by Antibiogram and Pulsed-Field Gel Electrophoresis Following *SmaI* Digestion. *Food Contrl* **19**: 200-207.

Bischoff WE, Wallis ML, Tucker KB, Reboussin BA and Sherertz RJ (2004). *Staphylococcus aureus* nasal carriage in a student community: prevalence, clonal relationships, and risk factors. *Infect Control Hosp Epidemiol* **25:** 485-91.

Centres for Diseases Control (CDC) (2019). Methicillin Resistant *Staphylococcus aureus.* URL: https://www.cdc.gov/mrsa/lab/index.html

Chakraborty SP, Mahapatra SK and Roy S (2011). Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates. *Asian Pac J trop biomed* **1**: 212-216.

Clinical and Laboratory Standard Institute (2013). M_{100}-$S_{23\ p}$erformance standards for antimicrobial susceptibility testing; twenty-third informational supplement. CLSI Wayne, USA **33**: 1-199.

Crago B, Ferrato C, Drews SJ, Svenson LW, Tyrrell G and Louie M (2012). Prevalence of *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) in food samples associated with foodborne illness in Alberta, Canada from 2007 to 2010. *F Microbiol* **32:** 202–205.

Hassoun A, Linden PK and Friedman B (2017). Incidence, prevalence, and management of MRSA bacteremia across patient populations-a review of recent developments in MRSA management and treatment. *Crit Care* **21**: 211-221.

Huber H, Koller S, Giezendanner N, Stephan R and

Zweifel C (2010). Prevalence and characteristics of meticillin resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin, Switzerland, 2009. *Euro Surveill* **15:** 19542.

Normanno G, Corrente M, La Salandra G, Dambrosio A, Quaglia NC and Parisi (2007). Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in Italy. *Int J Food Microbiol* **117**: 219-222.

Sakr A, Bregeon F, Mege JL, Rolain JM and Blin O (2018). *Staphylococcus aureus* basal colonization: An update on mechanisms, epidemiology, risk factors and subsequent infections. *Front Microbiol* **9**: 2419-2434.

Antibiogram and Biofilm Formation Among Carbapenem Resistant *Klebsiella pneumoniae*

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ABSTRACT

Objectives: This cross-sectional study was designed to detect the carbapenemase producing *K. pneumoniae* along with biofilm producers from different clinical specimens and to compare antibiotic susceptibility pattern of biofilm producing carbapenem resistant *Klebsiella pneumoniae* and biofilm non-producing carbapenem resistant *Klebsiella pneumoniae*.

Methods: A total of 1475 non-repetitive clinical samples were included on this study. Antibiotic Sensitivity Testing (AST), Modified Hodge Test (MHT) and Modified Carbapenem inactivation method (mCIM) were performed for detection of carbapenemase production and Congo red agar method (CRA) along with Microtitre plate method were performed for detecting biofilm production.

Results: Among the clinical specimens cultured, growth positivity was 62.71%. *E. coli* was most predominant organism followed by *K. pneumoniae* (17.89%). Among the 110 *K. pneumoniae*, 57 were found to be carbapenemase producer. Majority of the carbapenemase producing *K. pneumoniae* were isolated from sputum (45.61%), in the specimen collected from age group 61-70 (28.07%) and in out-patient department (50.88%). Similarly, 65.45% *K. pneumoniae* out of 110 were found to be biofilm producer by Congo red agar method while among those 72, 73.59% isolates were found to be quantitatively biofilm producer in Microtitre plate assay. Out of 57 carbapenemase producer, 35.08% were strongly biofilm producer while among 53 carbapenemase non-producer 30.18% were strongly biofilm producer from Congo red agar method. Moreover, Microtitre plate assay evidenced that, out of 57 carbapenemase producer, 40.35% were highly biofilm producing and among the 15 carbapenemase nonproducer 66.66% were highly biofilm producer.

Conclusion: Biofilm formation is highly prevalent with varying degree of resistance among different antibiotics including carbapenems that further augments antibiotic resistance. The study showed carbapenemase producers are stronger biofilm producer than the non-carbapenemase producer. Therefore, it is recommended to identify biofilm formation among carbapenemase producers for effective choice of antibiotics.

Key words: *Klebsiella pneumoniae*, Carbapenem, Biofilm, Carbapenemase

INTRODUCTION

Gram negative bacteria are major public health threat. They are becoming resistant to most of the antibiotic drug options available, creating reminiscent situation of the pre-antibiotic era. The most of Gram-negative infections occur in health care setting and are commonly caused by Enterobacteriaceae (mostly *Klebsiella pneumoniae*), *Pseudomonas aeruginosa*, and *Acinetobacter* species. *Klebsiella pneumoniae* is a Gram-negative bacterium belonging to the family Enterobacteriaceae, the most common cause of community-acquired and hospital-acquired infections. They are responsible in causing several health associated infections which include pneumonia, bloodstream infection, urinary tract infection, wound and surgical site infection, meningitis and so on. The trend of antibiotic resistance is continuously increasing in bacteria that cause either community infections or hospital acquired infections (Shaikh et al. 2014). It leads to increased mortality rate, economic burden, long stay on hospital

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and higher medical costs. The higher prevalence of antibiotic resistance and the lack of new antibiotic drug development have constantly reduced the available treatment options for bacterial infections. Emerging resistance in Enterobacteriaceae is a significant problem that requires immediate attention and action. This is a troubling trend, and one that requires vigilance and intensified measures to control the further spread of resistance by these important Gram-negative pathogens (David 2006). Carbapenem resistance is currently rare to most strains of Enterobacteriaceae, but some alarming signs have appeared (David 2006). The spreading of carbapenemase producing Gram negative bacteria in the hospital setting and community is a public health problem with major therapeutic and epidemiological consequences.

Biofilm formation is considered as an important virulence factor of *Klebsiella pneumoniae*. The majority of *Klebsiella pneumoniae* infections are associated with biofilm formation on host cells or abiotic surfaces such as indwelling catheter devices. Biofilm producing strains carry high morbidity and mortality in hospitalized patients more specifically patients of Intensive care units (ICU) (Sardi et al. 2013; Deorukhkar et al. 2014). In fact, biofilm formation is another effective way of bacteria for survival in presence of antibiotics (HallStoodley et al. 2004), especially for *A. baumannii* and *Klebsiella pneumoniae* which are the most common bacteria for developing biofilm related contamination of medical devices (Singhai et al. 2012). In developing nation like Nepal, where there is rampant irrational use of antibiotics, antibiotics can be easily purchased and used without medical authorization or supervision (Pokharel et al. 2006), the problem of antimicrobial resistance is growing day by day. This study could improve the knowledge regarding the rise and spread of the antimicrobial resistant bacteria in patients. Monitoring the presence of pathogenic and resistant bacteria would be significant in term of providing better and healthy quality of life to the patients with appropriate treatment facilities. Limited studies have been performed for the detection of carbapenem resistant and biofilm formation among the *K. pneumoniae* from clinical isolates in case of Nepal. This study would be very important step in identifying the prevalence of the carbapenem resistant and biofilm forming *K. pneumoniae* in Nepal. Further, this study would add additional information to existing information. The study was carried out for short duration including limited samples on the particular site only.

MATERIALS AND METHODS

This study was carried out at Microbiology department of Dhulikhel Hospital, Dhulikhel, Kavre, Nepal. The study was carried out from January to June 2019. A total of 1475 samples were processed out. Ethical approval was obtained from Institutional Review Committee (IRC) of Dhulikhel Hospital. The written and signed inform consents were taken from the patients and the guardian of patients.

Ethics approval and consent to participate: Ethical approval was obtained from Institutional Review Committee (IRC) of Dhulikhel Hospital with the protocol approval number of 103/19.

Sample type and collection methods: Different samples including pus, urine, sputum, catheter tip, tracheal aspirates, and bloods were collected from an outpatient department (OPD) and indoor patient department (IPD). Samples were collected in the sterile container. All the samples were analyzed in microbiology laboratory of the hospital.

Isolation and identification of *K. pneumoniae*: All the samples were cultured on the MacConkey agar and Blood agar. Growth on the media indicated presences of microorganism. Gram staining was performed for the differentiation of Gram negative bacteria from the Gram positive bacteria. Different biochemical test (indole test, MR-VP test, citrate test, TSIA test and urease test) were performed to identify *K. pneumoniae*. Confirmed *K. pneumoniae* isolates were stored for further analysis.

Antibiotic susceptibility testing of *K. pneumoniae*: Antibiotic susceptibility testing was done by applying Kirby Bauer disc diffusion method. During the process, *K. pneumoniae* isolates were plated on Mueller–Hinton agar and their susceptibilities to carbapenem were checked according to the Clinical and Laboratory Standard Institutes (CLSI) guidelines. Those isolates showing resistant to Meropenem (10 µg) were taken for the further confirmatory tests.

Modified carbapenem inactivation method (mCIM) and Modified Hodge test: Modified carbapenem inactivation method (mCIM) was performed following the AST to determine the percentage of isolates that inactivate the carbapenem with production of carbapenemase. To perform the mCIM, a suspension was made by suspending a full 1 µl inoculation loop

of culture, taken from a Mueller-Hinton or blood agar plate in 2 ml Trypticase soy broth (TSB). Subsequently, a susceptibility-testing disk containing 10 µg meropenem was immersed in the suspension and incubated for a minimum of four hours at 35°C in ambient air. After incubation, the disk was removed from the suspension using an inoculation loop, placed on a Mueller-Hinton agar plate inoculated with a susceptible *E. coli* indicator strain (ATCC 29522) compared with 0.5 MacFarland tube and subsequently incubated overnight at 35°C in ambient air, the zone of inhibition around the meropenem disk was measured and analyzed. Modified Hodge Test (MHT) is a confirmatory test for detection of presence of carbapenemase enzyme in bacteria. During the process, 0.5 McFarland dilution of the *E. Coli* ATCC 25922 in 5 ml of saline was prepared. Further, it was diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of saline. A lawn of diluent was streaked on MHA and allowed to dry 3-5 minutes. Meropenem disk (10 µg) was placed in the center of the test area. In a straight line, *K. pneumoniae* was streaked from the edge of the disk to the edge of the plate at 3 different places. Plate was incubated overnight at 35°C in ambient air for 16-24 hrs. After 16-24 hours of incubation the plate was checked for a clover leaf-type indentation at the intersection of the *K. pneumoniae* and the *E. coli* 25922, within the zone of inhibition of the carbapenem susceptibility disk.

Detection of biofilm production: Biofilm of all the *Klebsiella pneumoniae* isolates were detected using Congo Red Agar method and Microtitre plate assay. These methods indicated the qualitative and quantitative study of biofilm production respectively. All the isolates were categorized based upon the biofilm formation abilities. In case of Congo red agar, biofilm producer were categorized as strong, moderate, weak and non- producer. The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Similarly, in case of Microtitre plate assay, the biofilm producer were differentiated as high, moderate, weak and non-adherent based upon the comparison of optical density with positive control. In the study, the highest biofilm producer from the Congo red agar method was taken as the positive control for the Microtitre plate assay. Microtitre plate assay was carried out by inoculating a fresh colony of *K. pneumoniae* in 2 mL of Brain Heart Infusion broth (BHI). The broth was incubated overnight at 37 °C. Sterile individual plates with 96 flat-bottom polystyrene wells were filled with 200 µL of the diluted culture. The plate was incubated at 37 °C for 48 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline (pH 7.3) to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were fixed by 99% methanol for 5 minutes and stained with 0.1% crystal violet (CV) with 10-15 minutes. Excess stain was washed gently, and the plate was kept for drying. Around 100 µL of 95% ethanol was added on the well. The optical density of the stained adherent biofilm was measured using a ELISA auto-reader (CHROMATE) at a wavelength of 570 nm.

RESULTS

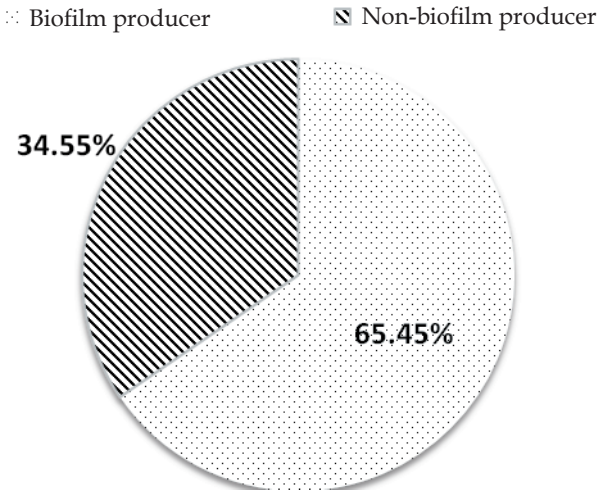


Figure 1: Biofilm producer in Congo red agar

Table 1: Comparison of biofilm producer and carbapenemase producer

	Biofilm formation abilities	Carbapenemase producer	Carbapenemase non-producer	p-value
Congo red agar method	Strong	20	16	
	Moderate	10	9	**0.040**
	Weak	9	8	
	Negative	18	20	
Microtitre plate assay	High	23	10	
	Moderate	8	2	
	Weak	8	2	
	Non-adherent	18	1	

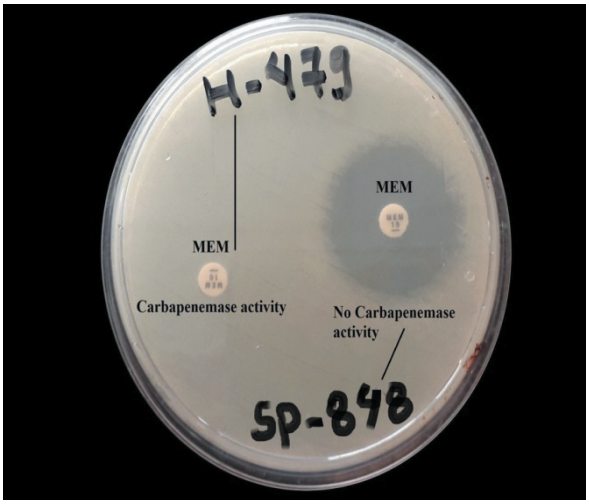
*p-value was calculated by using the Chi-square test.

Table 2: Antibiotic susceptibility pattern of *K. pneumoniae* isolates

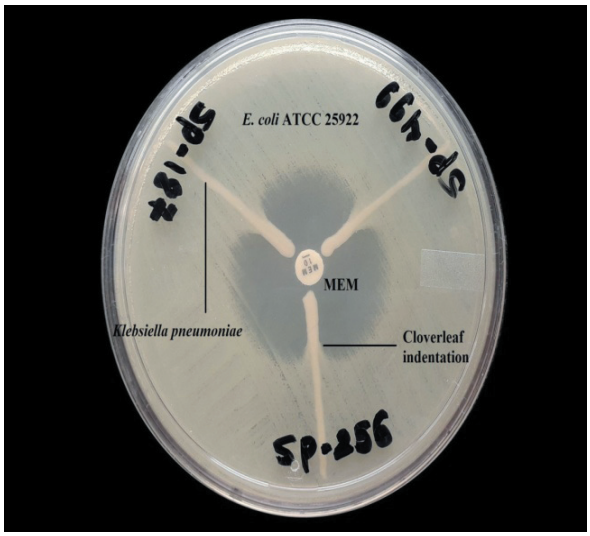
Antibiotics	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Amikacin	35	44.30%	8	10.12%	36	45.56%
Amoxycillin	36	45.56%	1	1.26%	42	53.16%
Cefoperazon	17	43.58%	0	0	22	56.41%
Cefoperazone/ Sulbactam	8	22.22%	0	0	28	77.77%
Cefixime	8	40.00%	2	10.00%	10	50.00%
Ceftriaxone	26	41.93%	0	0	36	58.06%
Cotrimoxazole	33	55.93%	0	0	26	44.06%
Ciprofloxacin	15	36.58%	1	2.43%	25	60.97%
Nitrofurantoin	2	12.50%	5	31.25%	9	56.25%
Norfloxacin	7	43.75%	0	0	9	56.25%
Piperacillin	5	12.50%	4	10.00%	31	77.50%
Gentamicin	21	47.78%	1	2.27%	22	50.00%
Sulphamethoxazole/ Trimethoprim	7	70.00%	0	0	3	30.00%
Levofloxacin	19	52.77%	0	0	17	47.22%
Cefepime	16	48.48%	2	6.06%	15	45.45%
Cefpodoxime	15	45.45%	0	0	18	54.54%
Meropenem	9	12.85%	0	0	61	87.14%
Imipenem	9	13.23%	1	1.47%	58	85.29%
Colistin	33	100%	0	0	0	0

During the study period, 1475 non-repetitive samples were processed and 62.71% showed growth. Out of those, *Klebsiella pneumoniae* were second most predominant isolates (17.89%) after *E. coli* (33.98%). Among the different antibiotics used against *K. pneumoniae* isolates, all the isolates were found to be 100% sensitive to Colistin. Thus it can be the drug of choice. 87.14% isolates were resistant to Meropenem and 85.29% were resistant to Imipenem. Those meropenem resistant *K. pneumoniae* were phenotypically confirmed to produce carbapenemase by Modified Hodge Test (MHT) and modified Carbapenem Inactivating Method (mCIM). 53.64% were screened as carbapenemase producer by mCIM and 51.82% by MHT. Among the carbapenemase positive *Klebsiella pneumoniae* isolates, highest percentage producers were observed in sputum (45.61%), in age group 61-70 (28.07%) and in out-patient department (50.88%). Further, among the total *K. pneumoniae*, 34.55% were biofilm producers on CRA. Also, among carbapenemase producer, 35.08% were strongly biofilm producer and among carbapenemase non-producer 30.18% were strongly biofilm producer in CRA. In addition to this Microtitre plate assay evidenced that, out of 57 carbapenemase producer, 40.35% were highly biofilm producing and among the 15 carbapenemase non-producer 66.66% were highly biofilm producer in Microtitrplate assay.

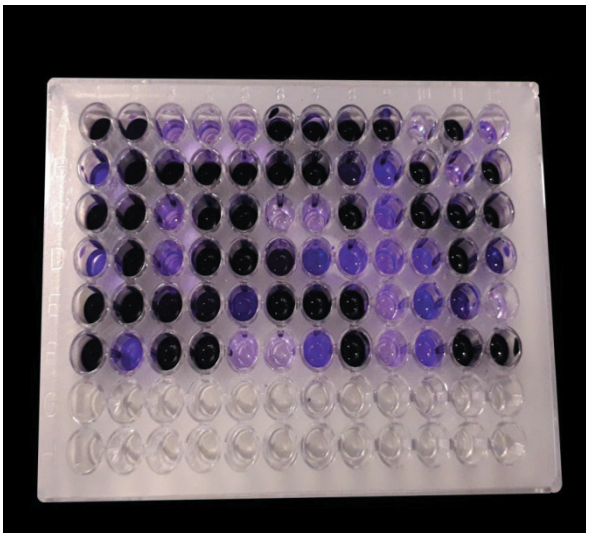
Photograph 1: Antibiotic Susceptibility Test (AST) of *Klebsiella pneumoniae* showing resistant to Carbapenems



Photograph 2: Modified Carbapenem Inactivation Method (mCIM) showing the carbapenemase activity and no carbapenemase activity (MEM- Meropenem



Photograph 3: Modified Hodge Test (MHT) showing the carbapenemase production by the isolates, indicated by cloverleaf indentation (Three different isolates were used for the test in the same plate).



Photograph 4: Detection of biofilm production by Microtitre plate assay (microtitre plate well indicate high, moderate, weak and non-adherent pattern of biofilm formation)

DISCUSSION

The resistance to carbapenem class of antibiotics which are considered as last resort antibiotics for bacterial infection have cause threat in the health sector. Biofilm is considered as one of the prominent factors for resistance. Hence this study has made attempt to investigate the relationship between carbapenemase producing and biofilm production. *Klebsiella pneumoniae* was second most predominat after *E. coli* in our study. However, Bina et al. (2015) and Dahiya et al. (2015) showed *Klebsiella pneumoniae* to be highly prevalent bacteria in hospital. These distribution and variation among the bacteria may be due to study location and geographical variation along with adaptation factor in environmental condition that comprises temperature, pH, humidity of the study site. *Klebsiella* has been associated with different types of infections and one of the important aspects of *Klebsiella* associated infections is their innate resistance to many antibiotics and the emergence of multidrug resistant strains particularly those involved in nosocomial diseases. AST showed 61 isolates to be meropenem resistant. Using the mCIM, 59 out of 61 isolate showed carbapenemase production with 53.64% while MHT showed carbapenemase

production among 57 out of 61 isolates with 51.82%. Those isolates which were negative for MHT and mCIM may have possibility of producing enzymes other than carbapenemase or due to mediation by other mechanism which favor carbapenem resistance or may be due to lesser amounts of carbapenemase production. Among the carbapenemase producer, highest was obtained from sputum. This result is contrast to the study by Henkhoneng et al. (2014) that showed, carbapenem resistant isolates were recovered primarily from urine. The reason for urine as primary samples could be urinary tract infection (UTI), being the most common hospital-acquired infection, which accounts for almost 40% of all nosocomial infections. But in our study sputum was found to be the key sample for the carbapenemase producer. It may be due to higher number of patient visiting hospital with respiratory tract infection or may be changing trend which substitute respiratory tract infection to be the more common hospital-acquired infection than the UTI. As infants and elderly patients are more prone to immunocompromised conditions, they are more prone to infection by carbapenemase producing isolates and other resistant microorganism that may follow failure antibiotic therapy. Our study also showed higher carbapenemase production in the elderly patients as significant case which might possibly be because of immunosuppressed condition and that may lead to failure of antibiotic application. Further, the oldest group are more likely to be hospitalized than the other ones and the immune status along with the underlying diseases and predisposing factors are more profound in the older age group (≥60 years) that makes them more prone to infection. This study showed a significant rate of carbapenem resistance among *K. pneumoniae* in hospitalized and OPD patients being higher in OPD cases, an indication by shift in infection from hospital acquired infection to community acquired infection. Biofilm-forming bacteria affect millions of people around the world every year, with a high mortality rate. The ability of nosocomial opportunistic microorganisms such as *K. pneumoniae* to produce biofilms on host-tissue surfaces is a critical stage in the development of infection. Biofilm formation affects the efficacy of antimicrobial therapies and the outcomes of subsequent infections. Thus, early determination of biofilm producer would be helpful to design and use of new and effective approaches which will ultimately aid in the treatment of biofilm-mediated infections and in the reduction of morbidity and mortality in patients suffering from life-threatening nosocomial infections. Our findings indicated that maximum proportion (more than 50%) of isolates were biofilm producing strains on Congo red agar (CRA). Among the 72 isolates which were biofilm producers in CRA, 53 isolates only were found to produce biofilm in quantitative assay analysis using Microtiter plate method. Moreover, the interpretation of Microtiter plate assay is optical density dependent and there are chances of subjective errors. However, despite the shortcomings of this method the Microtiter plate assay has been described as the most reliable method for the detection of biofilms (Djordjevic et al. 2002). Most carbapenemase producers are found to be strong biofilm producers. Those isolates which produced more carbapenemase were obtained from the specimen of the patients with higher line of antimicrobial therapy. Those patients have different implants and catheters and which are particularly susceptible to biofilm formation in *K. pneumoniae* because immune response are highly reduced in proximity to foreign objects (Houri et al. 2016). Proper management of biofilm is essential to control the infection related to carbapenemase production. Management of carbapenemase producing *K. pneumoniae* infection has become difficult due to limitation of treatment options. However, most of the last line antibiotics including colistin, tigecycline are stable antibiotics even in presence of carbapenemase enzyme. Therefore, this feature allows those antibiotics to be the first choice of the treatment for serious infections with carbapenemase *producing K. pneumoniae.*

CONCLUSION

This study identifies the carbapenemase producing *Klebsiella pneumoniae* and their association with biofilm production. The study revealed that carbapenem resistant isolates are strong biofilm producer than the non-carbapenemase producer. Early detection of biofilm production hence can be useful for clinical decision because of its suggestive

property for potential pathogenic capacity of *Klebsiella pneumoniae*. Implementation of early detection of carbapenem resistant and biofilm producing isolates would be useful, which may facilitate provision of individualized treatment regimen in patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Djordjevic D, Wiedmann M and McLandsborough LA (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol* **68**(6):2950–2958.

Houri H, Seifi K, Kazemian H, Heidari H, Rezagholizadeh F, Saee Y and Shirvani F (2016). Evaluation of biofilm formation among *Klebsiella pneumoniae* isolates and molecular characterization by ERIC-PCR. *Jundishapur J Microbiol* **9**(1): e30682.

Henkhoneng Mate P, et al. (2014). Prevalence of carbapenem resistance among Gram-negative bacteria in a tertiary care hospital in North-East India. I*OSR J Dent Med Sci* 13(12): 2279–2861.

Dahiya S, Singla P, Chaudhary U and Singh B (2015). Prevalence of *Klebsiella pneumoniae* Carbapenemase (KPC), metallo beta lactamases and AmpC beta lctamases in clinical isolates of Klebsiella species. *Hindawi* **4**(9): 170–176.

Bina M, Pournajaf A, Mirkalantari S, Talebi M and Irajian G (2015). Detection of the *Klebsiella pneumoniae* carbapenemase (KPC) in K. pneumoniae isolated from the clinical samples by the phenotypic and genotypic methods. *Iran J Pathol* **10**(3): 199–205.

Singhai M, Malik A, Shahid M, Malik MA and Goyal R. (2012). A study on device-related infections with special reference to biofilm production and antibiotic resistance. *J Glob Infect Dis* **4:** 193–198.

Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM and Mendes Giannini MJS (2013). Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of Medical Microbiology* **62**(1): 10–24.

Pokhrel BM, Koirala J, Mishra SK, Dahal RK and Tuladhar PKN (2006). Multidrug resistance and extended spectrum β-lactamase producing strains causing lower respiratory tract and urinary tract infection. *J Inst Med* **28:** 19-27.

Hall-Stoodley L, Costerton JW and Stoodley P (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**: 95–108.

Deorukhkar SC and Saini S (2014). Laboratory approach for diagnosis of candidiasis through ages. *Int J Curr Microbiol Appl Sci* **3**(1): 206–218.

David PL (2006). Resistance in gram-negative bacteria: Enterobacteriaceae. *Am J Infect Control* **34**(5): S20-S28.

Shaikh S, Fatima J, Shakil S, Rizvi DMS and Kamal MA (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi J Biol Sci* **22**(10): 90-101.

Bacterial Contamination of Street Vended Food Pani Puri Available in Janakpurdham, Dhanusha

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ABSTRACT

Objectives: To determine the level of bacterial contamination in food (Pani puri) available in market of Janakpur, Dhanusha, Nepal.

Methods: Total 120 samples of Pani puri (60 solid matters and 60 masalaa Pani) collected aseptically from the market were transported to the microbiology laboratory and processed for microbial count by serial dilution technique. Microbial load and presence of pathogen were detected in each sample.

Results: Analysis of the food samples revealed that 70% of Pani puri samples had high loads of bacterial pathogens such as *Escherichia coli, Staphylococcus aureus*, K*lebsiella* spp*, Pseudomonas* spp*, Bacillus* spp.

Conclusion: This study depicted that the street vended food Pani puri of Janakpur are highly contaminated with pathogenic bacteria which can cause health risk to consumers. Thus, it is suggested that regular monitoring of the quality of street foods must be practiced to avoid any food bone illness in future.

Key words: Pani puri, Hygiene, Bacteria, Contamination

INTRODUCTION

Street - vended foods are foods from street vendors which are ready to eat food (RTF) and drink prepared on the streets or at home and also sold in street or other public places, such as School, College, Universities, Market on fair, often from a portable food booth or food cart and are consumed on the streets without further preparation (Tambekar et al. 2011). Pani puri is very popular street food which is consumed by large amount of population of different age groups. The pani puri in Nepal has different names as Gol Gappa in Delhi, Phuchka in Bengal, Gup Chup in Chattisgarh or Pani Puri in Maharashtra evokes the same love at any part of the country. Gol Gappa is hollow puri, fried crisp and filled with a mixture of flavoured water (commonly known as imli pani), tamarind chutney, chilli, chaat masala, potato, onion and chickpeas. Pani puri is crowned as king of evening snack. This snack consists of three separate items i.e. pani, puri and masala. Wheat flour is used to make puri in masala boiled or mashed potatoes mixed with spicies are used. The pani is sour water to which spices likes salt, pepper, mango powder, jalijeera etc. are added. In every puri, masala are added after making a hole in it and then spicy water is filled in this puri and served to the consumers in plate on site (Saxena and Agarwal 2013).

The most popular street foods in Nepal are Pani puri among consumers. Although it is very popular, easily available and cheap, it is frequently associated with various food borne diseases. Food borne illness associated with the consumption of street foods has been reported in several places in Nepal. Selling the foods on road side, unhygienic preparation and handling, in sufficiency in water supply for cleaning purposes, make the street food more contaminated and major sources of food borne diseases (Abdussalam and Kaferstein 1993).

Microbial contamination of ready-to-eat foods sold by

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street vendors and hawkers has become a major health problem as they are associated with diarrhoeal diseases due to their improper handling and serving practices (Barro et al. 2007). Street food vendors are mostly uninformed of good hygiene practices (GHP) and causes of diarrhoeal diseases (Mensah et. al 2002), which can increase the risk of street food contamination. The vendors can be carriers of pathogens like *E. coli, Salmonella* spp, *Camplyobacter* spp and *S. aureus* who eventually transfer these foods borne hazards to consumers (Mankee et al. 2003, Dawson and canet 1991). The conditions of street food preparation and vending raise many concerns for consumer's health are consumed by huge population and frequently associated with diarrhoeal diseases due to their microbial contamination.

Street vended Pani puri is popular and sold in almost all the cities throughout Nepal. Janakpur, popularly known as Janakpurdham is a sub-metropolitan city in Dhanusha District of Province No. 2 of Nepal which is famous for religious and cultural tourism. There are various pani puri stalls in the streets and chowks of Janakpur in the crowdy and clumsy areas where has huge crowd of consumers during the business hours. Golgappas particularly in summer and monsoons are the season that conducive to bacterial growth in food items. Unclean location, unhygienic serving practice, storage of aaloo and masala, unhygienic water, stalls present in dust and dirt area and the source of transfats in Pani puri are major concerns of health issues in the front of the eye of Janakpur municipality. But no any permanent strategy has been planned to establish the Pani Puri stalls in hygienic places with hygienic services.

A general lack of factual knowledge about the epidemiological significance of many streets vended foods, poor knowledge of streets vendors in basic food safety measures and inadequate public awareness of hazards posed by certain foods has severely hamperd the development of a precise. Although there is a growing demand for these food products, enough information is not available regarding the microbiological quality of these products. Therefore, this study was aimed to determine the level of microbial burden in Pani puri to establish the hygienic status of street vended food Pani puri and their impact in street foods contamination at different parts of Janakpur, Nepal.

MATERIALS AND METHODS

The present study was conducted in microbiology laboratory of Model Multiple College, Janakpurdham from February to July in 2018 AD. The different samples of Pani puri were collected from different Pani puri stalls of local areas as Ramanand Chowk, Shiv chowk, Janak chowk, Ram chowk, Bhanu chowk and Railway station sites of Janakpur market. A total of120 samples were collected. Each sample of Pani puri was fragmented into two different segments (the liquid masala pani and solid matter masala) and were collected in sterile plastic containers which were sealed and transported aseptically to microbiology laboratory for further processing.

For the microbiological analysis, serial dilution technique was used. After serial dilution, pour plate technique was applied on nutrient agar for bacteria. After solidifying, Petri plates were incubated at 37^0C for 24 to 48 hours for the growth of bacteria in inverted position as per given in a monograph. After completion of the incubation period, the colonies appeared on the surface of NA media was counted in the petri plates. Total colony forming unit (CFU) was determined. The isolated colonies of organism were transferred into various selective and differential media and were identified by performing various biochemical tests.

RESULTS

A total of 120 Pani puri samples were analyzed. All the samples were processed for total count and pathogen identification and then result was interpreted. All the samples were examined for presence of microbial pathogens by pour plate technique. Among these 50(41.67%) samples were collected from the crowded vendors and 70 (58.33%) samples from non-crowded.

The total viable count of bacteria in masala pani was found between 90-182x10^5 and 50-121x10^5 and solid matter masala is varied between 80-130x10^1 and 46-118x10^5 from the crowded and non-crowded vendors respectively. The majority of samples were found to contaminate with different species of pathogenic microorganisms. Both pani masala 46(76.67%) and solid matter 38(63.33%) were found to be highly contaminated. Out of 120 samples analyzed, 84(70%) were found to be contaminated by pathogenic bacteria. The common contaminants isolated and identified were *E. coli* 32(38.09%), *Salmonella* 26(30.95%), *Staphylococcus aureus* 38(45.23%), *Pseudomonas* spp 18(21.42%), *Bacillus* 24(20.00%) etc. were the common isolates. *Staphylococcus aureus* and *E. coli* were the major contaminants of Pani puri.

Table 1: Percentage of positive sample among total sample

Sample	Panimasala (60)	Solid matter (60)	Growth in Panimasala (46)	Growth in solid matter (38)
Ramanand chowk	5	5	04(80.00%)	03(60%)
Ram chowk	8	8	06(75.00%)	05(62.5%)
Janak chowk	17	17	12(70.58%)	09(52.94%)
Shiv chowk	16	16	13(81.25%)	10(62.50%)
Railway station	14	14	11(78.57%)	11(78.57%)

Table 2: Bacterial contamination in crowded and non-crowded sample

Bacteria isolated	Crowded samples (50)	Non-crowded samples (70)	Total
E. coli	22(44%)	10(14.28%)	32(38.09%)
Salmonella spp.	15(30%)	11(15.71%)	26(30.95%)
Staphylococcus aureus	30(60%)	8(11.43%)	38(45.23%)
Pseudomonas spp.	10(20%)	8(11.43%)	18(21.42%)
Bacillus spp.	20(40%)	4(05.71%)	24(20.00%)

Distribution pattern of contaminated samples of pani puri

Out of 120 samples analyzed, 84(70%) were found to be contaminated by pathogenic bacteria and 30% were non-contaminates.

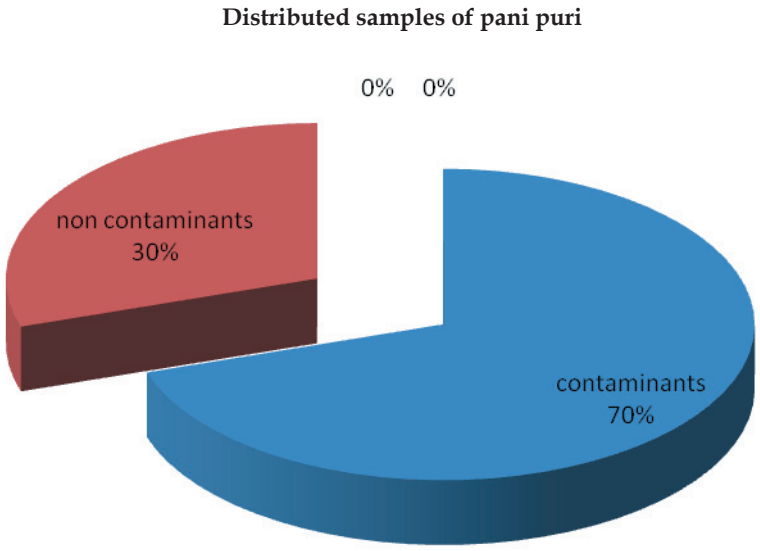

Figure 1: Distribution pattern of contaminated samples of pani puri

Bacteriological contamination of pani puri

Total five bacterial species were identified. Among them *E. coli 32(38.09%), Salmonella 26(30.95%), Staphylococcus aureus 38(45.23%), Pseudomonas 18(21.42%) and Bacillus 24(20.00%)* etc. were the common isolates. *Staphylococcus aureus* and *E. coli* were the major contaminants of Pani puri.

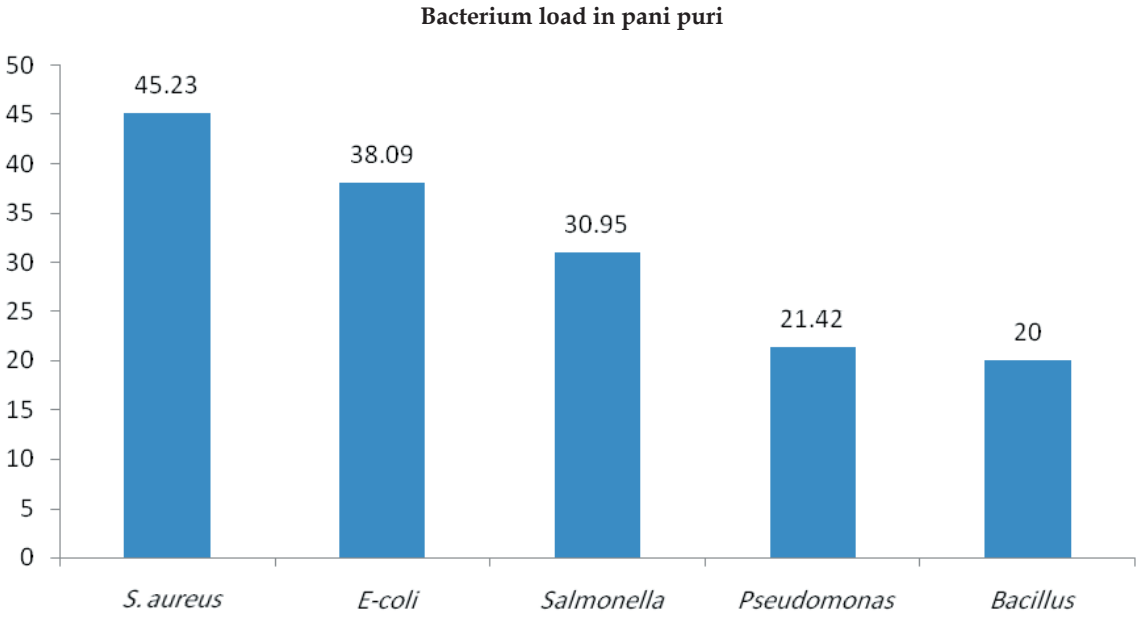

Figure 2: Bacteriological contamination of pani puri

Total viable count of bacteria (CFU)

The total viable count of bacteria in masala pani was found between 90-182x10^{5}CFU and 50-121x10^5 CFU and solid matter masala varied between 80-130x10^5 CFU and 46-118x10^5 CFU from the crowded and non-crowded vendors respectively.

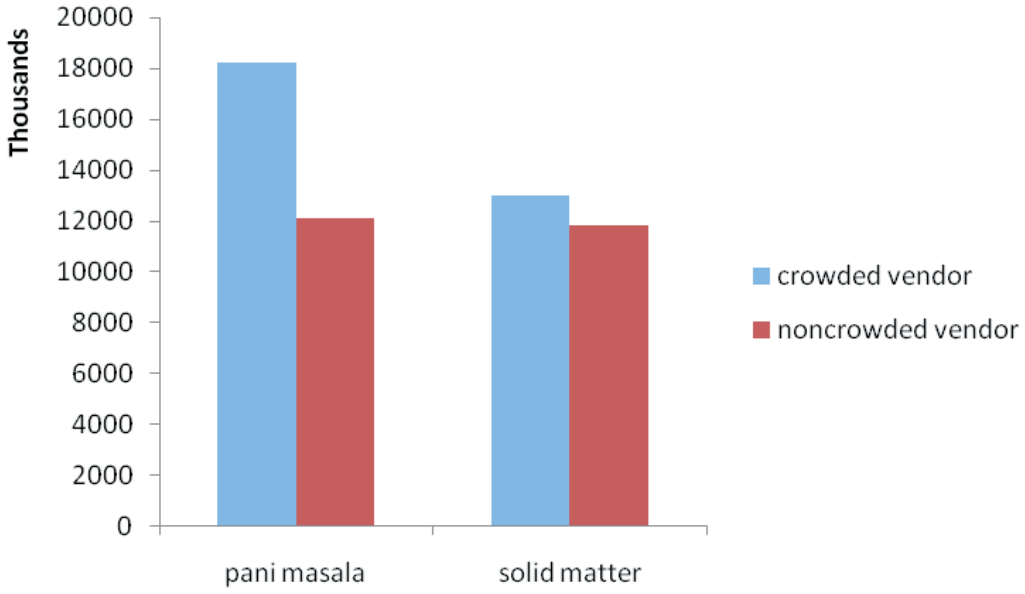

Figure 3: Total viable count of bacteria (CFU) in pani masala and solid matter

DISCUSSION

Hygienic quality of street food vending has become an important public health issue and a great concern to everybody. Microbial contamination of ready-to-eat foods and beverages sold by street vendors and hawkers has become a global health problem. In developing countries, fruit juices, drinks, meals and sold by street food vendors are widely consumed by millions of people (Tambekar et al. 2007).

Overall study indicated that most of the Pani puri samples were contaminated with variety of pathogenic bacterial contaminations. Many people have worked on the fact that Pani puri was contaminated with different bacterial pathogens because of various sources like improper handling of street foods, washing of utensils, dish cloths, stalls are at crowded place and movable stalls (Tamberkar et al. 2007). Microbial contamination may be linked to factors such as equipment and utensils with inadequate hygienic condition, uncovered utensils and garbage bin, irregularity of hand washing, in appropriate processing incomplete heating, use of contaminated water during preparation and washing or secondary contamination via contact with contaminated equipment's such as chopping boards, knives and serving wares (Adesiyun and Balbirsingh 1996). This might also implicate the processing and rinsing water as possible sources of contamination of pani puri sold by street vendors (Das et al. 2012). Similar results were obtained from the research conducted at Bharatpur (Khadaka et al. 2018).

In central Taiwan, 274 outbreaks of food-borne illness including 12,845 cases and 3 deaths were reported during 1991 to 2000. Majority (62.4%) of the outbreaks were caused by bacterial pathogens. The main etiologic agents were *Bacillus cereus*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*. The important contributing factor was improper handling of food. The implicated foods included seafood, meat products and cereal products (Chang & Chen, 2003). In a study carried out from October 2004 to October 2005 in Catalonia, Spain, 181 outbreaks were reported; 72 were caused by *Salmonella* and 30 by Norovirus (NoV) (Crespo et al., 2005). In 2002, in 31 the Netherlands a national study of food-borne illness outbreaks was performed. A total of 281 food borne illness outbreaks were included. Most of these outbreaks were reported from nursing homes, restaurants, hospitals and day-care centres. The causative agents included Norovirus (54%), Salmonella spp. (4%), Rotavirus (2%), and Campylobacter spp. (1%) (Duynhoven et al. 2005). A study conducted in Qassim province, Saudi Arabia, analyzed the food-borne illness surveillance data for the year 2006. During the study period, 31 food-borne illness outbreaks comprising of 251 cases, were reported. The most common etiologic agent was *Salmonella* species, followed by *Staphylococcus* aureus. The previous findings are almost in accord with this study.

The bacterial contamination in Pani puri is because of the conditions under which it is prepared and vended. In most of the cases running water is not available at vending sites and thus hand and dish washing are usually done in buckets & sometimes without soaps. (Das et al. 2012). *E. coli, Staphylococcus, Klebsilella, Salmonella etc* could be due to inadequate hand washing by food workers and the absence of good manufacturing practices. The occurrence of *P. aeruginosa* might be due to improper personal hygiene, unhygienic surrounding, vehicular transmission, and sewage. The presence of *S. aureus* was severe contamination through handling (Tambekar et al. 2007).

Crowded areas have a greater number of pathogens than non-crowded areas. There is potential health risks associated with initial contamination of foods by pathogenic bacteria as well as sub sequent contamination by vendors during preparation, handling, and cross contamination (Mosupye & van Holy 2000). From all above discussion it was concluded that sample of Pani puri was contaminated with *E. coli,* Staphylococcus *species. , Salmanella species., Pseadomonas species., Klebsilella pneumonia* which can cause various food borne infection.

The study cannot be related with the whole Dhanusha District and Janakpur Zone as it was carried specifically in town area of Janakpur only. Samples from all pani puri stalls in every street were not located due to impermanency of Pani puri stall and short availability of time during this research.

CONCLUSION

The present study concludes high level of contamination in different locations in one of the most popular Nepalese street food, Pani puri sold in Janakpur. *Staphyloccoccus aureus* and *E. coli* were the major contaminants of Pani puri. Foods sold by street vendors in Janakpur are contaminated with pathogenic bacterial organisms, which are likely to pose a potential hazard

to consumers, an issue that needs to be addressed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abdussalam M, Kaferstein FK (1993). Safety of street foods. *World Health Forum* **14:**191–194.

Adesiyun AA and Balbirsingh V (1996). Microbiological analysis of 'black pudding', a Trinidadian delicacy and health risk to consumers. *Int J Food Microbiol* **31:** 283–299.

Barro N, Razack BA, Yollande I, Aly S, Tidiane OCA, Philippe NA, Comlan DS and Sababenedjo TA (2007). Street vended foods improvement: contamination mechanisms and application of food safety objective strategy: critical review. *Pak J Nutr* **6**(1): 1–10.

Chang JM, and Chen TH (2003). Bacterial Foodborne Outbreaks in Central Taiwan, 1991- 2000. *Journal of Food and Drug Analysis* **11**(1): 53-59.

Crespo PS, Hernández G, Echeíta A, Torres A, Ordóñez P and Aladueña A (2005). Surveillance of foodborne disease outbreaks associated with consumption of eggs and egg products: Spain, 2002-2003. *European Communicable Disease Bulletin* **10**(6): E050616.2

Das M, Rath CC and Mohapatra UB (2012). Bacteriology of a most popular street food (pani puri) and inhibitory effect of essential oils on bacterial growth. *Journal of Food Science and Technology* **49**(50): 564-571.

Dawson RJ and Canet C (1991). International activities in street foods. *Food Control* **(2):** 135–139.

Duynhoven Van PHTY, Jager DMC, Kortbeek ML, Vennema H and Koopmans GPMH, et al. (2005). A One-Year Intensified Study of Outbreaks of Gastroenteritis in the Netherlands *Epidemiol Infect* **133**(1): 9-21.

Gormley FJ, Little CL, Rawal N, Gillespie IA, Lebaigue S and Adak GK (2011). A 17-year review of foodborne outbreaks: describing the continuing decline in England and Wales (1992-2008). *Epidemiol Infect* **139**(5): 688-99.

Khadka S and Adhikari S et al. (2018). Bacterial contamination and risk factors associated with street vended Pani puri sold in Bharatpur, Nepal. *Int J Food Res* **5**: 32-38.

Mankee A, Ali S, Chin A, Indalsingh R and Khan R, et al. (2003). Bacteriological Quality of doubles sold by street vendors in Trinidad and the attitudes, knowledge and perceptions of the public about its consumption and health risk. *Food Microbiol* **20:** 631–639.

Mensah P, Manu DY, Darko KO and Ablordey A (2002). Streets foods in Accra, Ghana: how safe are they? *Bulletin of World Health Org* **80**(7)**:** 546-554.

Mosupye FM and Van HA (2000). Microbiological hazard identification and exposure assessment of street food vending in Johannesburg, South Africa. International Journal of *Food Microbiol* **61:** 137-145.

Saxena G and Agarwal M (2013). Microbial quality assessment of street-vended Golgappa and Bhelpuri sold in Jaipur city of Rajasthan. *Int J Food Nutr Sci* **2**(1): 71-77.

Tambekar DH, Shirsat SD, Suradkar SB, Rajankar PN, and Banginwar YS (2007). Prevention of transmission of infectious disease: Studies on hand hygiene in health-care among students. *Continental Journal of Biomedical Sci* **1:** 6-10.

Comparison of Biofilm Producing and Non-producing *Escherichia coli* Isolated from Urine Samples of Patients Visiting a Tertiary Care Hospital of Morang, Nepal

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ABSTRACT

Objectives: The main objective of this study was to determine the prevalence of *Escherichia coli* among urinary tract infection (UTI) suspected patients visiting tertiary care hospital and to assess the biofilm producing ability of *E. coli* isolates.

Methods: A prospective cross-sectionalstudy was carried out in Biratnagar Metropolitan city, Eastern Nepal from December 2018 to May 2019. During the study 400 urine samples were collected from UTI suspected patients visiting a tertiary care hospital ofBiratnagar. Urine samples were cultured by using semi-quantitative culture technique and identified. Antibiotic susceptibility testing was done by Kirby-Bauer Disk Diffusion method according to CLSI (2011) guidelines.Biofilm assays were performed by microtitre plate method.

Results: This study reported 15% prevalence of *E. coli* out of 400 urine samples. 100% of *E. coli* isolates showed resistance to both Ampicillin and Amoxicillin while 100% were sensitive to Chloramphenicol. 70% (42/60) isolates wereMulti Drug Resistance (MDR)*E. coli*. The maximum isolates (86.66%) were found to be biofilm producers by microtitre plate method. Resistance to other antibiotics such as Nalidixic acid (71.11% vs 46.66%), Norfloxacin (53.33% vs 46.66%), Cotrimoxazole (42.22% vs 26.66%) was comparatively higher among biofilm producers than non-biofilm producers. There was a significance of association between biofilm and MDR ($p<0.05$).

Conclusion: There is relation between the ability of biofilm formation and drug resistance in the bacterium resulting to the failure of antibacterial drugs.

Key words: *E. coli,* Biofilm producer, Multidrug resistance (MDR), UTI

INTRODUCTION

Escherichia coli is Gram negative, facultative anaerobic and coliform bacterium which is common colonizer of lower intestine of warm-blooded animals (Tenaillon et al. 2012). Among all the members of *Enterobacteriaceae* family, *E. coli* is the most common pathogen (80-85%) involved in urinary tract infection (UTI) (Nicolle 2008; Bhatta et al. 2012). In case of UTI, fecal bacteria colonize urethra and spread up the urinary tract and finally to the bladder while sometimes to the kidneys causing pyelonephritis or the prostrate in males (Nicolle 2008).

During the lifetime approximately 10% of the humans acquire UTI at some time (Karki et al. 2004). The incidence of UTI is age and sex dependent example women are more prone to UTI than men (Nicolle 2008). Females falling within the age group 21-30 years experiences UTI more frequently (Baral et al. 2012).

Biofilm formation is a phenomenon which is produced by microorganisms to survive in harsh environment or for establishing bacterial infection in humans (Neupane et al. 2016). This protects bacteria from antibiotics and host defenses which as a result makes the treatment of infection more difficult (Anderson et al. 2003). The interaction between the bacterial cells within a biofilm can lead to the exchange of plasmid, drug resistance marker genes and hence enhances antimicrobial resistance (Watnick et al. 2000; Kostakioti et al. 2013). Thus, biofilm mode of living is advantageous for

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uropathogens to withstand stress and antibiotic drugs in urinary tract environment (Pramodhini et al. 2012).

According to the centers for disease control and prevention, multidrug resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al. 2011). The emergence of multidrug resistance *E. coli* in urinary tract infection has become a global concern (Mashwal et al. 2017). Study has reported *E. coli* being resistant against trimethoprim-sulfamethoxazole, fluoroquinolones and other antibiotics including ciprofloxacin (Karlowsky et al. 2006). The diagnosis of UTI is usually based on a quantitative urine culture yielding greater than 10^5 colony forming units per mL (Kass et al. 1957). However, several studies suggest that more than one third of symptomatic women show CFU counts below this level (low-coliform-count infection) and that a bacterial count of 100 CFU per mL of urine has a high positive predictive value for cystitis in symptomatic women (Komaroff et al. 1986; Kunin et al. 1993).The main aim of this study was to determine the prevalence of *E. coli* among UTI suspected patients visiting tertiary hospital and to assess the biofilm producing ability of *E. coli* isolates.

MATERIALS AND METHODS

Study site: This research was performed from December 2018 to May 2019 after receiving ethical approval from Nepal Health Research Council (NHRC), Kathmandu. During the study 400 urine samples were analyzed. All the works related to research were performed in microbiology laboratory of tertiary care hospital and of Central Campus of Technology, Dharan. The urine samples were taken from urinary tract infection suspected patients visiting a tertiary hospital of Biratnagar.

Sample collection: The midstream urine samples were collected from UTI suspected patients in sterilized screw-cap propylene bottles following standard guidelines (Isenberg 2004). The samples were then processed in microbiology laboratory as soon as after the collection. The containers were labeled with patient's name, ID number, specimen type and date of collection. In case of any delay in processing for more than 2 hours, samples were refrigerated at 4°C.

Isolation and identification: Urine specimens were cultured by using semi-quantitative culture technique as described by Kass (1962). A loopful of well-mixed sample was inoculated using standard calibrated loop onto Cystine-Lactose-Electrolyte-Deficient Agar (CLED) (HiMedia, India) and incubated aerobically at 37°C for 24 hours. After overnight incubation, colony counts yielding bacterial growth of $\geq 10^5$ were taken as being significant for UTI. For identification of isolates, at first colony characteristics of isolated bacteria were observed on agar plates and Gram staining was done. Gram negative isolates were then further identified by performing different biochemical tests including catalase, oxidase, indole utilization test, citrate test, methyl red, VP test, carbohydrate fermentation test and triple sugar iron utilization test. Isolates other than *E. coli*were not considered for this study.

Microtitre plate method for detection of biofilm: This method was performed as described by Borucki et al. (2003). Each culture was individually grown overnight in 10 mL of Trypticase Soy Broth (TSB) (HiMedia, India) at 37°C for 24 hours and diluted to 1:40 in TSB containing 0.25% glucose. Then 200µl of diluted culture was inoculated in a sterile microtitre well. The plates were incubated at 37°C for 24 hours for biofilm production. After incubation, content of each well was removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (PBS with pH 7.4) for four times and finally stained with 0.1% crystal violet solution for 30 minutes. After rinsing thrice with the sterile distilled water and subsequent drying, the stain taken up by the adherent biofilm was extracted by using 95% ethanol at 4°C. The content of each well was transferred to another microtitre well and the absorbance was measured at 595nm by ELISA plate reader(Loncare LR-620 microplate reader, Medical Technology Co., Ltd.).The experiment was performed in triplicate.Interpretation was made on OD by subtracting OD of control wells from OD of test wells. The optical density (OD_s) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD_{nc}). The following classification was used for the determination of biofilm formation: no biofilm production ($OD_s \leq OD_{nc}$), weak biofilm production ($OD_{nc} < OD_s \leq 2.OD_{nc}$), moderate biofilm production ($2.OD_{nc} < OD_s \leq 4.OD_{nc}$) and strong biofilm production ($4.OD_{nc} < OD_s$) (Stepanovic et al. 2007).

Antibiotic susceptibility test (AST): Antibiotic susceptibility of *E. coli* was evaluated against antibiotics ampicillin, chloramphenicol, sulfonamides, tetracycline,

ciprofloxacin, trimethoprim-sulfamethoxazole, cefotaxime and nalidixic acid by Kirby Bauer disc diffusion method following CLSI (2011) guidelines. Sub-cultured colonies were taken from nutrient agar plates and turbid suspension was made as per 0.5 McFarland standards by emulsifying colonial growth in Luria-Bertani broth (LB) (HiMedia, India). A sterile cotton swab was dipped into LB and the swab was streaked on the entire surface of Mueller Hinton agar (HiMedia, India) three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums. Finally, swab was done all around the edge of the agar surface. Using sterile tweezers, antibiotic discs were placed aseptically on the surface of Mueller Hinton agar plates. The plates were then incubated at 37°C for 24 hours.

Data analysis: The information was collected from questionnaire and finally tabulated. The data were analyzed by SPSS version 16. The p value less than equal to 0.05 was considered statistically significant.

RESULTS

Prevalence of *E. coli*

Out of 400 samples, 15% were positive for *E. coli*.

Table 1: Prevalence of *E. coli* in urine samples from UTI suspected patients

E. coli in urine samples		Prevalence	
Positive		60 (15%)	
Negative		340 (85%)	
Gender wise prevalence of *E. coli*			
Gender	**Number of subjects**	**UTI by *E coli***	**p-value**
Male	9	1(11.11%)	<0.05
Female	391	59(15.08%))	

Antibiotic susceptibility pattern of *E. coli* isolates

The most effective drugs for *E. coli* were found to be Chloramphenicol (100%), Cephoxitin (78.33%) and Ofloxacin (78.33%). *E. coli* were resistant to Ampicillin (100%), Amoxicillin (100%) and Nalidixic acid (65%).

Table 2: Antibiotic susceptibility pattern of *E. coli* isolates

Antibiotics	Resistant (%)	Sensitive (%)	p-value
Amoxicillin	60(100)	-	-
Ampicillin	60(100)	-	-
Cefotaxime	16(26.66)	44(73.33)	<0.001
Ceftriaxone	15(25)	45(75)	<0.001
Cephoxitin	13(21.66)	47(78.33)	<0.001
Chloramphenicol	-	60(100)	-
Ciprofloxacin	12(20)	48(60)	<0.001
Co-Trimoxazole	23(38.33)	37(61.66)	0.018
Gentamycin	14(23.33)	46(76.66)	<0.001
Nalidixic acid	39(65)	21(35)	0.01
Norfloxacin	31(51.66)	29(48.33)	0.584
Ofloxacin	13(21.66)	47(78.33)	<0.001
Tetracycline	26(43.33)	34(56.66)	0.201
Trimethoprim	22(36.66)	38(63.33)	0.060

Multidrug resistant (MDR) *E. coli*

Uropathogenic *E. coli* which showed resistance to three or more than three antibiotics were considered as multidrug resistant. 42 (70%) isolates of *E. coli* were MDR out of 60 isolates.

Table 3: Multidrug resistant (MDR) *E. coli*

Samples	Uropathogenic *E. coli*
Total samples	60
Multidrug resistant	42 (70%)

Antibiogram of biofilm producer and non-producer *E. coli*

Ampicillin and Amoxicillin were resisted by all isolates of *E. coli*. The biofilm producing *E. coli* showed high resistance to all antibiotics as compared to biofilm non-producer *E. coli*

Table 4: Antibiogram of biofilm producer and non-producer *E. coli*

Antibiotics	% of Biofilm Producing Resistant to antibiotics	% of non-biofilm Producing Resistant to antibiotics	P-value
Amoxicillin	100	100	-
Ampicillin	100	100	-
Cefotaxime	31.11	13.33	0.004
Ceftriaxone	31.11	6.66	0.001
Cephoxitin	24.44	13.33	0.019
Chloramphenicol	-	-	-
Ciprofloxacin	24.44	6.66	0.006
Cotrimoxazole	42.22	26.66	0.002
Gentamycin	28.88	6.66	0.002
Nalidixic acid	71.11	46.66	<0.001
Norfloxacin	53.33	46.66	0.002
Ofloxacin	24.44	13.33	0.019
Tetracycline	44.44	40	0.007
Trimethoprim	42.22	20	0.001

DISCUSSION

The overall prevalence of *E. coli* in urine samplesfrom UTI suspected patients was15% (60/400). Neupane et al. (2016) and Khatri et al. (2017) showed very similar report of 15.5% and 14.1% respectively. In this study the prevalence of UTI by *E. coli* was higher in female population than in male population which was statistically significant ($p<0.05$) which is consistent with many other studies.

In this study out of total 400 samples, 71 (17.77%) urine samples showed significant growth of uropathogens ($\geq 10^5$ cfu / mL) in which *E coli* was isolated from 60 (15%) urine samples Ponnusamy et al. (2012) and Sherchan et al. (2016) reported comparatively higher percentage of *E. coli* 23.49% and 87.9% of UTI casesrespectively. According to a research done by Neupane et al. (2016), 18.8% of the sample population showed significant growth of bacteria which is very similar to our result. All *E. coli* isolates were sensitive to Chloramphenicol and resistant to Amoxicillin and Ampicillin. A very close similarity was revealed by Sharma et al. (2013) and Ouno et al. (2013).

In our study, 70% *E. coli* were MDR. Baral et al. (2012) recorded 41.1% of MDR *E. coli* isolates in his investigation which was very less in comparison to our work. As per the experimentation done by Dehbanipour et al. (2016) and Poursina et al. (2018) multidrug resistant *E. coli* were73% and 68% respectively and it was very close to our analysis. Multidrug resistance has become a major problem in the treatment of diseases. The resistance of UTI causing bacteria towards commonly used antibiotics is escalating both in developing and developed countries (Elsayed et al. 2017).

Among 60 *E. coli* isolates, 31.66% were strong biofilm producers, 21.66% moderately positive, 21.66% were weak ones and 25% were biofilm non-producers by using microtitre plate method which were in accordance with the findings of Neupane et al. (2016) and Khatri et al. (2017).

Biofilm producing microorganisms shows resistance to large number of antibiotics increasing antibiotic resistance up to 1000 folds and hence, higher concentration of antimicrobial is required to treat such microorganisms (Stewart et al. 2001). Inadequate

amount of antibiotics reaching some areas of biofilm and inactiveness of bacteria located at the base of biofilm may be the reason for such resistance (Soto et al. 2014). In this investigation, both the biofilm producing and non-producing *E. coli* were resistant to Amoxicillin and Ampicillin (100%). However, resistance to other antibiotics such as Nalidixic acid, Norfloxacin and Cotrimoxazole was comparatively higher among biofilm producers than biofilm non-producers. Furthermore, this study there was a statistical significance ($p<0.05$) between biofilm formation and multidrug resistance (MDR) which was also reported by Murugan et al. (2011) and Kulkarni et al. (2018).

CONCLUSION

High prevalence of Multidrug resistant *E. coli* in UTI suspected patients alarms the need of prescribing antibiotics based only on culture and sensitivity reports. There is relation between the ability of biofilm formation and antibioticresistance in the bacterium resulting to the failure of antibacterial drugs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J and Hultgren SJ (2003). Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* **301**(5629): 105-107.

Baral P, Neupane S, Marasini BP, Ghimire KR, Lekhak B and Shrestha B (2012). High prevalence of multidrug resistance in bacterial uropathogens from Kathmandu, Nepal. *BMC Research Notes* **5**: 38.

Bhatta CP, Shrestha B, Khadka S, Swar S, Shah B and Pun K (2012). Etiology of urinary tract infection and drug resistance cases of uropathogenes. *Journal of Kathmandu Medical College* **1**(2): 114-120.

Borucki MK, Peppin JD, White D, Loge F and Call DR (2003). Variation in biofilm formation among strains of Listeria monocytogenes. *Environ Microbial* **69**: 7336-7342.

CLSI (2011). Performance standards for antimicrobial susceptibility testing. M100-S21. *Clinical and Laboratory Standards Institute*, Wayne PA.

Dehbanipour R, Rastaghi S, Sedighi M, Maleki N and Faghri J (2016). High Prevalence of multidrug-resistance uropathogenic *E. coli* strains, Isfahan, Iran. *Journal of Natural Science* **7**(1): 22-26.

Elsayed TI, Ismail HAF, Elgamal SA and Gad AHA (2017). The occurrence of multidrug resistant *E. coli* which produce ESBL and cause urinary tract infections. *Journal of Applied Microbiology and Biochemistry* **1**: 6-8.

Isenberg HD (2004). Clinical Microbiology Procedures Handbook (2nd edition). ASM press Washington, D.C.

Karki A, Tiwari BR and Pradhan SB (2004). Study of bacteria isolated from urinary tract infection and their sensitivity pattren. *Journal of the Nepal Medical Association* **154**(43): 200-203.

Karlowsky JA, Kelly LJ, Thornsberry C, Jones ME and Sahm DF (2002). Trends in antimicrobial resistance among urinary tract infection isolates of *E. coli* from female outpatients in the United States. *Antimicrob Agents Chemother* **46**: 2540–2545.

Kass EH (1962). Pyelonephritis and bacteriuria A major problem in preventive medicine. *Ann Intern Med* **56**: 46-53.

Kass EH (1957). Bacteriuria and the diagnosis of infections of the urinary tract: with observations on the use of methionine as a urinary antiseptic. *AMA Archives of Internal Medicine* **100(5)**: 709-714.

Khatri S, Pant ND, Neupane S, Bhandari S and Banjara MR (2017). Biofilm production in relation to extended spectrum beta-lactamase production and antibiotic resistance among uropathogenic *E. coli*. *Janaki Medical College Journal of Medical Sciences* **5**(1): 61-63.

Komaroff AL (1986). Diagnostic decision: Urinalysis and urine culture in women with dysuria. *Annals of Internal Medicine* **104**(2): 212-218.

Kostakioti M, Handjifrangiskou M and Hultgren SJ (2013). Bacterial biofilms: development, dispersal and therapeutic strategies in the dawn of the post-antibiotic era. *Cold Spring Harb Perspect Med* **3**(4): a010306.

Kulkarni SR and Peerapur BV (2018). Analysis of antibiotic sensitivity profile of biofilm-forming uropathogenic *E. coli*. *Journal of Natural Science, Biology and Medicine* **9**(2): 175-179.

Kunin CM, Hua TH, Krishnan C, Van Arsdale WL and Hacker J (1993). Isolation of a nicotinamide requiring clone of E. coli 018: K1: H7 from women with acute cystitis: resemblance to strains found in neonatal meningitis. Clin Infect Dis 16(3): 412-416.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falages ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Liljequist BO, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT and Monnet DL (2011). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **18**(3): 268-281.

Mashwal FA, Safi SHEI, George SJ, Adam AA and Jebakumar AZ (2017). Incidence and molecular characterization of the extended spectrum betalactamase-producing *E. coli* isolated from urinary tract infections in Eastern Saudi Arabia. *Saudi Medical Journal* **38**(8): 811-815.

Murugan S, Devi PU and John PN (2011). Antimicrobial susceptibilty pattern of biofilm producing *E. coli* urinary tract infections. *Current Research in Bacteriology* **4**(2): 73-80

Neupane S, Pant ND, Khatiwada S, Chaudhary R and Banjara MR (2016). Correlation between biofilm formation and resistance towards different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic *E. coli* isolated from the patients suspected of urinary tract infection visiting Shree Birendra Hospital, Chhauni, Kathmandu, Nepal. *Antimicrobial Resistance and Infection Control* **5**: 5.

Nicolle LE (2008). Uncomplicated urinary tract infection in adults including uncomplicated pyelonephritis. *Urologic Clinics of North America* **35**: 1-12.

Ouno GA, Korir SC, Cheruiyota JC, Ratemo OD, Mabeya BM, Mauti GO, Mauti EM and Kiprono SJ (2013). Isolation, identification and characterization of urinary tract infectious bacteria and the effect of different antibiotics. *Journal of Natural Sciences Research* **3**(6): 2224-3186.

Ponnusamy P, Natarajan V and Sevanan M (2012). Invitro biofilm formation by uropathogenic *E. coli* and their antimicrobial susceptibility pattern. *Asian Pacific Journal of Tropical Medicine* 210-213.

Poursina F, Sepehrpour S and Mobasherizadeh S (2018). Biofilm formation in Non-multidrug-resistant *E. coli* isolated from patients with urinary tract infection in Isfahan, Iran. *Advanced Biomedical Research* **7**: 40.

Pramodhini S, Niveditha S, Umadevi S, Kumar S and Stephen S (2012). Antibiotic resistance pattern of biofilm-forming uropathogens isolated from catheterized patients in Pondicherry, India. *Australasian Medical Journal* **5**(7): 344-348.

Sharma AR, Bhatta DR, Shrestha J and Banjara MR (2013). Antimicrobial susceptibility of *Escherichia coli* isolated from urinary tract infected patients attending Bir hospital. *Nepal Journal of Science and Technology*, **14**(1): 177-184.

Sherchan JB, Gurung P, Karkee P, Shrestha N and Ohara H (2016). Microbiological and clinical profile of uropathogenic *E. coli* isolates in Kathmandu University Hospital. *J Nepal Health Res Counc* **14**(32):33-38.

Soto SM (2014). Importance of biofilms in urinary tract infections: new therapeutic approaches. *Advances in Biology* Article ID 543974, 13 pages.

Stepanovic S, Vukovic D, Hola V, Bonqventura GD, Djukic S, Clrkovic I and Ruzicka F (2007). Quantification of biofilm in microtitre plates: Overview of testing conditions and practical recommendation for assessment of biofilm production by *Staphylococci*. *Journal Compilation APMIS* **115**: 891-899.

Stewart PS and Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. *The Lancet* **358**(9276): 135-138.

Tenaillon O, Rodriquez VA, Gaut RL, McDonald P, Bennett AF, Long AD and Gaut BS (2012). The Molecular diversity of adaptive convergence. *US National Library of Medicine National Institutes of Health* **335**(6067): 457-61.

Watnick P and Kolter R (2000). Biofilm, city of microbes. *Journal of Bacteriology* **182**(10): 2675-2679.

Comparative Study of Antibacterial Activity of Juice and Peel Extract of Citrus Fruits

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ABSRTACT

Objectives: This study was aimed to compare the antibacterial activity of peel and juice extracts of citrus fruits.

Methods: The antibacterial activity of juice and peel extract of five selected citrus fruits [C1- *Citrus reticulata* (mandarin orange), C2- *Citrus limetta* (sweet lime), C3- *Citrus aurantifolia* (lime), C4- *Citrus limon* (lemon) and C5- *Citrus maxima* (pommelo)] on three Gram positive and five Gram negative bacterial strains were examined. The juice and peel extract of citrus fruits was obtained by using absolute ethanol. The obtained extracts were dissolved in 1ml 10% v/v DMSO which was taken to carry out the antibacterial susceptibility assay using agar well diffusion method.

Results: The peel extracts of C1 and C2 were found to be more effective against the given Gram-positive bacteria than the juice extracts with maximum zone of inhibition (20.33±1.527) and (15.33±0.577) against *Bacillus* spp respectively. While the juice extracts of C3 and C4 were more effective against both Gram positive and negative bacteria than their peel extracts with maximum zone of inhibition (26.66±1.15) against *S. aureus* ATCC 25923 and (20±1) against *E. coli* ATCC 25922 respectively. The juice extract of C5 was found to be more effective against both Gram positive and negative bacteria than its peel extract with maximum zone of inhibition (18.66±1.154) against *E. coli* ATCC 25922 except for *Bacillus* spp in which peel extract showed maximum zone of inhibition i.e. (15±1)

Conclusion: This study suggests that these juice and peel extracts may have beneficial antibacterial roles that can be exploited in controlling unwanted bacterial growth.

Key words: Citrus fruits, Phytochemicals, Antibacterial susceptibility

INTRODUCTION

Citrus fruits belong to the family of Rutaceae. They consist of about 140 genera and 1,300 species, and are one of the major fruit tree crops grown throughout the world. Citrus juices are consumed not only because of their nutritional value but also due to their special flavor. Fruit juice consumption is beneficial for the maintenance of good health and prevention of diseases. The positive health benefits of juices have been ascribed in part to Vitamin C (ascorbic acid), the major vitamin found in fruits and vegetables (Boudries et al. 2012; Rekha et al. 2012). Citrus fruits are also known to contain phytochemicals (bioactive compounds) such as phenolic, flavonoids, vitamins, and essential oils. Multiple solvents can be employed for the desired yield of phytochemicals. These phytochemicals are believed to be responsible for wide range of protective health benefits including anti oxidative, anti-inflammatory, antitumor, and antimicrobial activities (Aruoma et al. 2012; Karimi et al. 2012). Traditionally it is used to soothe sore throats, indigestion, relieve intestinal gas and bloating, resolve phlegm and as an additive for flavoring to our foods (Nicolosi et al. 2000; Adham et al. 2000).

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Bacterial infections are one of the prominent causes of health problems, physical disabilities and mortalities around the world. Due to the presence of wide range of phytochemicals, medicinal plants are believed to provide a safer and cost-effective way of treating bacterial infections. The antibacterial activity of natural products from medicinal plants is applicable for the treatment of bacterial, fungal and viral diseases. Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased (Bernhoft 2010). Secondary metabolites or phytochemicals such as phenols, flavonoids, alkaloids, terpenoids, and essential oil have proved to be responsible for the antimicrobial activity of plants (Hwang et al. 2001). Phenol and polyphenol group of compounds consist of thousands of diverse molecules with heterogeneous structure with common feature of having one or more phenol ring. Several workers have reported that phenolic compounds such as gallic acid, coumarins, polyphenols, caffeic acid, cinnamic acid, pyrogallol, eugenol show antimicrobial activity against virus, bacteria and fungi (Saify et al. 2005).The present study aimed to investigate antibacterial properties of juice and peel extract of *Citrus reticulata, Citrus limetta, Citrus aurantifolia, Citrus limon* and *Citrus maxima* using agar well diffusion method against some Gram positive bacteria (*Bacillus* spp, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* ATCC 25922, *Escherichia coli*, *Salmonella* Typhi, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa*)

MATERIALS AND METHODS

Five different varieties of unripe raw citrus fruits named as Lime (*Citrus aurantifolia*), Lemon (*Citrus limon*), Pommelo (*Citrus maxima*), Sweet lime (*Citrus limetta*), and Mandarin orange (*Citrus reticulata*) was collected from the local market of Lagankhel, Lalitpur in the month of November. Eight microorganisms were used in this study, consisting of three Gram positive (*Staphylococcus aureus*ATCC 25923, *Staphylococcus aureus* and *Bacillus* spp), and five Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli* ATCC 25922, *Escherichia coli*, *Klebsiella pneumonia*e ATCC 13883, and *Salmonella* Typhi). All microorganisms were obtained from MED Micro Lab, Kathmandu, Nepal.The study was conducted in the microbiological laboratory, Department of Microbiology, DAV College. The study was conducted from 12th November-18th February (3 months) 2018/19.

Method described by Hegazy and Ibrahium (2012) was followed with slight modification. The fruits were washed thoroughly after collection by in distilled water. After washing, the peels were separated and oven dried at 55°C and after they were completely dried, they were finely crushed into coarse powder with the help of blender and preserved at room temperature (RT) for future use. On the other hand, pulp of fruits was blended using blender to obtain juice. Both the samples i.e. juice and peel powder were extracted by absolute ethanol (10gm powder mixed with 100ml ethanol for peel extract and 10ml juice in 90ml ethanol for juice extracts) and the mixture was kept at 30°C for 72 hours with constant agitation and the extract was then filtered through Whatman No.1 filter papers. The filtrate was then centrifuged at 4000rpm for 15 min. The solution was then evaporated at 50°C until a sticky mass was obtained that was weighed and dissolved in an aliquot of 1.0 ml of 10% v/v Dimethyl sulfoxide (DMSO). The mass was stored at 4°C until further use. The samples were coded as [C1: Mandarin orange, C2: Sweet lime, C3: Lime, C4: Lemon, and C5: Pomelo]. Yields were estimated according to the method described by Prasani et al. (2005).

The agar well diffusion method was performed to analyze the antibacterial activity against the test organisms. The plant extract sample was prepared by dissolving 1000mg of plant extract into 1ml of 10% DMSO and for control sample 10% DMSO was used. The plates were incubated at 37°C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition (ZOI) around the well. The assay was repeated thrice for concurrent readings.

The yields of extracts were calculated in percentage and the mean and standard deviation of the three readings of antibacterial susceptibility assay was calculated using Microsoft excel 2007. The data are characterized in the form of bar diagram and in tables.

RESULTS

Percentage yield of extracts

Using ethanol as solvent, the greatest yield of phytochemical extract from juice was obtained from C5 (13.43%) and least from C4 (8.8%). From the peel, the greatest yield obtained was of C1 (25.7%) and the least was of C5 (16.6%).

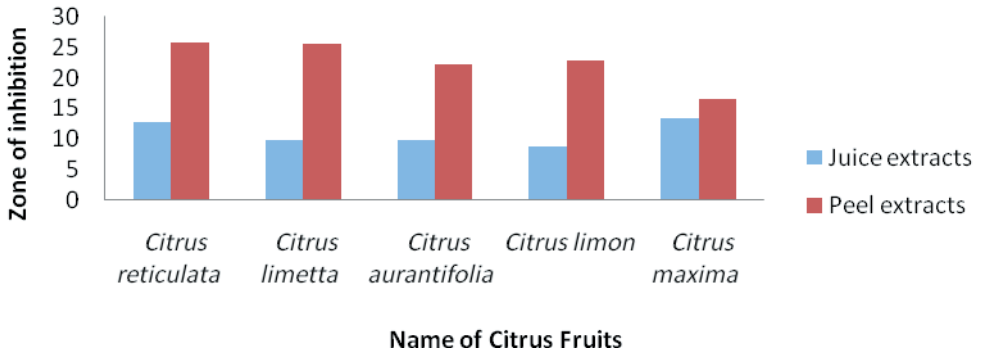

Figure 1: Graph showing yield of extract of citrus fruits

Antibacterial activity of *Citrus reticulata* extracts against selected bacterial strains

The peel extract of C1 was found to be more effective against the Gram-positive bacteria than its juice extract with maximum zone of inhibition (20.33±1.527) against *Bacillus* spp. Among the Gram-negative bacteria, the juice extract of C1 was found to be more effective than its peel extract with maximum zone of inhibition (11.33±1.154) against *Klebsiella pneumoniae* ATCC 13883 while 10% DMSO didn't show any zone of inhibition.

Table 1: Antibacterial activity of *Citrus reticulata* extracts against selected bacterial strains

Organism	Diameter of zone of inhibition in mm	
	Juice extracts (Mean±S.D)	Peel extracts (Mean±S.D)
Bacillus spp	11.67±0.577	20.33±1.527
S. aureus ATCC 25923	8.33±0.577	12±1
S. aureus	10.33±1.528	14.66±1.154
E. coli ATCC 25922	11±1	0
E. coli	9.33±0.577	0
S. Typhi	9.66±1.527	0
P. aeruginosa	9.33±0.577	0
K. pneumoniae ATCC13883	11.33±1.154	10.33±1.527

Antibacterial activity of *Citrus limetta* against selected bacterial strains

The peel extract of C2 was found to be more effective against the Gram-positive bacteria than its juice extract with maximum zone of inhibition (15.33±0.577) against *Bacillus* spp. Among the Gram negative bacteria, the juice extract of C2 was found to be more effective than its peel extract against the four other bacteria chosen with maximum zone of inhibition (8.33±2.081) against *E. coli* ATCC 25922, except *Klebsiella pneumoniae* ATCC 13883 on which the peel extract was more effective with zone of inhibition (10.33±1.527) while 10% DMSO didn't show any zone of inhibition.

Table 2: Antibacterial activity of *Citrus limetta* against selected bacterial strains

Organism	Diameter of zone of inhibition in mm	
	Juice extracts (Mean±S.D)	Peel extracts (Mean±S.D)
Bacillus spp	10.33±0.577	15.33±0.577
S. aureus ATCC 25923	6.33±1.527	11.33±1.154
S. aureus	7.66±0.577	11.33±1.154
E. coli ATCC 25922	8.33±2.081	0
E. coli	7±1	0
S. Typhi	5±1	0
P. aeruginosa	6.33±1.527	0
K. pneumoniae ATCC13883	0	10.33±1.527

Antibacterial activity of *Citrus aurantifolia* against selected bacterial strains

The juice extract of C3 was found to be more effective against the Gram-positive bacteria than its peel extract with maximum zone of inhibition (26.66±1.15) against *S. aureus* ATCC 25923. Among the Gram-negative bacteria, the juice extract of C3 was found to be more effective than its peel extract with maximum zone of inhibition (25±1) against *E. coli* ATCC 25922 while 10% DMSO didn't show any zone of inhibition.

Table 3: Antibacterial activity of *Citrus aurantifolia* against selected bacterial strains

Organism	Diameter of zone of inhibition in mm	
	Juice extracts (Mean±S.D)	Peel extracts (Mean±S.D)
Bacillus spp	23±1	23.33±1.154
S. aureus ATCC 25923	26.66±1.15	21.33±1.154
S. aureus	24±1	21.66±1.527
E. coli ATCC 25922	25±1	21.33±1.154
E. coli	21.33±1.527	8.33±0.577
S. Typhi	21.33±1.154	20.66±1.154
P. aeruginosa	20.66±1.154	9.33±1.154
K.pnuemoniae ATCC13883	17±1	18±2

Antibacterial activity of *Citrus limon* against selected bacterial strains

The juice extract of C3 was found to be more effective against the Gram-positive bacteria than its peel extract with maximum zone of inhibition (19.33±0.577) against *S. aureus* ATCC 25923. Among the Gram-negative bacteria, the juice extract of C3 was found to be more effective than its peel extract with maximum zone of inhibition (20±1) against *E. coli* ATCC 25922 while 10% DMSO didn't show any zone of inhibition.

Table 4: Antibacterial activity of *Citrus limon* against selected bacterial strains

Organism	Diameter of zone of inhibition in mm	
	Juice extracts (Mean±S.D)	Peel extracts (Mean±S.D)
Bacillus spp	19.66±1.527	16±1
S. aureus ATCC 25923	19.33±0.577	17.66±0.577
S. aureus	18.33±0.577	9.33±1.154
E. coli ATCC 25922	20±1	9±1
E. coli	18.66±0.577	8.66±0.577
S. Typhi	15.33±0.577	11±1
P. aeruginosa	17.66±0.577	7±1
K. pneumoniae ATCC13883	15.66±0.577	11.33±1.154

Antibacterial activity of *Citrus maxima* against selected bacterial strains

Among Gram positive bacteria, the juice extract of C5 was found to be more effective against the *S. aureus* ATCC 25923 and *S. aureus* while its peel extract was more effective against *Bacillus* spp. Among the Gram-negative bacteria, the juice extract of C5 was found to be more effective than its peel extract with maximum zone of inhibition (18.66±1.154) against *E. coli* ATCC 25922 while 10% DMSO didn't show any zone of inhibition.

Table 5: Antibacterial activity of *Citrus maxima* against selected bacterial strains

Organism	Diameter of zone of inhibition in mm	
	Juice extracts (Mean±S.D)	Peel extracts (Mean±S.D)
Bacillus spp	3±2.645	15±1
S. aureus ATCC 25923	17.33±1.154	6.66±1.154
S. aureus	14.66±0.577	7.66±0.577
E. coli ATCC 25922	18.66±1.154	0
E. coli	12.66±1.154	0
S. Typhi	14±1	0
P. aeruginosa	13±1	0
K. pneumoniae ATCC13883	13.33±1.527	10.66±1.154

DISCUSSION

In this study, peel extracts of *Citrus reticulata* was found to be more effective than its juice extracts against the Gram-positive bacteria with highest zone of inhibition against the *Bacillus* spp (20.33±1.527) while it did not show any zone of inhibition against *E. coli*, *E. coli* ATCC 25922, *S.* Typhi and *P. aeruginosa*. In case of juice extracts, *Bacillus* spp showed highest zone of inhibition (11.67±0.577) and the least zone was showed by *E. coli* ATCC 25922 and *P. aeruginosa* (9.33±0.577). Similar finding was observed from the study by Zainab et al. (2017) who reported the highest zone of inhibition exhibited by peel extract of *Citrus reticulata* was against *S. aureus* (28mm) while *E. coli*, *S.* Typhi, *P. aeruginosa* were found to be resistant against the peel extracts. The highest zone of inhibition by juice extracts was against *S. aureus* (22mm) but it did not show any zone of inhibition against *P. aeruginosa* (0mm). In mandarin peels, flavanone glycosides were present in a decreasing sequence, as follows: narirutin>hesperidin>naringin. The presence of higher amount of such flavanone in peel might be the reason behind the effectiveness of peel extract than that of juice (Levaj et al. 2009).

The peel extract of *Citrus limetta* was more effective against the Gram-positive bacteria than its respective juice extracts. Highest zone of inhibition shown by peel extract was against *Bacillus* spp (15.33±0.577) and its effect was absent on *E. coli* ATCC 25922, *E. coli*, *S.* Typhi and *P. aeruginosa*. Juice showed the highest effectiveness against *Bacillus* spp (10.33±0.577) and was not effective against *K. pneumoniae* ATCC 13883(0mm). The juice extract was found to be more effective against all Gram-negative bacteria chosen except for *K. pneumoniae* ATCC 13883 in which the peel extract was found more effective. Javed et al. (2013) investigate that the tested *C. limetta* had shown nearly equal antimicrobial effects on both Gram positive (*B. subtilis*, *S. aureus* ATCC 25923, *B. cereus* ATCC 14579) and Gram negative (*E. coli* ATCC 25922, *S. typhimurium* ATCC 14028) bacterial strains in culture media. However, in this finding most of the Gram-negative bacteria were found to be resistant against peel extract. The reason behind this might be due to the difference in the cell structure of Gram positive and Gram-negative bacteria. Gram negative bacteria possess outer membrane which can act as a barrier for antimicrobials to enter to the cells. Reviews suggest that flavonoids and phenolics were significantly greater in peel than the juice which might be the reason for effectiveness of peel.

The present study found that the juice extract of *Citrus aurantifolia* was more effective than its respective peel extracts against both Gram positive bacteria and Gram-negative bacteria. The highest zone of inhibition shown by juice extract was against *S. aureus* 25923 (26.66±1.15 mm) and the least against *K. pneumoniae* ATCC 13883 (17±1) while the highest zone of inhibition shown by peel extract was against *Bacillus* spp. (23.33±1.154) and the least against *E. coli* (8.33±0.577). Similar finding on juice extract of *Citrus aurantifolia* was observed from the study by Aibinu et al. (2007) who reported that the juice extract of *Citrus aurantifolia* showed highest zone of inhibition against *Staphylococcus aureus* (35mm) and the least against *K. pneumoniae* ATCC 13883 (16mm). Similarly, Alfarraj et al. (2018) in his experiment found that the peel extract of *C. aurantifolia* showed highest zone of inhibition against *S. aureus* (18.5mm) and the least against *E. coli* (15mm). Pathan et al. (2012) observed that the phytochemicals such as flavonoides, steroids and tannins were absent in ethanolic extract of peel whereas they were present in juice. The presence of these chemical constituents underscores the effectiveness of juice extract of *Citrus aurantifolia* than that of peel.

In this study, the juice extract of *Citrus limon* was more effective than its respective peel extracts against both Gram positive and Gram-negative bacteria. The highest zone of inhibition shown by juice extract was against *Bacillus* spp (19.66±1.527) and the least was against *K. pneumonia* ATCC 13883(15.66±0.577) while highest zone of inhibition shown by peel extract was against *S. aureus* ATCC 25923 (17.66±0.577) and the least against *P. aeruginosa* (7±1). Okeke et al. (2015), in his study found that the zone of inhibition by *C. limon* juice extract against *Bacillus* spp was 19mm. Present study is in agreement with the above findings. In the study carried out by Sokovic et al. (2007), the highest zone of inhibition shown by the peel extract of *C. limon* was against *S. aureus* (16mm) and *P. aeruginosa* was found resistant to it. His finding was quite similar to that of present study. Pandey et al. (2011) in his study reported that the peel of *C. limon* consists of tannins, reducing sugars, flavonoides but there is absence of saponins and phlobatannins. The juice extract contained all of these phytochemicals and thus the juice extract showed maximum inhibition than peel.

The juice extract of *C. maxima* had highest zone of inhibition against *E. coli* ATCC 25922 (18.66±1.154) whereas lowest zone of inhibition against *Bacillus* spp(3.0±2.645) while the highest zone of inhibition shown by peel extract was against *Bacillus* spp (15±1) and there was no zone of inhibition against *E. coli*, *E. coli* ATCC 25922, *S.*Typhi, *P. aeruginosa.* Swarnami et al. (2013), in his study found that the zone of inhibition by *C. maxima* juice extract against *E. coli* was 22mm and against *Bacillus* spp was 24mm. The zone of inhibition shown by *Bacillus* spp didn't match to that of present findings. This difference might be due to the difference in the species of *Bacillus* chosen for study as the different species have their own susceptibility against the different antibacterial substances.

CONCLUSION

Antibacterial activity shown by the juice and peel extracts of Citrus fruits guided that more pure form of these extract can be more effective agent and can be used as alternative for the treatment of infections associated to the studied microorganisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adebayo Tayo BC, Akinsete T and Odeniyi OA (2016). Phytochemical composition and comparative evaluation of antimicrobial activities of the juice extract of *Citrus aurantifolia* and its silver nano particles. *Nigeria Journal of Pharmacology* **12**(1): 59-64.

Adham T, Andrea V and Nadia N (2000). Comparative Antimicrobrial activity of peel and juice extract of citrus fruits growing in Kurdistan/Iraq. *American Journal of Microbiological Research* **3**(5): 155-159.

Alfarraj D, Manal M, Al K and Nadine MSM (2018). Correlation of phenolic content and antibacterial activity of dried lime extracts against human pathogen. *Biomedical Research* **29** (16): 3239-3242.

Altemimi A, Lakhsassi NB, Ahar louei A, Watson DG and Lightfoot DA (2017). Phytochemicals: extraction, isolation and identification of bioactive compounds from plant extracts. *Plants* (6): 42-43.

Aruoma OI, Landes B, Ramful D, Bourdond E., Neergheen V and Wagnerf K (2012). Functional benefits of citrus fruits in the management of diabetes. *Prev Med* **54**: S12–S16.

Boudries H, Madani K, Touati N, Souagui S, Medouni S and Chibane M (2012). Pulp antioxidant activities, mineral contents and juice nutritional properties of Algerian Clementine Cultivars and Mandarin. *Afr J Biotechnol* **11**: 4258–4267.

Burkill HM (1997). The useful plants of West Tropical Africa. Royal Botanic Gardens, Kew, UK, **4**: 969.

Chakraborty A, Devi RK, Rita S, Sharatchandra K and Singh TI (2004). Preliminary studies on ant-inflammatory and analgesic activities of Flavonoids Spilanthesacmella in experimental animals. *Indian J Pharmacol* **36**: 148-150.

Do QD, Awijaya AE, Njuyen PLT, Huynhl H, Soetaredjo FE, Ismadji S and Ju YH (2014). Effect of extraction solvents on total phenol content, total flavonoids content and antioxidant activity of *Limnophila aromatic. Journal of Food and Drug Analysis* **22**(3): 296-302

Dubey D, Balamurugan K, Agrawal RC, Verma R and Jain R (2011). Evaluation of antibacterial and antioxidant activity of methanolic and hydromethanolic extract of sweet orange peels. *Recent Res Sci Technol* **3**(11): 22-25.

Fessenden RJ and Fessenden JS (1982). Organic chemistry. Willard Grant Press, Boston, Mass U. K. flavonoids in Pulp and Peel of Mandarin Fruits, ACS 2.

Geissman TA (1963). Flavonoid compounds, tannins, lignins and related compounds. *Comprehensive Biochemistry* **9**: 213-250.

Ghasemi K, Ghasemi Y and Ebrahimzadeh AM (2009). Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. *Pak J Pharm Sci* **22**(3):277-281.

Hegazy AE and Ibrahium MI (2012). Antioxidant activities of orange peel extracts. *World Applied Sciences Journal* **18** (5): 684-688.

Hwang SL, Shih PH and Yen GC (2012). Neuroprotective

effects of citrus flavonoids. *J Agric Food Chem* **60**: 877-885.

Karimi E, Oskoueian E, Hendra R, Oskoueian A, and Jaafar HZE (2012). Phenolic compounds characterization and biological activities of *Citrus aurantium* bloom. *Molecules* **17**: 1203–1218.

Khan AA, Mahmood T, Siddiqui HH and Akhtar J (2016). Phytochemical and pharmacological properties on *Citrus limetta* (Mosambi). *Journal of Chemical and Pharmaceutical Research* **8(**3): 555-563.

Klangpetch W, Phromsurin K, Hannarong K, Wichaphon J and Rungchang S (2016). Antibacterial and antioxidant effects of tropical citrus peel extracts to improve the shelf life of raw chicken drumettes. *International Food Research Journal* **23**(2): 700-707.

Koffi E, Sea T, Dodehe Y and Soro S (2010). Effect of solvent type on extraction of polyphenols from twenty-three ivorian plants. *J Anim Plant Sci* 550–558.

Levaj B, Dragovic V, Bursac D, Krasnici N (2009). Determination of Flavonoids in Pulp and Peel of Mandarin Fruits. *Agriculturae Conspectus Scientificus* **74** (3): 221-225.

Li Y, Guo C, Yang J, Wei J, Xu J and Cheng S (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chem* **96**: 254-260.

Mishra RP, Yadav S and Anjali K (2012). Study of Antimicrobial Activities of *Citrus limetta*. *Journal of Pharmaceutical and Biomedical Sciences* **19** (15):1-4.

Okeke M, Okoli A, Eze E, Ekwume G, Okosa E and Iroegbu C (2015). Antibacterial activity of *Citrus limonum* fruit juice extract. *Pak J Pharm Sci* **28**(5): 1567-1571.

Okwu DE (2005). Phytochemicals, vitamins and minerals contents of two nigerian medicinal plants. *International J Molecular Medicine and Advance Sciences* **1**: 375-381.

Pathan R, Gali P, Pathan P, Gowtham T and Pasupuleti S (2012). In vitro antimicrobial activity of *Citrus aurantifolia* and its phytochemical screening. *Asian Pacific Journal of Tropical Disease* S328-S331.

Prashani ME, Geun TP, Young DL, Sejae K, Sang J and Jehee L (2005). Antioxidative activity extract from fenugreek seeds. Pak J Anak Environ Chem **9**(2): 78-83.

Ravi KU, Dwivedi P and Ahmad S (2010). Screening of antibacterial activity of six plants essential oils against pathogenic bacterial strains. *Asian J Med Sci* **2**: 152-158.

Roger GDP (2002). Encyclopedia of medicinal plants, education and health library. Safeliz SL Spsin, 1: 153-154.

Saify ZS, Farhad J, Mushtaq N and Noor F (2005). Antibacterial activity of 1-methyl-7- methoxybeta-carboline and its phenacyl and coumarin analogues. *Pakistan Journal of Pharmaceutical Science* **18**(3): 39-41

Silalahi J (2002). Anticancer and health protective properties of Citrus fruit components. *Asia Pac J Clin Nutr* **11**: 79-84.

Soković M, Marin P, Brkić D and Griensven L (2007). Chemical composition and antibacterial activity of essential oils of ten aromatic plants against human pathogenic bacteria. *Global Science Books* **1**(1): 2-5.

Swarnamoni D, Mukundam B and Shagufa A (2013). Antibacterial activity of the ethanolic extract of leaves of citrus maxima (Burm,) Merr on *Escherichia coli* and *Pseudomonas aeruginosa*. *Asian Journal of Clinical and Pharmaceutical Research* **6**(4): 136-139.

Wong PYY and Kitts DD (2006). Studies on the dual antioxidant and antibacterial properties of parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) extracts. *Food Chem* **97**: 505–515.

Zainab AGC and Kadhim NK (2013). Antimicrobial activity of different aqueous lemon extracts. *Journal of Applied Pharmaceutical Science* **3**(6): 74-78.

Antimicrobial Susceptibility Pattern of Gram-Negative Bacterial Isolates from Raw Chicken Meat Samples

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ABSTRACT

Objectives: This research aims to study the microbial quality of chicken meat available in retail shop of Kathmandu valley.

Methods: This study was conducted from June to December 2018 in three different districts of Kathmandu Valley. Samples were collected in sterile plastic bags, labeled properly and stored in an icebox and transported to the Food Microbiology laboratory of Golden Gate International College.

During sample preparation, 25 grams of each sample was taken and transferred to sterile flasks containing 225 ml of buffered peptone water. Potential pathogenic Gram-negative bacteria were isolated by using respective selective media and identified by biochemical test. Antibiotic susceptibility profile of isolates was carried out by Kirby-Bauer disc diffusion method according to CLSI 2017 guideline.

Results: Of total 81 chicken meat samples processed, 201 Gram negative bacteria were isolated. *E. coli* (100%) was the dominant Gram-negative isolates, followed by *Citrobacter* spp (62.96%), *Pseudomonas* spp (40.74%), *Proteus* spp (19.75%), *Salmonella* spp (16.04%) and *Klebsiella* spp (8.64%) respectively. No any multidrug isolates were detected.

Conclusion: The study showed that the raw chicken meat samples of Kathmandu valley was highly contaminated with Gram negative potential pathogenic bacteria. Antimicrobial resistance pattern shown by the isolates may indicates that there is not overuse of drug in animals and the less chance of risk of increasing antimicrobial resistance.

Key words: Chicken meat, Antimicrobial susceptibility test, Gram negative bacteria

INTRODUCTION

Poultry meat is the combination of muscle tissue, attached skin, connective tissue, and edible organs, comprising about two-thirds of the total meat production in the world (Ruban et al. 2010). Chicken meat is considered as a healthy food, due to high protein content and reduced fat content, as well as a higher proportion of polyunsaturated fatty acids (PUFA), when compared to other species meats (Riovanto et al. 2012). In Asian countries, chicken meat was periodically included in the diets of consumers in the past. However, with the rapid economic growth and globalization of the food industry, the amount of meat production and consumption has grown rapidly in recent years (Nam et al. 2010).

Due to its high percentages of nitrogenous compounds of various degrees of complexity, high moisture, abundant supply of minerals, accessory growth factors and some fermentable carbohydrates (glycogen) of a suitable pH, meat are regarded as a perfect culture medium for most of the microorganisms (Holck et al. 2017). The hygiene often fails during slaughtering,

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scalding, evisceration, plucking, bleeding, washing, and rinsing, and increase the health risk associated with the consumption of this meat (Coulibaly et al. 2010). Similarly, animals can also become infected from water or food contaminated with wastes of human or animal origin or with human carrier workers (Da Silva et al. 2011). One of the possible ways of entry of various microbes could be the handling of meat and meat products by adopting improper hygienic measures during handling and processing (Kiranmayi et al. 2011). It has been pointed out that during slaughter, dressing, and cutting, microorganisms come chiefly from the exterior of the animal and its intestinal tract but more are added from knives, cloths, air, workers, carts, boxes, and equipment in general (Bhandare et al. 2007). *Escherichia, Enterobacter, Klebsiella, Proteus, Citrobacter, Serratia, Salmonella, Shigella* and *Yersinia*, are among the most important causes bacterial infections in humans (Jarzab et al. 2011).

Antibiotics are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food producing animals. Many findings suggest that inadequate selection and abuse of antimicrobial may lead to resistance in various bacteria sand make the treatment of bacterial infections more difficult (Kolar et al. 2001).

Poor personal hygiene is prime cause of food borne illness. Hand washing is the crucial measure to prevent propagation of diseases and to cut back the transmission of bacteria among individuals and to food as well (Lambrechts et al. 2014). Resistant bacteria are transmitted to human through direct contact with animal, by exposure to animal manure, through consumption of uncooked meat, and through contact with meat surfaces (Marshall and Levy 2011). The expansion of antibiotic resistance in bacteria is also an emerging public health hazard due to the compromised efficacy in the treatment of infectious diseases (Helmy et al. 2017).

MATERIALS AND METHODS

Study was conducted from June to December 2018. Random, purposive sampling was fconducted for the collection of sample to study. A total of 81 raw chicken meat samples were collected from different retail meat shops at Kathmandu, Lalitpur and Bhaktapur districts.

Samples were collected in sterile plastic bags. Samples collected from different butcher shops at Lalitpur, Kathmandu and Bhaktapur districts were labeled properly and stored in an icebox until delivery and transported to the Food Microbiology laboratory of Golden Gate International College. Raw chicken meat was collected from different meat shops and studied. Packaged, cold stored or chemically treated meat was not collected (Da Silva et al. 2013).

During sample preparation, 25 gram of each sample was taken and transferred to sterile flasks containing 225 ml of buffered peptone water. Samples were being homogenized using a meat grinder under aseptic condition and kept in rotatory shaker at 80 rpm for 30 minutes (Shrestha et al. 2017).

For the enrichment, 1ml of the sample from the flask was suspended in 9ml of Selenite Broth and incubated for 24 hours at 37°C (Shrestha et al. 2017). Loopful of sample from Selenite broth was streaked on Xylose Lysine Deoxycholate (XLD) agar plates, plates were then incubated at 37°C for 24 hours (Da Silva et al. 2013).

Plates were examined for presence of typical *Salmonella* colonies. *Salmonella* colonies may appear pink colonies with black center (Shrestha et al. 2017). Isolated colony was taken and streaked in Nutrient agar and incubated for 24 hours at 37°C. Isolated colony from NA was transferred to Triple Sugar Iron Agar (TSIA) and was incubated at 37°C for 24 hours for the confirmation of *Salmonella* (Shrestha et al. 2017). For the enrichment, 1ml of the sample from the flask was suspended in 9ml of Nutrient Broth and incubated for 24 hours at 37 °C (Shrestha et al. 2017). Loopful of sample from Nutrient Broth was streaked on M-endo agar. The plates were incubated at 37 ℃ for 24 hours. Colonies exhibiting metallic sheen on M-endo agar was sub cultured on NA to obtain pure culture. Pure cultures were confirmed to be *E. coli* by biochemical tests (IMViC, TSI, O/F and Urease) (Da Silva et al. 2013).

Isolation and identification of *Pseudomonas* spp. was done by streaking loopful sample from NA to cetrimide agar plate. Colonies showing yellowish green color was tested for conformation of *Pseudomonas* (Estepa et al. 2015).

Colonies showing pink color in Mac conkey agar plate were conformed as Gram negative isolates and species were confirmed by following CLSI guidelines 2017.

Testing for antibiotic sensitivity was done by the

Modified Kirby-Bauer Disc diffusion method by using Muller Hilton Agar. The bacterial suspensions made in normal saline were compared with 0.5 McFarland solutions and swabbed on MHA plate using sterile cotton swab. Antibiotic disc were then placed on the swabbed MHA plate. After overnight incubation at 37°C the zone of inhibition was observed around the antibiotic disc. Using the CLSI guidelines, the susceptibility or resistance of the organism to each drug tested was determined. For each drug, the recording sheet whether the zone size is susceptible (S) or resistant (R) based on the interpretation chart was indicated. The results of the Kirby-Bauer disk diffusion susceptibility test were reported only as susceptible or resistant (CLSI 2017). Statistical analysis was done by using SPSS version 16.

RESULTS

Of the total of 201 potential bacterial pathogens isolates, meat samples from the Kathmandu district show the highest contamination and samples from the Lalitpur least as shown in Table 1.

Table 1: Percentage occurrence of bacterial pathogens in Kathmandu valley

Districts	Number	Percentage
Kathmandu	84	41.79
Bhaktapur	61	30.34
Lalitpur	56	27.86

Among 81 samples examined from three different districts, Gram negative bacterial isolates identified were *E. coli, Salmonella* spp, *Proteus* spp, *Citrobacter* spp, *Pseudomonas* spp, and *Klebsiella* spp. respectively. Among them, *E. coli* was isolated from all the samples 81 (100%), *Salmonella* spp. from 13 (16.04%) samples, *Pseudomonas* spp. from 33 (40.74%) samples, *Proteus* spp. from 16 (19.75%) samples, *Klebsiella* spp. from 7 (8.64%) samples and *Citrobacter* spp. from 51 (62.96%) samples as shown in Table 2. Among the 27 meat samples collected from each district, *E. coli* was isolated from all the samples, whereas *Klebsiella* was not isolated from sample of Bhaktapur and s*almonella* from sample of Lalitpur district.

Table 2: Percentage occurrence of the isolated bacteria from chicken sample

Bacteria	No. of isolates	Percentage
E. coli	81	100.00
Salmonella ssp	13	16.04
Pseudomonas ssp	33	40.74
Proteus ssp	16	19.75
Klebsiella ssp	7	8.64
Citrobacter ssp	51	62.96

Antibiotics susceptibility testing showed all isolates of *E. coli* were resistant to Ampicillin and sensitive to Amikacin, Gentamicin, Ceftriaxone and Cotrimoxazole (Table 3).

Table 3: Sensitivity pattern of *E. coli* (n=81)

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin	-	81(100)
Ciprofloxacin	78(96.29)	3(3.71)
Gentamicin	81(100)	-
Chloramphenicol	80(98.76)	1(1.24)
Tetracycline	9(11.11)	72(88.89)
Cotrimoxazole	81(100)	-
Amikacin	81(100)	-
Ceftriaxone	81(100)	-

All the isolates of *Salmonella* spp. isolates were sensitive to Cotrimoxazole, Ciprofloxacin, Amikacin and Ceftriaxone and resistant to Ampicillin (Table 4).

Table 4: Sensitivity pattern of *Salmonella* spp. (n=13)

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin	-	13(100)
Ciprofloxacin	13(100)	-
Gentamicin	11(84.62)	2(15.38)
Chloramphenicol	9(69.23)	4(30.77)
Tetracycline	3(23.07)	10(76.93)
Cotrimoxazole	13(100)	-
Amikacin	13(100)	-
Ceftriaxone	13(100)	-

All the isolates of *Pseudomonas* spp. isolates were subjected to AST and found sensitive to Gentamicin, Ciprofloxacin, Amikacin, Ceftriaxone and Cotrimoxazole and resistant to Ampicillin (table 5).

Table 5: Sensitivity pattern of *Pseudomonas* spp. (n=33)

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin	-	33(100)
Ciprofloxacin	33(100)	-
Gentamicin	33(100)	-
Chloramphenicol	8(24.24)	25(75.76)
Tetracycline	10(30.30)	23(69.70)
Cotrimoxazole	22(66.67)	11(33.33)
Amikacin	33(100)	-
Ceftriaxone	33(100)	-

All the isolates of *Citrobacter* spp isolates were found to be sensitive to Chloramphenicol, Ciprofloxacin, Amikacin, Gentamicin and Ceftriaxone. All the isolates of *Proteus* spp. were sensitive to Gentamicin, Cotrimoxazole, Amikacin and Ceftriaxone and resistant to Tetracycline. All the isolates of *Klebsiella* spp were sensitive to Amikacin and Ceftriaxone. No any MDR isolates were identified.

DISCUSSION

Out of 81 samples collected from different meat shops, a total of 201 potential pathogenic Gram negative bacterial isolates were detected among which, all samples showed growth with multiple isolates. Similar result were reported from other studies conducted in North East India, *E. coli* (98%), *Citrobacter* (52%), *and Salmonella* (20%) (Sharma 2012).

In this study all the samples showed presence of *E. coli*, which is very high as compared to previous study (4.8%) by Shrestha et al. 2017 and Thanigaivel and Anandhan 2015 (70%). The reason behind this huge difference could be due to the use of contaminated water during slaughtering, washing and in other handling processes, and also due to *E. coli* being common inhabitants of animal and human guts (Cabral 2010). As this study does not focus on the water quality of the water used in the meat shops and the parameters on the water quality are not studied either. Total of 62.96% of occurrence of *Citrobacter* spp was seen, which is less as compared to result presented from other studies, (Shrestha et al. 2017) i.e. 44.7% and (Gwida 2014) in Egypt which is 13.3%. The higher percentage of *C. freundii* presence in foods can be ascribed to secondary contamination (Ryan et al. 2004; Raphael and Riley 2017).

Thirteen (16.04%) isolates were detected as *Salmonella* spp which is much lower than the result presented in other similar studies from Egypt, Azez (2013) i.e. 44% and Nigeria i.e 33.33% (Adeyanju and Ishola 2014).

About 41 % (33) isolates were *Pseudomonas* spp. which is much higher than 1.9% reported (Shrestha et al. 2017) in Bharatpur, Nepal and 10% (Thanigaivel and Anandhan 2015) in India but somewhat similar to 46.66% found in Dharan (Bantawa et al. 2018). *P. aeruginosa* is one of the most relevant opportunistic human pathogens, although there are also reports that show clinical cases caused by environmental *Pseudomonas*, such as *P. mendocina* and P. *fulva* (Nseir et al. 2011). Differences in prevalence rates from this study to another might be attributed to the unhygienic processing and poor sanitation of meat shops. It showed that meat retailers were found to be unaware of the basic requirements of

basic guidelines regarding meat (Almuzara et al. 2010 and Seok et al. 2010).

Percentage of *Proteus* spp. isolates were more similar to study by Shrestha et al. (2017) but lower than study by Al-Mutairi (2011) from Saudi Arabia. *Proteus* spp. is considered as an indicator of contamination of meat during any of the processing, handling, storage stages. If the optimal condition for the isolated *Proteus* were existed, typical cases of food poisoning, urinary infection and other *Proteus* related human illnesses could happen due to rapid proliferation of the pathogen (Al-Mutairi 2011).

Klebsiella spp had least occurrence as compared to other isolates i.e.7 (8.64%), which is very less as compared to the result shown (Kim et al. 2005) in Oklahoma which is 30% but similar to Al-Mutairi 2011 (10.66%). *Klebsiella* spp is a colonizing opportunistic pathogen of humans and animals, and a common contaminant of retail meat (Kim et al. 2005). In animals, *Klebsiella* spp. causes disease in cows, horses, and other animals (Bersisa et al. 2019 and Ewers et al. 2014). In humans, *Klebsiella* spp. frequently colonizes the gut and sporadically causes extra intestinal infections (Podschun and Ullmann 1998).

All *E. coli* isolates were resistance to Ampicillin, 88.89%, 3.71% and 1.24% were resistant to Tetracycline, Ciprofloxacin and Chloramphenicol respectively. All of isolates were sensitive to Cotrimoxazole, Amikacin, Gentamicin and Ceftriaxone followed by 98.76%, 96.29% and 11.11% sensitivity to Chloramphenicol, Ciprofloxacin and Tetracycline respectively. In the study carried at Nigeria 83% of *E. coli* isolates were resistant to Tetracycline, 89% resistant to Cotrimoxazole and 28.3% resistant to Gentamicin (Adeyanju and Ishola 2014). Thanigaivel et al. 2015 in India reported 26% resistivity of *E. coli* to Tetracycline. Somda et al. 2018 in Burkina Faso, reported 100% sensitivity to Ciprofloxacin, Gentamicin, and Chloramphenicol. However, resistance was observed with Ceftriaxone (10.71%), Ampicillin (42.86%), and tetracycline (64.3%).

A potential health hazard to consumers can be expected from resistant bacteria. If the organism is resistant to antibiotics, then initial treatment may be ineffective both in man and animals and an alternative treatment need to be applied (cotterill et al. 1977).

Total of 13 isolates of *Salmonella* spp., 100% were sensitive to Ciprofloxacin, Cotrimoxazole, Amikacin, and Ceftriaxone and 100% were resistant to Ampicillin. Only 84.62%, 69.23%, and 23.07% sensitivity were shown towards Gentamicin, Chloramphenicol and Tetracycline respectively. The study conducted in Egypt (Moawad et al. 2017) reported that 87% and 40% *Salmonella* spp were resistant to Ampicillin and Tetracycline, 53% sensitive to Chloramphenicol and 67% sensitive to Ciprofloxacin.

Similarly, all *Pseudomonas* spp. isolates were found sensitive towards Ciprofloxacin, Gentamicin, Amikacin and Ceftriaxone and completely resistant to Ampicillin. Among which 24.24%, 30.30% and 66.67% were sensitive to Chloramphenicol, Tetracycline and Cotrimoxazole respectively. According to result reported by Estepa et al. 2015 in Spain, *Pseudomonas* spp. showed 100% sensitivity to Amikacin, Gentamicin, Kanamicin, and Tobramycin.

Citrobacter spp. showed sensitivity towards many antibiotics such as, Ciprofloxacin, Gentamicin, Chloramphenicol, Amikacin and Ceftriaxone. Liu et al. 2017 in China reported that all *Citrobacter* spp. isolates were sensitive to Amikacin, 66.67% were resistant to Ampicillin, 16.67% resistivity to Ciprofloxacin, 5.56% resistivity to Gentamicin, 44.45% resistivity to Tetracycline and 27.78% resistivity to Cotrimoxazole.

All *Proteus* spp. isolates were found sensitive towards Gentamicin, Amikacin, Cotrimoxazole and Ceftriaxone and resistant to Tetracycline. Shrestha et al. 2017 in Bharatpur, Nepal reported 11.7% of *Proteus* spp. resistant to Ciprofloxacin.

All *Klebsiella* spp. isolates were found sensitive towards Amikacin and Ceftriaxone. They showed 14.29%, 85.71%, 71.43%, 42.86%, 57.14% and 71.43% sensitivity to Ampicillin, Ciprofloxacin, Gentamicin, Chloramphenicol, Tetracycline and Cotrimoxazole respectively. According to the report of Kim et al. 2005, the study conducted in Oklahoma, 100% of *Klebsiella* isolates were found resistant to Ampicillin, Tetracycline and also Ceftriaxone. Davis et al. (2015) also reported 100% resistivity towards Ampicillin and 100% sensitivity to Amikacin.

Antimicrobial are used even in the absence of illness to prevent diseases when animals are susceptible to infection (Turtura et al. 1990). In slaughterhouse, resistant strains from the gastrointestinal tract may infect chicken carcasses and, as a result, chicken meats are often related to antimicrobial-resistant

microorganisms (Reza et al. 2014). Therefore, these antimicrobial-resistant bacteria from poultry can infect humans directly and indirectly with food. Though rarely, these resistant bacteria may colonize in the human gastrointestinal tract and may also transfer resistance bacteria to human endogenous flora (Reza et al. 2014). However, the rate of MDR for all the isolates was nil whereas, 79.6% prevalence of MDR bacteria was found in chicken meat in Bharatpur (Shrestha et al. 2017).

Poor hygienic practices during slaughtering and marketing of meat are one of the major contributing factors for unsafe meat in Nepal (Joshi et al. 2003). Slaughtering animals in open and unhygienic places, use of dirty water during slaughtering process, and selling meat in open and non-refrigerated places are some of the unhygienic practices being used by the sellers (Sharma 2012).

CONCLUSION

Out of 81 samples, all the samples showed the presence of potential pathogenic bacteria with *E. coli* being the dominant. Antibiogram studies of all isolates against 8 different antibiotics showed that majority of isolates were sensitive. And rate of MDR was nil for each isolate. Among the three districts, Lalitpur showed comparatively satisfactory hygienic condition in relative to Bhaktapur and Kathmandu. Slaughtering of animals in unhygienic place , not maintaining the good hygiene practices during cutting, handling, transportation results to the poor quality of the meat and furthermore, use of polluted water contaminate the meat with different microorganism which can be harmful for the health of the consumers and which can increase the risk of food-borne illness and epidemic diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adeyanju GT and Ishola O (2014). Salmonella and Escherichia coli contamination of poultry meat from a processing plant and retail markets in Ibadan, Oyo State, Nigeria. *Springer Plus* **3**: 139.

Almuzara MN, Vazquez M, Tanaka N, Turco M, Ramirez MS, Lopez EL, Pasteran F, Rapoport M, Procopio A and Vay CA (2010). First case of human infection due to *Pseudomonas fulva*, an environmental bacterium isolated from cerebrospinal fluid. *J Clin Microbiol* **48**: 660–664.

Al-Mutairi MF (2011). The incidence of enterobacteriaceae causing food poisoning in some meat products. *Advance Journal of Food Science and Technology* **3**(2): 116-121

Bantawa K, Rai K, Subba Limbu D and Khanal H (2018). Food-borne bacterial pathogens in marketed raw meat of Dharan, Eastern Nepal. *BMC Res Notes* **1** (1): 1-5

Bersisa A, Tulu D and Negera C (2019). Investigation of bacteriological quality of meat from abattoir and butcher shops in Bishoftu, Central Ethiopia. *International Journal of Microbiology* **1**: 1-8.

Bhandare SG, Sherikarv AT, Paturkar AM, Waskar VS and Zende RJ (2007). A comparison of microbial contamination on sheep/goat carcasses in a modern Indian abattoir and traditional meat shops. *Food Control* **18**: 854-868.

Cabral Joao PS (2010). Water Microbiology. Bacterial pathogens and water. *Int J Environ Res Public Health* **7**(10): 3657-3703.

Cotterill OJ, Glauert HP and Russell WD (1977). Microbial counts and thermal resistance of *Salmonella oranienburg* in ground turkey meat. *Poult Sci* 56(6):1889-92.

Da Silva DCF, De Arruda AMV and Gonçalves AA (2017). Quality characteristics of broiler chicken meat from free-range and industrial poultry system for the consumers. *J Food Sci Technol* **54**(7): 1818–1826.

Da Silva N, Taniwaki MH, Junqueira VCA, Silveira NFA, do Nascimento MS and Gomes RAR (2013). Microbiological examination methods of food and water: a laboratory manual. London: Taylor & Francis Group

Estepa V, Rojo-Bezares B, Torres C and Saenz Y (2015). Genetic lineages and antimicrobial resistance in *Pseudomonas* spp. isolates recovered from food samples. *Food-borne Pathogens and Disease* **1**: 1-6.

FAO (2009). Food and Agriculture Organization article on egg. Food and Agriculture Organization of United Nations 2004- 03-07.

Helmy MO and Kashef TM (2017). Different phenotypic and molecular mechanisms associated with Multidrug resistance in Gram-negative clinical isolates from Egypt. *Infect Drug Resist* **10**: 479–498.

Holck A, Axelsson L, McLeod A, Rode TM and Heir E (2017). Health and safety considerations of fermented sausages. *Hindawi Journal of Food Quality* **1**: 1- 25.

Joshi DD, Maharjan M, Johansen MV, Willingham AL and Sharma M (2003). Improving meat inspection and control in resource-poor communities: the Nepal example. *Acta Tropica* **87** (1): 119-127.

Kiranmayi CB, Krishnaiah N, Subhashini N, Amaravathi P, Maheswari M and Ramya P (2011). PCR analysis of mutton and chicken samples for the presence of Shiga toxigenic *E. coli*. *Arch Clin Microbiol* **2**: 2-4

Kolar M, Urbanek K and Latal T (2001). Antibiotic selective pressure and development of bacterial resistance. *Int J Antimicrob Agents* **17**: 357-63.

Lambrechts AA, Human IS, Doughari JH and Lues JFR (2014). Bacterial contamination of the hands of food handlers as indicator of hand washing efficacy in some convenient food industries. *Pak J Med Sci* **30**(4)**:** 755-758.

Liu X, Jayasena DD, Jung Y, Jung S, Kang BS, Heo KN, Lee JH, and Jo C (2012). Differential proteome analysis of breast and thigh muscles between Korean native chickens and commercial broilers. *Asian Australas J Anim Sci* **25**: 895-902.

Marshall BM and Levy SB (2011). Food animals and antimicrobial: impacts on human health. *Clin Microbiol Rev* **24**: 718–33.

Moawad AA, Hotzel H, Awad O, Tomaso H, Neubauer H and Hafez HM (2017). Occurrence of *Salmonella enterica* and *Escherichia coli* in raw chicken and beef meat in northern Egypt and dissemination of their antibiotic resistance markers. *Gut Pathog* **9**(1): 57

Podschun R and Ullmann U (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**: 589–603.

Reza T, Mehdi K, Faham K and Mohammad RF (2014). Multiple antimicrobial resistance of *Escherichia coli* isolated from chickens in Iran. *Veterinary Medicine International Article* ID 491418, 4 pages

Riovanto R, Marchi M, Cassandro M and Penasa M (2012). Use of near infrared transmittance spectroscopy to predict fatty acid composition of chicken meat. *Food Chem* **134(4)**: 2459–2464.

Ruban SW, Thiyageeswaran M and Sharadha R (2010). Isolation and identification of *Salmonella* spp. from retail chicken meat by polymerase chain reaction. *Int J Microbiol Res* **1(**3**)**: 106-109.

Ryan KJ and Ray CG (2004). *Sherris Medical Microbiology, 4th ed. McGraw Hill p 370.*

Sharma Bajagai Y (2012). Food safety regulation in Nepal and issues in regulation of meat safety. Available at https://www.foodandenvironment.com/2012/11/food-safety-regulation-in-nepal.html

Seok Y, Shin H, Lee Y, Cho I, Na S, Yong D, Jeong SH and Lee K (2010). First report of bloodstream infection caused by *Pseudomonas fulva*. *J Clin Microbiol* **48**: 2656–2657.

Shrestha A, Bajracharya AM, Subedi H, Turha RS, Kafle S, Sharma S and Chaudhary DK (2017). Multidrug resistance and extended spectrum beta lactamase producing Gram negative bacteria from chicken meat in Bharatpur Metropolitan, Nepal. *BMC Res Notes* **10(**574**)**: 1-5

Somda NS, Traorore O, Bonkoungou O JI, Bassole IHN, Traore Y, Barro N and Savadogo A (2017). Serotyping and antimicrobial drug resistance of *Salmonella* isolated from lettuce and human diarrhea-samples in Burkina Faso. *African Journal of Infectious Diseases* **11(2)**: 024–030.

Thanigaivel G and Anandhan AS (2015). Isolation and characterization of microorganism from raw meat obtained from different market places in and around chennai. *Journal of Pharmaceutical, Chemical and Biological Sciences* **3(2)**: 295-301

Turtura GC, Massa S and Ghazvinizadeh H (1990). Antibiotic resistance among coliform bacteria isolated from carcasses of commercially slaughtered chickens. *International Journal of Food Microbiology* **11(**3–4**)**: 351–354.

Bacterial Profile and Their Antibiogram Isolated from Cell Phones

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ABSTRACT

Objectives: The present study aimed to identify bacteria profile of cell phones used by different people from different profession of Bhaktapur and to access their antimicrobial resistance.

Methods: Forty-nine mobile swab samples were collected from 7 different profession category (7 samples each from student, butcher, cook, pani puri vendor, health workers, and dairy employee). Samples were cultured and processed by standard Microbiological procedures. All the isolates were further subjected to antibiotic susceptibility testing using modified Kirby Bauer disc diffusion method as describe in CLSI guidelines. The rate of multiple drug resistant (MDR) bacteria was also determined.

Results: Out of 49 sample, *Bacillus* spp (20.4%) was the most predominant isolate, followed by *Staphylococcus aureus* (10.6%) and *Pseudomonas* spp (10.6%). Higher variety of bacterial isolates was found in the cell phones swabs from butcher followed by cook, farmer and pani puri vendor group. The data from the questionnaire showed that handkerchief or tissue paper were mostly used by respondents to clean their mobile. All Gram-positive and Gram-negative isolates were resistance to Cefoxitin (100%) except *Micrococcus* spp and *Neisseria* spp. Gram positive (18.2%) and Gram-negative (36.9%) isolates were identified as MDR. All *S. aureus* and coagulase negative staphylococci were methicillin resistant

Conclusion: The cell phones of people from different profession were found to possess many different bacterial pathogens including multi drug resistant strains which could be the possible pathogens for food borne infections and opportunistic infections.

Key words: Cell phones, Antimicrobial susceptibility test, MDR, Standard microbiological procedure

INTRODUCTION

Microorganisms live almost everywhere on Earth from the liquid water, including hot springs and the ocean floor, rocks deep inside Earth's crust. A huge load of microorganisms is also present in the daily life stuffs like electronic devices, ornaments, study materials etc. Electronic devices include mobile phone, television, laptop etc (Madigan and Martinko 2006). Cell phones might act as fomites as they are carried to the places such as toilets, hospitals and kitchens where they are loaded with microorganisms (Bhoonderowa et al. 2014).

The recent evaluation of microbial contamination in mobile phone of dental and engineering schools in Iran reported the higher percentage of mobile phones were contaminated and mainly by *S. aureus* (Fard et al. 2017). Similarly, recent bacterial isolates from mobile phone surface of students of University of Kufa, Iraq found *Bacillus* spp, as the predominant ones followed by *S. aureus* and *S. epidermis* (Harmoosh et al. 2017). Likewise,

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the research at University of Gondar, Ethiopia, showed higher number of *E. coli, E. aerogenes*, *Streptococcus* spp and *S. aureus* isolates in the mobile phones of students and employee (Verma et al. 2015).

In an urban teaching hospital in Durban, South Africa, the study had showed that Gram-positive microorganisms were more frequently cultured from Caregivers phones and predominantly contaminated with *Staphylococcus* spp. (Bobat et al. 2017). It was found that mobile phones are ten times dirtier than toilet seats. Due to carelessness about the pathogenic organisms, people don't give a second thought to use their cell phone everywhere from morning commute to the dinner table, to doctor office to respective workplace (Abrams 2017). In the study of hospital survey in Nepal, Health care workers used their cell phone in the hospital and many of them never cleaned their cell phone. Among them, 20% didn't even practice hand washing before or after attending patients and used their cell phones immediately (Chawla et al. 2009). In 2017, Adhikari et al. reported the presence of *S. aureus* and Methicillin resistant *S. aureus* associated with mobile phones (Adhikari et al. 2017). The research on mobile phone as a possible vector of bacterial transmission in hospital setting was conducted in Dhulikhel hospital, Nepal and revealed that bacterial growth was positive for pathogenic organisms in 89 out of 124 mobile phones (Karkae et al. 2017).

Use of mobile phones in sensitive areas is the subject of controversy as there are no guidelines for disinfection of mobile phones that meet standards and some people are unaware of the fact that mobile phones act as the fomite for transmission of pathogens. So, the focus should be on how to use mobile phones sensibly, getting their benefits and minimizing their risks (Ulger et al.2009). Hence, the present study was undertaken to examine the mobile phones of respective profession people for presence of bacteria, as mobile phones transmitted infections possess a constant threat to lives of people living around. This study was also aimed to find the correlation between the organisms and if any behavioral/ professional traits determines the types of organisms to be found in the phones. Moreover, we assessed the antibacterial resistance pattern of bacteria isolated from these phones along with MDR load.

MATERIALS AND METHODS

Sample collection

Forty-nine cell phone swabs were collected people of seven different profession (farmer, cook, butcher, student, pani puri installer, dairy employee and health workers) in Bhaktapur. Sterile swab moistened with normal saline were used for the sample collection.

Bacterial identification

The sample collected were immediately transported to laboratory of Sainik Awasiya Mahavidhyalaya and processed to identify the bacterial isolates by using standard microbiology procedures including Gram staining and biochemical tests.

Antibiotic susceptibility testing

Antibiotic sensitivity testing was performed for those isolates using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to CLSI guidelines (CLSI 2012). Antibiotics; cefoxitin, vancomycin, chloramphenicol, ampicillin, norfloxacin, azithromycin, tetracycline and nalidixic Acid were used for Gram-positive isolates while cefoxitin, vancomycin, chloramphenicol, ampicillin, norfloxacin, erythromycin and polymyxin b were tested against Gram-negative isolates. Those which were resistant to 3 or more different classes of antibiotics were categorized as multi drug resistant strains.

RESULTS

Bacterial contamination of cell phones

Out of forty-nine samples from cell phones, 48(98%) showed bacterial growth while only one sample (2%) from health worker's cell phone wasn't contaminated. A total of 146 bacterial isolates were isolated in which 79(54.1%) was Gram positive and 67(45.9%) Gram negative.

The cell phone of health workers was the least contaminated with bacteria (7.7%) with high Gram-positive isolates (7%) whereas those from butcher group had higher bacterial isolates (19.8%) followed by cook (18.4%), farmer (16.9%), pani puri vendor (16.2%) etc.

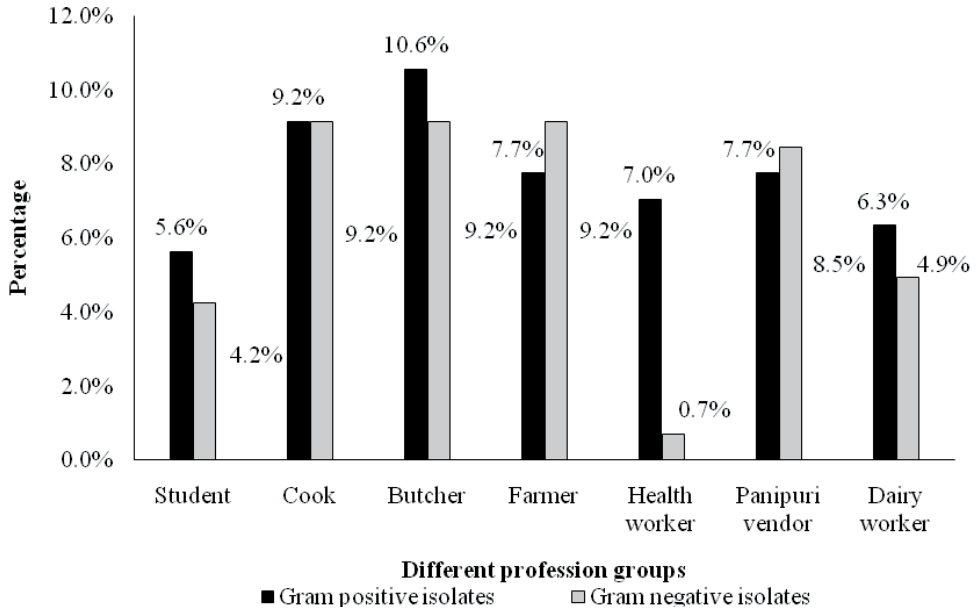

Figure 1: Gram positive and Gram-negative isolates on different groups

Bacterial profile

Among the different bacterial isolates from cell phones, *Bacillus* spp (20%) was the most predominate Gram-positive isolate followed by *S. aureus* (11.0%). Similarly, among Gram-negative isolates, *Pseudomonas* spp (10.3%) was mostly commonly isolated followed by *Proteus* spp (9.7%) (Table 1).

Table 1: Frequency of bacterial isolates from cell phones

Bacterial isolates	Percent (%) of isolates from cell phone of professions							Total
	Student	Cook	Butcher	Farmer	Health worker	Pani puri vendor	Dairy worker	
Bacillus spp	3.4	4.1	2.8	2.8	1.4	3.4	2.1	**20.0**
S. aureus	0.7	0.7	3.4	1.4	1.4	1.4	2.1	**11.0**
Pseudomonas spp	0	2.1	2.1	3.4	0	0.7	2.1	**10.3**
Proteus spp	0	3.4	1.4	2.1	0	2.1	0.7	**9.7**
Corynebacterium spp	0	0.7	0.7	3.4	2.8	1.4	0.7	**9.7**
Citrobacter spp	2.8	0.7	1.4	1.4	0	2.1	0	**8.3**
Klebsiella spp	0.7	1.4	2.1	1.4	0	1.4	0.7	**7.6**
S. epidermis	0	1.4	2.1	0	0	0	0.7	**4.1**
Micrococcus spp	0.7	2.1	0	0	0	0.7	0	**3.4**
E. coli	0	0.7	0	0	0.7	1.4	0	**2.8**
Enterobacter spp	0.7	0	0.7	0.7	0	0.7	0	**2.8**
Streptococcus spp	0	0	2.1	0	0	0	0	**2.1**
Providencia spp	0	0	0.7	0	0	0	0	**0.7**
Shigella spp	0	0	0	0	0	0	0.7	**0.7**
Salmonella spp	0	0.7	0	0	0	0	0	**0.7**
Unidentified	0.7	0	1.4	0.7	1.4	0.7	1.4	**6.2**
Total bacterial isolates	**9.7**	**17.9**	**20.7**	**17.2**	**7.6**	**15.9**	11.0	100

Antibiotic susceptibility pattern of Gram-positive isolates

Almost all Gram-positive isolates were resistance to cefoxitin (100%) except *Micrococcus* spp. All the Gram-positive isolates were sensitive to norfloxacin followed by chloramphenicol. 56% of Gram-positive isolates were found to be tetracycline resistant (Table 2).

Table 2: Antibiotic susceptibility pattern of Gram-positive isolates

Antibiotic used / Gram positive isolates	No of Gram-positive isolates resistant to (%)							
	CX	Va	C	AMP	Nx	AZMI	TET	NV
Bacillus spp (n=29)	29 (100)	29 (17.2)	0	0	0	0	2 (6.9)	0
S. aureus (n=16)	16 (100)	4 (25)	0	0	0	16 (100)	13 (81.3)	16 (100)
Corynebacterium spp (n=14)	14 (100)	3 (21.4)	0	6 (42.9)	0	0	14 (100)	0
S. epidermis (n=6)	6 (100)	3 (50)	0	0	0	6 (100)	1 (16.7)	6 (100)
Streptococcus spp (n=3)	3 (100)	0	0	2 (66.7)	0	0	3 (100)	0
Lactobacillus spp (n=7)	7 (100)	0	3 (42.9)	0	0	2 (28.6)	7 (100)	0
Micrococcus spp (n=5)	0	2 (40)	0	4 (80)	0	1 (20)	5 (100)	0

Note: Cx: Cefoxitin, Va: Vancomycin, C: Chloramphenicol, Amp: Ampicillin, Nx: Norfloxacin, Azmi: Azithromycin, Tet: Tetracycline, Nv: Nalidixic Acid.

Antibiotic susceptibility pattern of Gram-negative isolates

Major Gram-negative isolates were resistance to cefoxitin (100%) except *Neisseria* spp. Bacteria were resistant to polymyxin B followed by vancomycin, erythromycin and ampicillin (Table 3).

Table 3: Antibiotic susceptibility pattern of Gram-negative isolates

Antibiotic used / Gram negative isolates	No. of resistant isolates/Total no. of Gram-negative isolates						
	PB	E	C	Nx	Va	AMP	Cx
Klebsiella spp (n=11)	11 (100)	11 (100)	0	1 (9.1)	1 (9.1)	3 (27.3)	11 (100)
Citrobacter spp (n=12)	0	10 (83.3)	4 (33.3)	0	0	3 (25.0)	12 (100)
Enterobacter spp (n=4)	2 (50)	4 (100)	0	0	2 (50)	3 (75)	4 (100)
Pseudomonas spp (n=15)	13 (86.7)	10 (66.7)	13 (86.7)	0	10 (66.7)	11 (73.3)	15 (100)
Proteus spp (n=14)	14 (100)	0	10 (71.4)	1 (7.1)	8 (57.1)	6 (42.9)	14 (100)
Salmonella spp (n=1)	1 (100)	1 (100)	0	0	1 (100)	1 (100)	1 (100)
E. coli (n=4)	4 (100)	3 (75)	0	0	4 (100)	0	4 (100)
Providencia spp (n=1)	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	1 (100)
Neisseria spp (n=2)	2/2 (100)	2/2 (100)	0	0	2 (100)	0	1 (50)
Shigella spp (n=1)	1 (100)	1 (100)	0	0	1 (100)	1 (100)	1 (100)

Note: Cx: Cefoxitin, Va: Vancomycin, C: Chloramphenicol, Amp: Ampicillin, Nx: Norfloxacin, E: Erythromycin, PB: Polymyxin B.

Multi drug resistant strains

Out of 145 isolates, 50 isolates (34.5%) were observed to be MDR isolates. Among them, twenty-six isolates were Gram positive and twenty-four were Gram negative (Figure 2).

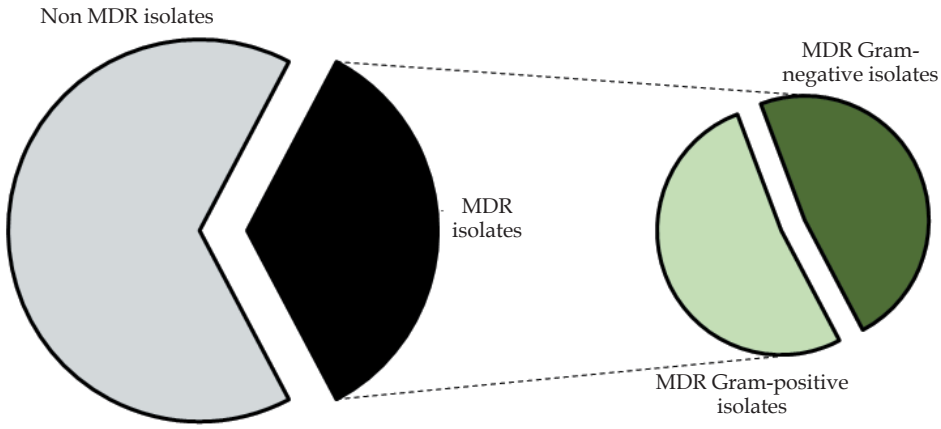

Figure 2: Multidrug resistant isolates

DISCUSSION

In present study, 98% of sample mobile phones showed bacterial contamination. Rate of contamination of mobile phones in present study seemed to be higher than previous studies. Kotris et al. (2016) reported 84% of mobile phone of students were contaminated. According to Adhikari et al. (2018), 56% of mobile phones of students and staff contained bacterial agents. Mobile phones of 98% students of dental school were contaminated by bacterial agents. The higher percentage of bacterial contamination in mobile phones of health worker and students might be due to over use of mobile phones at work place, washroom and even at dining with lack of hand washing practice.

According to Karkee et al. (2017), Gram positive bacteria (79.81%) dominant over Gram negative (20.19%-) in the survey done at Dhulikhel hospital. Ramesh et al. (2008) reported that 46% of mobile phones of medical staff and students were positive culture with 15% belonging to Gram-negative pathogens and 85% were Gram positive. According to international journal of infection control, 56.67% of bacterial isolates were Gram positive whereas 43.33% were Gram negative isolates. Akinyemi et al. (2009) also mentioned huge percentage of Gram-positive isolates i.e. 83.87% and low Gram-negative isolates (16.13%). This study showed similar results as compared to other results. The higher rate of Gram positive might be originated from skin normal flora.

Bacillus spp was found to be the most predominant one (20.4%) of total microbial population from the 49 swabbed samples followed by *S. aureus* (11.0%). This might be due to its presence everywhere in nature and its spore forming characteristics. *Bacillus* spp with a 100% frequency of occurrence has been identified as an important organism in food spoilage by Dave and Shende (2015) in Chhattisgarh region, India. The research carried out in University of Peradeniya, Sri Lanka of veterinary undergraduate students by Viveka (2017) reported that dominant organisms were *S. epidermis* (87.5%), *Bacillus* spp (60%), *Pseudomonas* spp (50%), *S. aureus* (22.5%) etc. Results of this investigation show the potential of cell phones to participate as fomites and a vehicle of different types of microorganisms.

All the Gram-positive isolates were observed to be resistant to cefoxitin (100%) except *Micrococcus* spp but most of all isolates were sensitive to norfloxacin and chloramphenicol. *Bacillus* spp were resistant to vancomycin (17.2%) and tetracycline (6.9%). These findings were similar to resistant pattern of bacterial isolates in sachet water sold in the streets of Cape Coast (Tagoe et al. 2011). The survey from Dhulikhel hospital reported that most of the Gram-positive cocci were sensitive to vancomycin and ciprofloxacin. *S. epidermis* and *Micrococcus* spp were reported to be most sensitive to vancomycin (81.36%, 75%). *S. aureus* were not reported as resistant to vancomycin and erythromycin was 80%. In the survey performed in Bangladesh, 27.3% of *S. aureus* were resistant to azithromycin, 36.4% to tetracycline and 31.8% to chloramphenicol. Also 37.5% and 68.8% of *S. epidermis* were resistant with tetracycline and ampicillin respectively Debnath et al. (2018).

Most of all the Gram-negative isolates were resistant to polymyxin b, erythromycin, vancomycin, ampicillin and cefoxitin. *Klebsiella* spp were identified as polymyxin b (100%), erythromycin (100%) and cefoxitin (100%) resistant. In study conducted by Akinyemi et al. 80.7% of total bacteria were sensitive to ciprofloxacin. Debnath et al. (2018) reported 45.5% of *Pseudomonas* spp were resistant to Tetracycline and 50% and 35.7% of *E. coli* were resistant to chloramphenicol and tetracycline respectively. *Salmonella* spp were resistant to chloramphenicol (50%) and tetracycline (16.7%).

In this study, after *Bacillus* spp; *Staphylococcus* spp were found to be high (i.e. 13.9%) which were all resistant to norfloxacin, cefoxitin and azithromycin. As all the *Staphylococcus* species were resistant to cefoxitin, it could be assumed to be MRSA. This assumption was supported by Jain et al. (2008) which stated that use of disc diffusion test for both oxacillin and cefoxitin could help in more accurate prediction of methicillin resistant. MRSA is of particular importance in the medical community, as it has evolved resistance to β-lactam antibiotics (Jonathan et al. 2010). 59% of Methicillin resistant *S. aureus* and 37.7% of Methicillin resistant CoNS were also reported by Worku et al. (2018) in Mizan- Tepi university teaching hospital, Southwest Ethiopia.

From the antibiotic sensitivity testing, it was observed that most of the isolates obtained from cell phones of different profession groups were showing growth of multi drug sensitive organisms. A total of 18.2% of Gram positive MDR isolates were secured among 77 of Gram-positive isolates whereas 36.9% of Gram negative MDR isolates were secured among 65 of total Gram-negative isolates. Multidrug resistant *S. aureus, Klebsiella, Proteus* spp, *Pseudomonas* spp and *E. coli* were reported by Worku et al. (2018) with a percentage of 21%, 53.8%, 44.4%, 30% and 7.1% respectively. Loyola et al. (2018) reported that 2.9% of *Pseudomonas* spp and 46.7% of *S. aureus* were reported as MDR from the health care workers cell phones at Peruvian hospital.

Many of the previous studies revealed that people do not consider mobile phones to be contaminated items and rarely disinfect their phones (Ramesh et al. 2008). Hand washing is the most effective method for the prevention of bacterial transmission. Although there are strict rules on hand hygiene in hospitals, it is not possible to provide de-contamination, disinfection or sterilization of each device used personally. Periodic disinfection of instruments and surfaces that often come into contact with the hands, such as computer keyboards and mouse, was recommended by the Centers for Disease Control and Prevention (CDC)'s guidelines (Boyce and Pittet 2002).

CONCLUSION

The Cell phones of people from different profession were found to possess many different bacterial pathogens including multi drug resistant strains which could be the possible pathogens for food borne infections and opportunistic infections. Based on this study, it seems to be essential to aware people about the possible risk of transferring MDR while using mobile phone at workplace.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abrams A (2017). Time health: your cell phone is 10 times dirtier than a toilet seat. Here' what to do about it. Retrieved from http://time.com/4908654/cell-phonebacteria/

Adhikari S, Khadka S, Sapkota S and Shrestha P (2018). Methicillin Resistant *Staphylococcus aureus* associated with mobile phones. *SOJ Microbiol Infect Dis* **6**: 1-6.

Akinyemi KO, Atapu AD, Adetona OO and Coker AO (2009). The potential role of mobile phones in the spread of bacterial infections. *J Infect Dev Ctries* **3**: 628–632.

Bhoonderowa A, Gookool S and Biranjia-Hurdoyal SD (2014). The importance of mobile phones in the possible transmission of bacterial infections in the community. *J Community Health* **39**(5): 965–967.

Bobat R, Archary M, Lawler M, Mawlana S, Naidoo KL, Maphumulo S and Coovadia Y (2017). The presence and spectrum of bacteria colonizing mobile phones of staff and caregivers in high disease burden paediatric and neonatal wards in an urban teaching hospital in Durban, South

Africa. *Southern African Journal of Infectious Diseases* **32**: 9-11.

Boyce J.M and Pittet D (2002). Guideline for hand hygiene in health-care settings. *MMWR Recomm Rep* **51: 1–45.**

Chawla K and Mukhopadhayay C, Gurung B, Bhate P and Bairy I (2009). Bacterial 'Cell' Phones: Do cell phones carry potential pathogens? *Online Journal of Health and Allied Sciences* **8**: 1-5.

CLSI (2012). Performance Standards for Antimicrobial Susceptibility Testing, Vol. **32**, Clinical and Laboratory Standards Institute, Wayne, Pa, USA, Twenty-second informational supplement, M 100-S22.

Dave S and Shende K (2015). Toxicology and food technology. *Journal of Environmental Science* **1**(6): 71-73

Debnath T, Bhowmik S, Islam T and Chowdhury HM (2018). Presence of multidrug-resistant bacteria on mobile phones of healthcare workers accelerates the spread of nosocomial infection and regarded as a threat to public health in Bangladesh. *Journal of Microscopy and Ultrastructure* **6**: 165–169.

Fard RH, Moradi M and Hashemipour MA (2017). Evaluation of the cell phone microbial contamination in dental and engineering schools: Effect of antibacterial spray. *J Epidemiol Global Health* **8**(3-4):143-148.

Harmoosh AL, Mutlaq R, Mutlaq N, Alabassi M, Alabassi N, Shamari M, Shamari MA, Khafaji A, Khafaji MA and Hussein. (2017). Surface of Mobile Phone: As a Carrier of Pathogenic Bacteria. *Research Journal of Pharmacy and Technology* **10**(10):1827-1830.

Jonathan W, Holmes MD, Mark D and Williams MD (2010). Methicillin-resistant *Staphylococcus aureus* screening and eradication in the surgical intensive care unit: Is it worth it? *The American Journal of Surgery* **200**(6) :827-831.

Karkee P, Madhup SK, Humagain P, Thaku N and Timilsina B (2017). Mobile phone: A possible vector of bacterial transmission in hospital setting. *Kathmandu Univ Med J* **59**(3): 217-21.

Kotris I, Drenjančević D, Talapko J and Bukovski S (2016). Identification of microorganisms on mobile phones of intensive care unit health care workers and medical students in the tertiary hospital, Zagreb, Croatia **14**(1): 85- 90.

Loyala S, Gutierrez L, Avendarno E, Severino N, Tamariz J (2018). Multidrugs resistant bacteria isolated from cell phones in five intensive care units: Exploratory dispersion analysis. *Germs.* **8**: 85-91.

Madigan M and Martinko J (2006). Brock Biology of Microorganisms (13th ed.). Pearson Education. p. 1096.

Ramesh J, Carter AO, Campbell MH, Gibbons N, Powlett C and Moseley H (2008). Use of mobile phones by medical staff at Queen Elizabeth Hospital, Barbados: evidence for both benefit and harm. *J Hosp Infect* **70**:160–165.

Tagoe DN, Gyande VK and Ansah EO (2011). Bacterial contamination of mobile phones: When your mobile phone could transmit more than just a call. *Web Med Central Microbiology* **2**(10): WMC002294.

Ulger F, Esen S and Dilek A (2009). Are we aware how contaminated our mobile phones with nosocomial pathogens? *Ann Clin Microbiol Antimicrob* **8**:7

Verma D, Barasa A, Dara D, Medehen H, Asrat H, Demissie N, Tegenaw K, Sendeku W and Berhane N (2015). Isolation and characterization of bacteria from mobile phones of students and employees at university of Gondar, Ethiopia. *Bulletin of Pharmaceutical Research Institute* **5**(3):96-100.

Viveka VA (2017). Isolation and identification of common bacterial contaminants in mobile phones qwned by veterinary undergraduate students. *Journal of Health, Medicine and Nursing* **35**: 93-105

Worku T, Abateneh D and Kumalo A (2018). Bacterial profile and antimicrobial susceptibility pattern of the isolates from stethoscope, thermometer, and inanimate surface of Mizan-Tepi University Teaching Hospital, Southwest Ethiopia. *International Journal of Microbiology Article* ID **9824251**: 1-7.

Effective Use of Penicillin to Improve Culture Yield for *Mycobacterium tuberculosis*

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ABSTRACT

Objectives: To compare LJ media and LJ media with penicillin for the growth of *Mycobacterium tuberculosis* and contamination, in pulmonary tuberculosis (PTB) suspected patients.

Methods: A total of 300 PTB suspected cases at National Tuberculosis center (NTC) for analyzed for culture and contamination. Early morning sputum samples were collected in sterile leak-proof falcon tube. Digestion, decontamination and homogenization of sputum were done using NALC-NaOH (Modified Petroff method). The sputum sample was processed on LJ media and penicillin added LJ media and incubated at 37°C. Cultures were examined after 8 weeks.

Results: All the PTB suspected cases were compared in LJ media and LJ media with penicillin, 29.7% (89) were positive, 21% (63) were contaminated on LJ media whereas 41% (123) were positive, 3.7 % (11) were contaminated on penicillin added LJ media. Also, 25 (8%) were 1^+ grading, 14 (4.7%) were 2^+ grading, whereas 81 (27%) and 45 (15%) were 3^+ grading LJ + Penicillin and LJ media respectively.

Conclusion: Contamination is reduced with the addition of penicillin to LJ media and isolation of total positive cultures of Mycobacterium tuberculosis enhanced.

Key words: Culture, Penicillin, Contamination, Pulmonary tuberculosis (PTB), LJ media

INTRODUCTION

Tuberculosis (TB) is a specific chronic infectious disease caused by *Mycobacterium tuberculosis* and occasionally by *M. bovis* and *M. africanum*. It is characterized by formation of granuloma in the infected tissue. This organism usually enters the body by inhalation through lungs. They spread from initial location in the lungs to other body parts via blood stream, the lymphatics system, via the air ways or duct extension to other organs (Park 2005). TB is potentially fatal & contagious disease that can infect any part of the body but most importantly the lungs. TB is caused by any of *Mycobacterium tuberculosis* complex (MTC) organism as well as Non-tuberculous Mycobacteria (NTM) (NTP 2014). TB now ranks alongside HIV as a leading cause of death worldwide. It is estimated by World Health Organization (WHO) that between 2000 and 2020, nearly one billion people will be newly infected, 200 million will get sick and 35 million will die from TB if global control is not strengthened. Nepal is currently considered an immediate TB burden country with 45% of total TB being infected (NTP 2014).

Culture still relies on relatively cumbersome and lengthy process starting with collection of clinical specimens and their transport to the laboratory, decontamination of the clinical specimen, likely to be contaminated by commensal flora, inoculation and incubation of appropriate media for growth detection and mycobacterium identification (Asmar and Drancourt 2015). Lowenstein Jensen (LJ) Medium is used for the isolation and cultivation of mycobacteria and as bases for selective, differential and enriched media for mycobacteria. However, the effectiveness of culture is greatly undermined by contamination with bacteria and fungi. Contamination reduces the proportion of interpretable results there by limiting the diagnostic value of culture system. This hazard might be partly eliminated by use of penicillin, since

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the principle contaminants are penicillin sensitive whereas tubercle bacilli itself is relatively resistant. Penicillin is effective when incorporated into LJ media at concentration ranging from 10- 125 units per ml of the medium (Abbott 1951). Penicillin inhibits most of the Gram-positive bacteria (Hardy Diagnostic 2014). Low-level concentrations of penicillin (50.0 units/ml) and nalidixic acid (35.0 mg/ml) are included in the LJ Medium to inhibit Gram-positive as well as some Gram-negative bacterial contaminants. (Hi Media 2014).

Contaminated cultures are recognizable from various characteristics. Tubercle bacilli will not grow under these conditions and cultures should be discarded. If the contamination is present only in a part of the slant and the medium maintains its characteristics, the cultures can be retained until 8 weeks (NTP 2014). This study aims to compare the growth and contamination rate on penicillin treated LJ media & LJ media alone and will help to reduce the level of contamination to yield better growth colonies for identification.

MATERIALS AND METHODS

Study design

A comparative cross-sectional study was conducted between February to September 2015 at National Tuberculosis Center, Thimi, Bhaktapur, Nepal. A total of 300 samples were taken, consenting new and previously treated patients suspected of PTB, able to produce sputum, of any age and gender visiting NTC, were included in the study. All TB suspected were inoculated on both LJ with penicillin and LJ media.

Sample collection

The two consecutive early morning sputum samples were collected in sterile leak-proof, wide mouthed, screw-capped, transparent 50 ml single use plastic falcon tube labeled with laboratory serial number. The patients were given clear instruction about the quality and quantity of the samples and method of collection. The patients were suggested to cough deeply from the chest and spit out 3-5ml sputum in the given tube. The saliva, nasal secretions and specimen less than 3ml in volume were avoided. Similarly, sputum containing food particles residues and other extraneous matter were also rejected (STAC 2011).

Macroscopy

The sputum sample was examined macroscopically and characterized as purulent, mucopurulent, mucoid, salivary, mucosalivary or bloody.

Sputum processing

Digestion, decontamination and homogenization of sputum was done using NALC-NaOH (Modified Petroff's method) and concentrated by centrifugation at 3000×g for 15 minutes at 4°C (STAC 2011).

Primary culture of *Mycobacteria*

The sputum sample was further processed for culture on penicillin added LJ media and LJ media alone, in accordance to STAC 2011.

Inoculation and incubation

The centrifuged sediment sputum sample of 0.2-0.4 ml (2-4 drops) was inoculated to each of two slopes of LJ medium and LJ + Penicillin medium, each. The inoculum was spread evenly over the whole surface of each medium and the caps of the inoculated medium tubes were loosened at least for 1 week to ensure even distribution of the inoculums and the tubes were laid on the slanting bed with the slants facing upward. The inoculated slants were incubated at 37°C. After a week, the caps of the tubes were tightened securely and further incubated in upright position at 37°C for 8 weeks. All inoculations were done under BSC level II facility.

Culture examination and reporting

The cultures were examined at 48-72 hours after inoculation to detect gross contaminants. The culture was observed at one week for rapid growers and 3-4 weeks for positive cultures of *M. tuberculosis* as well as other slow growing Mycobacteria. If the colonies were not appeared at the 4th week, weekly observation was done till 8 weeks before discarding and reporting as negative. The grading of culture was done. The culture isolates were confirmed as *M. tuberculosis* by biochemical tests and interpreted. A patient was considered as a "TB positive subject" if the sputum specimen had a positive culture and as a "TB negative subject" if the sputum showed no growth (STAC 2011).

RESULTS

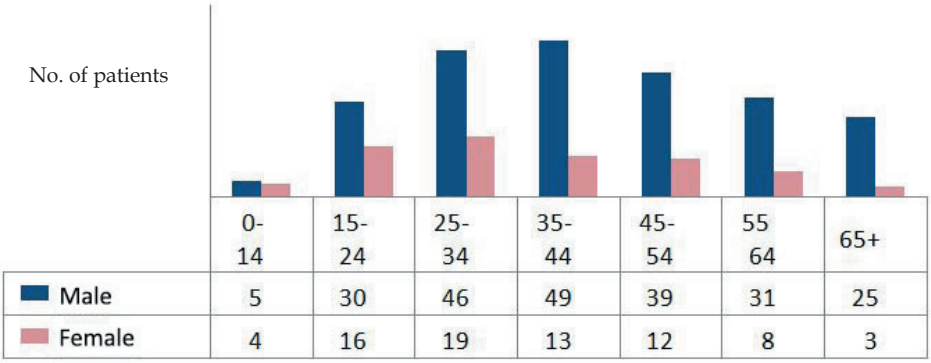

Figure 1: Demographic characteristics of study population

Among 300 PTB patients 225(75%) were male and 75(25%) were female. In this study, the maximum number of TB cases found between the productive age (15-54) was 224(74.7%), followed by above 55 years was 67(22.3%) and below 15 was 9(3%).

Table 2: Comparative isolation of *M. tuberculosis* on LJ & LJ + penicillin media

Growth/ Media	Positive growth	Negative growth	Contamination
LJ media	29.70%	49.30%	21%
LJ+P media	41%	55.30%	3.70%

* LJ + P media = LJ + Penicillin media Among 300 TB patients, 29.7% (89) were positive, 49.3% (148) were negative, 21% (63) were contaminated on LJ media whereas 41% (123) were positive, 55.3% (166) were negative and 3.7% (11) were contaminated on penicillin added LJ media

Table 3: Variation of growth on LJ + penicillin & LJ media

Colony count	Growth on LJ + penicillin N (%)	Growth on LJ N (%)
1+	25 (8.3%)	25 (8.3%)
2+	14 (4.7%)	14 (4.7%)
3+	81 (27%)	45 (15%)
Exact No.	3 (1%)	5 (1.7%)
Negative	166 (55.3%)	148 (49.3%)
Contamination	11 (3.7%)	63 (21%)

Out of 300 PTB patients, 25(8%) were 1^+ grading, 14(4.7%) were 2^+ grading, whereas 81(27%) and 45(15%) were 3^+ grading and 3(1%) and 5(1.7%) had exact number on penicillin treated LJ media and LJ media respectively. Similarly, 166(55.3%) and 148(49.3%) were negative and 11(3.7%) and 63(21%) were contaminated LJ + penicillin and LJ media respectively.

DISCUSSION

In this study, comparative study on rate of contamination and growth pattern on LJ media and penicillin added LJ media was done. A contamination rate of 3-5 % is considered a good balance between need to kill contaminating bacteria and the need to keep the majority of tubercle mycobacteria present in the sample.

A total number of 300 PTB patients from previously treated and new suspects were included. Males, 75% (n=225) were likely to suffer from TB than females 25% (n=75) which is higher with earlier findings by National Tuberculosis Program (64%) during the fiscal year 2012/13 but consistent with the other findings by Khati (2012) 71.65%. This finding is similar to other countries by Kamal et al. (2009) in Bangladesh 79%, Mubarak and Mohammad (2012) in UAE (79%), Feng et al. (2012) in

Taiwan, 77.3% and Range et al. (2012) 69.23%. Evidences show that males are more prone to get severe form of TB like cavity lesion and so forth. Meanwhile, the possible impact of sexual hormones and the differences between men and women in immunological reactions have also been proposed as factors causing men to be more susceptible to *M. tuberculosis* (Neyrolles 2009). Besides that, bias in sample size, behavioral and socio-economic factors may play important role (Sangare et al. 2010).

Out of 300 PTB cases, the isolation rate of *M. tuberculosis* was 41% (n=123) on LJ media with Penicillin and 29.7% (N=89) on LJ media alone. This finding is consistent to other findings by Lamsal (2012) 31% culture positive in Kathmandu, but not consistent with Affolabi et al. 2011(10.9%) in France, Kamal et al. 2009 (44%) in Bangladesh, Abd-El Aal et al. 2014 (54.5%) in Egypt, Kelfie (2014) 51% in Ethopia, 33.7% in Zambia. According to Kassaza et al. 2014, TB positive culture rate was 12.4% and 9.8% in penicillin treated LJ media and LJ media, respectively. Though the present result was much higher than of Kassaza et al. (2014), but on internal examination positivity rate on this study was 11.3% (N=34) higher in penicillin treated LJ media than LJ media alone. Culture identification is still the gold standard for diagnosis of pulmonary tuberculosis despite the fact *M. tuberculosis* is a slow growing organism and culture may take up to 4-8 weeks to provide a positive result (Castro et al. 2015). Penicillin containing media also demonstrated higher rates of *M. tuberculosis* isolation.

The culture contamination rate was 3.7% (N=11) on penicillin added LJ media and 21% (N=63) on LJ media. Contamination is greater than the recommended threshold of 5% on LJ media alone, while contamination rate on LJ + Penicillin was within threshold for the laboratories that receive freshly collected samples and 5-10% in cases on transportation of the samples. The contamination rate for LJ alone was approximately 31% and 9% for penicillin containing LJ media (Kassaza et al. 2014). In this study, contamination rate is higher than reports by Thakuri 2013 (12%) in Kathmandu for LJ media. Contamination rate was reported 14.9% by Zambian National Laboratory (Muyuyeta et al. 2009), 9% by Nagarajan et al. (2012) in India, 14.2% by 9.3% by Chihota et al. (2010) in South Africa, on LJ media alone. The contamination in this study might be due to delay in transportation of the sputum sample. A contamination rate of 0-1% may indicate too strong decontamination process. However, according to WHO guidelines the contamination rate 5-10% is acceptable in case of delay in transportation. As there was no provision on use of oral rinse solutions such as chlorohexidine and nystatin, penicillin is effective when incorporated into LJ medium, concentration ranging from 10125 units/ml (prior to inspissation) definitely reduce contamination, but the limitations of this method of using penicillin have yet been adequately tested. The high rate of contamination on LJ media could partly be due to the fact that this method used highly nutritious medium that can easily supports growth of other bacteria or may be due to enrollment of patients with cough for more than 2 weeks and other TB symptoms. Although, LJ contains malachite green, which has antibiotic properties, several other groups have reported similarly high contamination rates (Abott 1951; Kassaza et al. 2014).

CONCLUSION

Contamination was reduced by 17.3% with the addition of penicillin to LJ media. The isolation of total positive cultures was also enhanced by 11.3%. Thus, addition of penicillin on LJ medium ought to be better media for isolation of *M. tuberculosis,* as the cost of adding penicillin is low and effective in suppressing the contaminating bacteria and improving culture yield. This suggest that, LJ + penicillin is efficient than LJ media alone, as it saves time, cost and effort.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFRENCES

Abd-El Aal MA, Agha SA, Mohamed HE, Zaghloul MHE, Elshahawy HA, Azim DMA and Amal Fathy M (2014). DNA fingerprinting and drug resistance patterns of active pulmonary *Mycobacterium tuberculosis* in Mansoura hospitals, Egypt. *Egyptian Society of Chest Disease* **63**: 369-375

Abott JN (1951). Effective use of penicillin to reduce contamination in sputum concentrates to be examined for tubercle bacilli. American Public

Health Association Vol. **41**

Affolabi D, Akpona R, Odoum M, Alidjinou K, Wachinou P, Anogow S, Gninafon and Trebuca A (2011). Smear negative culture positive pulmonary tuberculosis among patients with chronic cough in Contow, Benin. *Int J Tuberc Lung Dis* **15**(1): 67-70.

Asmar S and Drancourt M. (2015). Rapid culture based diagnosis of pulmonary tuberculosis in developed and developing countries. *Front Micrbiol* **6**: 1184.

Castro AN, Madea M, Freitas S and Roxo PC (2015). Diagnostic yield of sputum, micrbiological analysis of pulmonary tuberculosis in a period of 10 year. *Rev Port Pneumol* **21**(4): 185-191.

Chihota VN, Grant AD, Fielding K, Neribongo B, Vanzyl A and Murinead D (2010). Liquid vs solid culture for tuberculosis: Performance & cost in a resource-constrained setting. *Int J Tuberc Lung Dis* **14**(8): 10241031..

T, Mendoza MD, Heide RS, Celada-Ong R, Carmela PE, Wilma Feng JY, Huang SF, Ting WY, Chen YC, Lin YY, Huan RM, Lin CH, Hwang JJ. Yu MC, Yuk W, Lee YC and Su WJ (2012). Gender differences in treatment outcomes of tuberculosis patients in Taiwan: a prospective observational study. *Clinical Microbiol Infect* **18**: E331-E337

Hardy Diagnostics 2014. Hardydiagnostics.com/cp_ prod/Content/hug o/LJMedia.htm Hi Media 2014. http://www.himedialabs.com

Kassaza K, Patrick O, Losla A, Bazira J, Nyehangane B, Page AL and Boum II Y (2014). Lowenstein-Jensen selective medium for reducing contamination in *Mycobacterium tuberculosis* culture. *J Clin Microbiol* **52**(7): 2671.

Lamsal A (2012). Direct Nitrate Reductase Assay (NRA) for detection of drug resistance in *Mycobacterium tuberculosis*: Rapid, Inexpensive method for low resource laboratories. M.Sc. Dissertation submitted to Department of microbiology, St. Xaviers College. Pp 34.

Mubarak SA and Muhammad HA (2010). Susceptibility Pattern and epidemiology of *Mycobacterium tuberculosis* in United Emirati Hospital. *The Open Microbiology Journal* **4**: 14.

Muyoyeta M, Schap JA, De HP, Mwanza W, Muvwimi MW, Godfrey-Faussett P and Ayles H (2009). Comparison of four culture systems for *M. tuberculosis* in Zambian National Reference Laboratory. *Int J Tuberc Lung Dis* **13**: 460-465

Nagaranjan P, Anbarasu S, Kumar V, Selvakumar N (2012). Recovery of M. tuberculosis from LJ medium contaminated with other organism. *Int J Tuberc Lung Dis* **6**(2): 230-231

National Tuberculosis Program (2014). Nepal Annual Report FY 2013/14. Ministry of Health and Population. National Tuberculosis Center, Thimi, Bhaktapur, Nepal.

Neyrolles O and Quanintana-Murci L (2009). Sexual Inequality in tuberculosis. *PLoS Med* **6**: e1000199.

Park K (2005). Park's textbook of Preventive and social medicine. 18 th ed. Mlsbarnarsidas.

Range N, Friis H, Mfaume S, Magnussen P, Chagalucha J, Kilale A A, Mugomela A and Anderson A (2012). Anti-tuberculosis drug resistance pattern among PTB patients with or without HIV infection in Mwanza, Tanzania. *Tanzania Journal of Health Research* **14**(4): 6.

SAARC TB and HIV/AIDS Center (STAC) (2011). SAARC training module for AFB smear and quality assurance in AFB microscopy. SAARC Tuberculosis and HIV/AIDS Center, Thimi, Bhaktapur, Nepal.

Sangre L, Diande S, Badoum G, Dingtoumda B and Traore AS (2010). Anti-tuberculosis drug resistance in new and previously treated pulmonary tuberculosis in Burkina-Faso. *Int J Tuberc Lung Dis* **14**(11): 1424-29.

Thakuri DM (2013). Anti-tuberculosis drug resistance among pulmonary tuberculosis patients attending reference laboratory, GENETUP, Kalimati, Kathmandu. M. Sc. dissertation submitted to Department of microbiology, Tri-Chandra Multiple Campus.

Urinary Tract Infection among Patients Visiting Ganesh Man Singh Memorial Hospital and Research Center, Lalitpur, Nepal

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ABSTRACT

Objectives: The objective of this study was to determine the prevalence of urinary tract infection (UTI) and antibiotic sensitivity pattern among the suspected UTI cases visiting at Ganeshman Singh Memorial Hospital Lalitpur, Nepal.

Methods: A total of 300 mid-stream urine, catheter and suprapubic aspirate from UTI suspected patients were included and processed for routine microscopy and culture and then identified by standard microbiological methods. Antibiotic susceptibility test was performed by Kirby- Bauer disc diffusion method.

Results: Out of 300 samples, 55(84.6%) mid-stream urine and 10(15.4%) catheter sample had significant bacterial growth. *E. coli* (32,49.2%) was the most common isolate followed by *Staphylococcus aureus* (10,15.3%), *Enterobacter* spp. (8,12.3%), *Klebsiella* spp. (7,10.7%), *Pseudomonas aeruginosa* (3,4.6%), *Proteus* spp. (3,4.6%), *Acinetobacter* spp. (1,1.5%) and *Enterococcus* spp. (1,1.5%). Most of the Gram-negative bacterial isolates were sensitive to Ceftriaxone (88.8%) followed by Gentamicin (72.2%), and Nitrofurantoin (64.8%) and resistant to Amoxicilin (68.5%) followed by Nalidixic Acid (53.7%). Gram positive isolates were sensitive to Amikacin (72.7%) followed by Imipenem (63.6%) and Gentamicin (63.6%) whereas resistant to Amoxycilin (72.7%) and Ciprofloxacin (63.63%).

Conclusion: The main cause of the UTIs was found as Gram negative bacteria. Prescription of antibiotics based on susceptibility tests would help in reduction of antibiotic resistance.

Key words: Antimicrobial susceptibility, *Escherichia coli*, *Staphylococcus aureus,* Urinary tract infection

INTRODUCTION

Urinary tract infection (UTI) is an infection caused by the presence and growth of microorganisms anywhere in the urinary tract. Urinary Tract Infection (UTI) remains the commonest bacterial infection in human population with a high rate of morbidity and financial cost as this disease encounter with both community and hospitalized patients of all age group. In contrast to men, women are more susceptible to UTI, and this is mainly due to short urethra, absence of prostatic secretion, pregnancy and easy contamination of urinary tract with faecal flora (Haider et al. 2010).

UTI is usually classified by the infection site: -bladder(cystitis), kidney(pyelonephritis), and urethra(urethritis). UTIs that occur in a normal genitourinary tract with no prior instrumentation are considered as "uncomplicated," whereas "complicated" infection is diagnosed in genitourinary tracts that have structural or functional abnormalities, including instrumentation such as indwelling urethral catheters (Haider et al. 2010; Taher et al. 2009).

The common pathogens that cause UTI are *E. coli*, *Klebsiella* spp, *Staphylococcus* spp and other pathogens. Other pathogens include *Pseudomonas*, *Streptococcus* and MRSA. More than 95% of UTI cases are caused by bacterial pathogens, among which *E. coli*, the

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most leading causative organism, is responsible for the urinary tract infection. More than 80% of urinary tract infections get caused by *Klebsiella* spp. (Ramesh et al. 2008). Other Gram-negative bacteria that cause infection include *Enterobacter* spp., *Pseudomonas aeruginosa*, *Proteus* spp., *Citrobacter* spp., *Morganella morganii*, and so on. The total account of Gram-positive bacteria to cause urinary tract infection is5 to 15% of the total bacteria, which include *Enterococcus* spp., *Staphylococci, and Streptococci* (Akram et al. 2007).

Majorities of UTIs are not life threatening and do not cause any serious disease to the human health. Nevertheless, when the bacterial pathogens that affect kidneys are involved, there is a risk of serious disease like tissue damage with an increased risk of bacteremia (Manikandan et al. 2011). Presence of bacteria, fungi and viruses, among others, could be involved most often to cause UTI. These bacteria enter the urethra and then travel to the bladder and kidneys (Benjamin 2009).

UTI is a common disease aliment among Nepalese population as well as one of the commonest nosocomial infection (Kattel et al. 2008). Nowadays, antimicrobial resistance is a global problem that threatens individual and social well-being. The changing patterns in the etiological agents of urinary tract pathogen and their sensitivities to commonly prescribed antibiotics are reported (Manikandan et al. 2011). The problems of antimicrobial resistance may be due to the fact that antibiotics can be obtained and used without medical authorization or supervision in developing countries (Pokhrel et al. 2006) such as Nepal.

This study was conducted to assess the bacteria causing UTI among the suspected patients visiting Ganeshman Singh Memorial Hospital and Research Center, Lalitpur, Nepal.

MATERIALS AND METHODS

This study was carried out among the patients visiting at Ganeshman Singh Memorial Hospital and Research Center, Lalitpur, Nepal. Three hundred samples, i.e. clinically suspected UTI defined by physician, were investigated from December 2016 to March 2017. The patients with age group ten years and more were included and mid-stream urine, catheter urine and suprapubic aspirate were collected for this study. Under macroscopic examination of urine, the specimens were observed for its colour and appearance and reported accordingly (Cheesbrough 2000).

During microscopic examination of urine, 10ml of urine sample was taken in a clean test tube and the sample was centrifuged at 3000 rpm for 5 minutes. Then the supernatant was discarded and sediment was used for the wet mount preparation to detect RBC, pus cell and epithelial cell. Culture of each urine sample was done into the MacConkey agar and blood agar medium by semi-quantitative method using standard sterile inoculating loop of standard dimension (0.001ml). The plates were then incubated at 37^0C for overnight. Samples showing $\geq 10^5$colony forming unit (CFU) per milliliter (ml) of urine were taken as significant. Low count significant bacteriuria (10^4-10^5CFU/ml) was taken into consideration if there was any indication which can lower the concentration of bacteria in the urine.

Identification of significant isolates was done based on morphological appearance of the colonies, Gram's staining reactions and different biochemical reactions.

Antibiotic susceptibility testing: Antibiotics sensitivity testing of bacterial isolates was done by modified Kirby Bauer disc diffusion method as recommended by CLSI (2014) on MHA.

Statistical analysis: Data were entered into SPSS version 19.0 and analyzed for descriptive statistics.

RESULTS

Among total samples received in laboratory for culture, 269(89.7%) samples were mid- stream urine, 30(10%) samples were catheter and remaining 1(0.3%) samples were suprapubic aspirate.

Out of 300 samples, 65(21.6%) samples had significant growth. Among 65 samples, 55(84.6%) were mid-stream urine samples (MSU) and 10(15.3%) were catheter samples. Out of 201 samples from outdoor patients, 45 (22.3%) samples and out of 99 samples from indoor patients, 20(20.2%) samples had significant growth respectively. Among 138 samples from male patients, 26 (18.8%) samples had significant growth. Similarly, 39 (24.1%) out of 162 samples from female patients showed significant growth. Among the 65 significant growth cultures, high percentage (35.8%) was obtained from age group 31-40 years.

Bacterial isolates causing UTI

Among the bacterial isolates, *E. coli* (49.2%) was found to be the most predominant organism followed by *Staphylococcus aureus* (15.3%), *Klebsiella* spp.(10.7%) and others (24.6%).

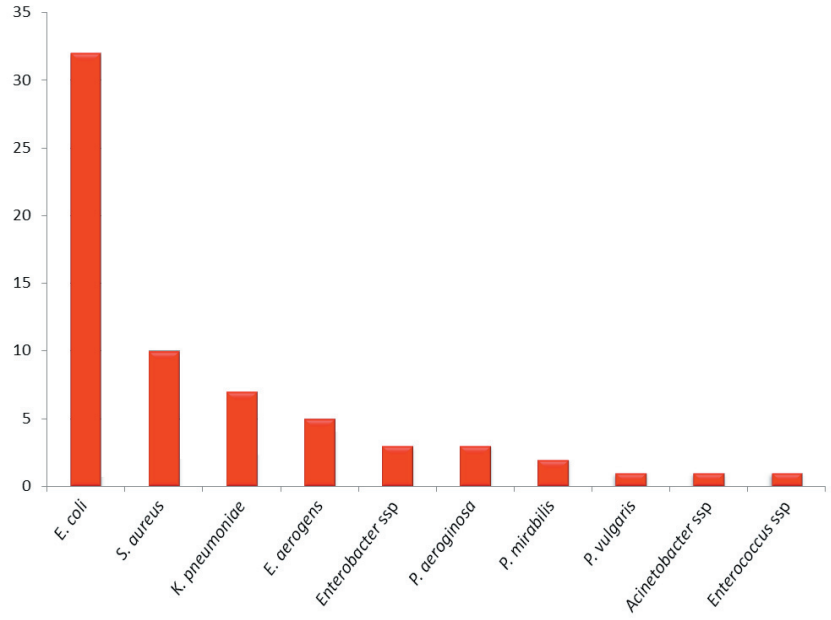

Figure 1: Pattern of bacterial isolates causing urinary tract infection

Antibiotic susceptibility profile

More proportion of *E. coli* was found to be sensitive towards Ceftriaxone (93.5%) followed by Nitrofurantoin (87.5%), Gentamicin (78.1%), Ciprofloxacin and Norfloxacin (71.8%), Imipenem and Chloramphenicol (65.6%), Cotrimoxazole (59.3%), and Cefixime (56.2%). E. coli was found resistant towards Nalidixic acid (84.3%) followed by Amoxycillin (75%) and Amikacin (50%).

S. aureus was found sensitive towards Amikacin (80%) followed by Imipenem and Gentamicin (70%) whereas it was found resistant to Amoxycillin (70%). All *Enterococcus* isolates were found sensitive towards Cotrimoxazole and Nitrofurantoin.

Klebsiella spp was found sensitive towards Imipenem and Ceftriaxone (71.4%) followed by Ciprofloxacin, Gentamicin (57.1%) whereas it was found resistant towards Amoxycilin (85.7%) followed by Norfloxacin (71.4%), Cotrimoxazole (57.1%) and Amikacin (57.1%).

Pseudomonas aeruginosa was sensitive towards Gentamicin whereas it was resistant towards Levofloxacin (66.6%) followed by Amoxycilin, Norfloxacin and Piperacilin (33.3%).

Acinetobacter spp. were sensitive to Norfloxacin, Ciprofloxacin and Ceftriaxone whereas were resistant towards Cotrimoxazole, Nitrofurantoin, Amoxycilin and Nalidixic acid.

Table 1: Antibiotic resistance pattern of isolated bacteria in percentage

Bacteria	Ceftriaxone	Nitrofurantoin	Gentamicin	Ciprofloxacin	Norfloxacin	Imipenam	Chloramphenicol	Cotrimoxazole	Cefixitin	Levofloxacin	Amoxicilin	Nalidixic Acid	Amikacin
E. coli	6.2	12.5	21.8	28.1	28.1	4.3	34.3	40.6	42.7	46.6	75	84.3	50
S. aureus	-	40	30	-	-	30	-	50	-	-	70	-	20
Enterococcus spp	-	0	-	100	-	100	-	0	-	-	100	-	100
Klebsiella spp	28.5	-	42.8	42.8	71.4	28.5	-	57.1	-	-	85.7	-	57.1
P. aeruginosa	-	33.3	-	-	33.3	-	-		-	66.6	33.3	-	-
Acenetobacter spp	0	100	-	0	0	-	-	100	-	-	100	100	-
Enterobacter spp	0	37.5	37.5	-	37.5	12.5	-	12.5	75	-	50	-	-
Proteus spp	33.3	33.3	33.3	66.6	0	-	-	66.6	-	-	33.3	33.3	-

DISCUSSION

Age group 31-40 years had got the high prevalence of UTI which is in contradictory to the study by Daniyan and Ojha (2013). More than one third patients of the total UTI positive cases were found in this age group which is similar to the study of Leigh (1990). Leigh (1990) had found that nuns and unmarried women have lower prevalence of UTI in compared to married women. In addition, use of spermicidal coated condoms dramatically alters the normal bacterial flora and has been associated with marked increase in vaginal colonization with *E. coli* and in the risk of UTI (Braunwald et al. 2001).

The majority cases were from outpatient department and 33% were from patients admitted to the hospital. In comparison to males, higher number of urine samples was collected from females which is also similar to other studies (Arjunan et al. 2010; Alzohairy and Khadri 2011). The increased incidence of the urinary tract infection in women is conditioned by favoring anatomic factors, by hormonal changes and by the urodynamic disturbance occurring with age (Bobos et al. 2010).

In this study, 21.6% urine specimens from suspected UTI patients gave significant growth. Similar results were reported by other investigators from Nepal (Chhetri et al. 2001; Kumari et al. 2005) and rest of the world (Bashir et al. 2009). Among the bacterial isolates, *E. coli* was found to be the most predominant organism followed by *S. aureus*, *Klebsiella* spp. and other which is similar to the study of Kattel et al. (2008). However, Aboderin et al. (2009) reported *Pseudomonas aeruginosa* and *Klebsiella* spp respectively as the predominant bacteria.

Majority of *E. coli* were isolated from outpatient department. *E.coli* accounts for 50% - 90% of all the uncomplicated urinary tract infections (Vgaarali et al. 2008). In our study, *Staphylococcus aureus* was found the second most common isolates. UTI due to *Enterococcus faecalis* are usually associated with the use of instrument or catheterization (Collier et al. 1998).

CONCLUSION

The main cause of the UTIs is Gram negative bacteria. Gentamicin and Ceftriaxone may be drug of choice for Gram negative bacteria whereas Amikacin and Ciprofloxacin may be drug of choice for Gram positive bacteria.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aboderin OA, Abdu A, Odetoyinbo BW and Lamikanra A (2009). Antimicrobial resistance in *Escherichia coli* strains from urinary tract infections. *Natl Med Assoc* **101**: 1268-1273.

Abou-Dobara MI, Deyab MA, Elsawy EM and Mohamed HH (2012). Antibiotic susceptibility and genotype patterns of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from urinary tract infected patients. *Pol J Microbiol* **59**(3): 207-212.

Alzohairy M and Khadri H (2011). Frequency and antibiotic susceptibility pattern of uro-pathogens isolated from community and hospital–acquired infections in Saudi Arabia- A prospective case study. *Br J Med Med Res* **1**(2): 45-56.

Arjunan M, Al-Salamah AA and Amuthan M (2010). Prevalence and antibiotic susceptibility of uropathogens in patients from a rural environment, Tamilnadu. *Am J Infect Dis* **6**(2): 29-33.

Bashir MF, Qazi JI, Ahmad N and Riaz S (2008). Diversity of urinary tract pathogens and drug resistant isolates of *Escherichia coli* in different age and gender groups of Pakistanis. *Trop J Pharm Res* **7**(3): 1025-1031.

Benjamin WD, Biran KP and Gary VD (2009). Lactobacillus deldrueckii as the cause of urinary tract infection. *J Clin Microbiol* **47**: 275-277.

Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL and Jameson JL (2001). *Principles of Internal Medicine*, McGraw-Hill, New York USA, 15 Ed. **2**: 620-625.

Cheesbrough M (2000). *District laboratory practice in tropical countries (Part 2)* Cambridge University press.

Chhetri PK, Rai SK and Pathak UN (2001). Retrospective study on urinary tract infection at Nepal Medical College Teaching Hospital, Kathmandu. *Nepal Med Coll J* **3**: 83-85.

Collier L, Balows A and Sussman M (1998). *Topley and Wilson's Microbiology and Microbial infections (9th ed)* London: *Oxford University Press* **3**: 601-621.

Daniyan SY and Ojha BA (2013). Prevalence and antimicrobial susceptibility of urine pathogen among patient in tertiary health institution in Ado Ekiti, Nigera. *International Journal of Biomedical and Advance Research* **4**(2): 841.

Forbes BA, Sham DF and Weissfeld AS (2007). Infections of the urinary tract. In Bailey and Scott's Diagnostic Microbiology, 12th edition. *St. Louis; Mosby Elsevier*: 842- 55.

Haider G, Zehra N, Afroze A and Haider A (2010). Risk factors of urinary tract infection in pregnancy. *J Pak Med Assoc* **60**: 213-216.

Kattel HP, Acharya J, Mishar SK, Rijal BP and Pokhrel BM (2008). Bacteriology of urinary tract infection among patients attending Tribhuvan University teaching hospital, Kathmandu, Nepal. *JNAMLS* **9**(1): 25-29.

Kumari N, Ghimire G, Magar JK, Mohapatra TM and Rai A (2005). Antibiogram pattern of isolates from UTI cases in Eastern part of Nepal. *Nepal Med Coll J* **7**: 116-118.

Leigh DA (1990). *UTI. In: Smith GR and Easmon CSF, In (eds) Toperly and Wilson,s Principles of Bacteriology, Virology and immunity, Bacterial Diseases, 8th ed.* Frome and London: *Bulter and Tanner Ltd*. **3**: 197-214.

Manikandan S, Ganesapandian S, Singh M and Kumaraguru AK (2011). Emerging of multidrug resistance human pathogens from urinary tract infections. *Current Research in Bacteriology* **4**(1): 9-15.

Manjunath GN, Prakash R, Vamseedhar A and Shetty K (2011). Changing trends in the spectrum of antimicrobial drug resistance pattern of uropathogens isolated from hospital and community patients with urinary tract infections in Tumkur and Bangalore. *Int J Biol Med Res* **2**(2): 504-507.

Mohammadi M (2007). Antimicrobial Resistance patterns of *E. coli* detected from hospitalized urine samples. *Asian Journal of Biomedical Sciences* **3**(4): 195-201.

Pokhrel BM (2006). *A handbook of clinical microbiology, 1st ed.* Gorakhnath Desktop and Printing Supports, Kathmandu, Nepal.

Ramesh N, Sumathi CS, Balasubramanian V, Palaniappan KR and Kannan VR (2008). Urinary tract infection and antimicrobial susceptibility pattern of extended spectrum of beta lactamase producing clinical isolates. *Advan Biol Res* **2**(5-6): 78-82.

Richards MJ, Edwards JR, Culver DH and Gaynes RP (1999). Nosocomial infections in medical intensive care units in the United States. *Crit Care Med* **27:** 887

Taher M, Mohamed F, Ghaith M and Ismail M (2009) Prevalence and predictors of asymptomatic bacteriuria among pregnant women attending primary health care in Quatar. *Middle East J Fam Med* **7**: 10-13.

Vgaarali MA, Karadesai SG, Patil CS, Metgud SC and Mutnal MB (2008). Haemagglutination and siderophore production as the urovirulence markers of the uropathogenic *Escherichia coli*. *Indian J Med Micro-biol* **26(**1): 68-70

Production of Garbage Enzyme from Different Fruit and Vegetable Wastes and Evaluation of Its Enzymatic and Antimicrobial Efficacy

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ABSTRACT

Objectives: To evaluate the enzymatic and antimicrobial efficacy of enzyme from garbage produced from different fruits and vegetable wastes.

Methods: This study was conducted from October-2018 to February-2019 in the laboratory of Padma Kanya Multiple College, Bagbazar, Kathmandu, Nepal. This study was carried for production, analysis of enzymatic and antimicrobial efficacy by using yeast (*Saccharomyces cerevisae)* and bacteria (*Bacillus* species) in 5 fruits peels, Mosambi (*Citrus limetta*), Pomegranate (*Punica granatum*), Pineapple (*Ananas comosus*), Papaya (*Carica papaya*) and mixed fruits collected from fresh fruit stall and vegetable peels collected from college's hostel. The fermentation mixture was made in the ratio 1:3:10 (1 part brown sugar, 3 parts fruits/vegetable peels and 10 parts water) and left for 3 months for fermentation.

Results: After fermentation, enzyme activity (amylase, protease, caseinase, cellulase and lipase) and antimicrobial efficacy (*S. aureus, S. aureus* (ATCC 25923), *Bacillus* spp, *Salmonella* Typhi, *E. coli, E. coli* (ATCC 25922), *Shigella* spp, *Pseudomonas aeruginosa*) were analyzed. All the samples showed amylase and caseinase enzyme activity, only Pineapple (*Ananascomosus)*, Papaya (*Carica papaya)* and Mixed fruit showed protease enzyme activity while only Pomegranate (*Punicagranatum)* showed lipase enzyme activity. In antimicrobial efficacy test, garbage enzyme produced from vegetable sample didn't show antimicrobial activity with bacteria used except *E. coli* (ATCC 25922)and *S. aureus* (ATCC 25923). Similarly, garbage enzyme produced from Mixed fruit and Papaya (*Carica papaya)* didn't show antimicrobial activity with *Salmonella* Typhi and *S. aureus* (ATCC 25923) respectively but garbage enzyme from other wastes showed antimicrobial activity with bacteria used in test.

Conclusion: Different fruits and vegetables wastes showed different enzyme activity and antimicrobial activity.

Key words: Garbage enzyme, fruits, vegetables, Antimicrobial susceptibility test.

INTRODUCTION

Garbage enzymes are the organic solution produced by the simple fermentation of fresh vegetable wastes, fruit wastes with addition of brown sugar and water by using the selective microorganisms like Yeast and Bacteria (Thirumurugan 2016). This fermentation creates a vinegar like liquid with natural proteins, mineral salts and enzymes that make it magnificently multipurpose in and out of the home. In 2006, a researcher from Thailand named Rosukun developed a solution from product using organic solid waste and named it garbage enzyme (Chelliah and Palani 2015). This enzyme is composite organic substance made up of organic acids, proteins chains (enzyme), and minerals salts produced by fermentation of waste vegetables, fruits, or its peels, sugars, and water. The garbage enzyme can be applied to compose, decompose, transform, and catalyze (Palanisamy and Palani 2017). The functions of garbage enzyme are to resolve (decompose), transform (change), and catalyze the reduction (Voet 2012).

Fruits and vegetable wastes are produced in large

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quantities in market and constitutes a source of nuisance in municipal landfills because of their high biodegradability (Virtrurtia et al. 1989). Garbage/citrus enzymes is different from fruit enzymes and is not for human consumption. It is a nutritious drink prepared through proper fermentation of fruits. Garbage/citrus enzymes is used as a natural household cleaner; air purifier; deodorizer; insecticides; detergent; body care; organic fertilizer etc. It removes odor and dissolve toxic air released from smoking, car exhaust, chemical resides, from household products etc. Enzymes that flow underground will eventually purify the river and the sea. It reduces mosquitos, flies, rats, cockroaches etc. It is a natural antiseptic for your home. It prevents drain pipe blockages (Pinang 2012).

MATERIALS AND METHODS

Sample size, sample site and duration of study

The study was done from October 2018 to February 2019. A total 6 samples including 5 fruits peels sample i.e. Lime (*Citrus aurantiifolia*), Pineapple (*Ananas comosus*), Pomegranate *(Punica granatum*), Papaya (*Carica papaya*) and mixed fruit and one vegetable peels sample was taken for the study. Fruit peels were collected from various fresh juice stall near Padma Kanya Campus. Similarly, vegetables peels were collected from the hostel's kitchen of Padma Kanya Campus.

Study design

Purposive/judgement sampling was performed for sample selection and cross-sectionals-descriptive study designed was performed.

Preparation of fermentation medium

The collected fruits and vegetable samples were mixed in 1:3:10 (1part molasses, 3 parts fruits/ vegetables peels and 10 parts water) for the fermentation process. Air tight plastic jars were used for fermentation process. In this mixture, 3 tea spoonful of yeast powder (*Saccharomyces cerevisiae*) and 10 ml of bacterial suspension *(Bacillus* species) was added. Then the jars were left for fermentation for 12 weeks (Thirumurugan 2016). After 12 weeks, enzyme activity test and antimicrobial efficacy test was performed. For the enzymatic and antimicrobial assay, the fermented mixtures were centrifuged at 5000 rpm for 10 minutes. The supernatant (crude garbage enzyme) was used to analyzed enzyme activity and antimicrobial efficacy test (Sarkar et al. 2011).

Screening of enzyme activity

Amylase

For amylase enzyme activity, agar-agar with 1% starch was prepared aseptically. With help of sterile cork borer, 4mm size wells were made in which 50µl of enzyme from garbage was inoculated then the plates were incubated for 48 hours at 37^0C. Hydrolysis of starch was visualized as clear zones around the wells of plates against deep blue brown for starch by flooded with iodine solution (Emimol et al. 2012). Diameter of the clear zone was measured and the activity level of the microorganisms was determined by the diameter of the clear zone formed.

Cellulase

The cellulase agar was prepared with 1% carboxy methyl cellulose aseptically. With the help of sterile cork borer of 4mm size, wells were made in plates in which 50µl of enzyme from garbage was inoculated in well and plates were incubated at 37^0C for 24 hours - 48 hours, the plates were flooded with 0.3% congo-red solution for 10 minutes. Then it was washed with water and flooded with 1N NaCl as distaining solution. Cellulase production is visualized by translucent zone around the colonies. Diameter of the translucent zone was measured and the activity level of the microorganisms was determined by the diameter of the translucent zone formed (Thirumurugan 2016).

Protease

The protease agar was prepared with 1% gelatin aseptically. With help of sterile cork borer of 4mm sizes, wells were made in plates in which 50µl of enzyme from garbage was inoculated then the plates were incubated at 37^0c for 24 hours-48 hours. After incubation plates were flooded with acidic mercuric chloride solution and were allowed to stand for 5-10 mins, excess solution was decanted. Appearance of a clear zone around the colonies demonstrated the positive result for the proteolytic hydrolysis of gelatin by the enzyme gelatinase. Diameter of the clear zone was measured and the activity level of the microorganisms was determined by the diameter of the clear zone formed. Unhydrolyzed and continuous opaque zone around the growth indicates the absence of gelatinase enzyme. Diameter of the clear zone was measured and the activity level of the microorganisms was determined by the diameter of the clear zone formed (Emimol 2012).

Caseinase

The casein hydrolysis test was done by inoculation

of the garbage enzyme to be tested on the agar plates containing 1% skimmed milk powder. With help of sterile cork borer of 4mm size wells, were made prepared in 50µl of enzyme from garbage was inoculated then the plates were then incubated at 37^{0}c for 24 hours-48 hours. After incubation plates were flooded with copper sulphate solution and excess solution was decanted off. Formation of a clear zone was observed around the well and the diameter of the clear zone was measured. Diameter of the clear zone was measured and the activity level of the microorganisms was determined by the diameter of the clear zone formed (Sazci et al. **1986**)

Lipase

1% Tween-20 hydrolysis agar medium was prepared. With help of sterile 4mm cork borer, wells were made one plate was. The wells were labelled by the name of the sample to be inoculated.50µl of each sample was added to well. The plates were at 37°C for 24 hours. After the incubation, the clear zone of hydrolysis was observed around well (Emimol 2012).

Antimicrobial efficacy test

The crude extract of garbage enzyme was screened for its antimicrobial activity i.e. determination of zone of inhibition against tested organisms by agar well diffusion method as given by Balouiri et al. (2016). According to CLSI 2012, 3- 4 fresh bacterial culture colonies was inoculated in nutrient broth and incubated for 4 hours then compared its turbidity standard 0.5 McFarland. Sterile cotton swab was dipped into the prepared inoculums, rotated and pressed against the upper inside wall of the tubes to express excess fluid. The entire agar plate was streaked 3 times, turning the plate at 60^o angle between each streaking. Inoculums was allowed to dry for 5-10 minutes. Then with the help 4mm sterile cork borer, wells were made in the inoculated media plates then 50µl of the suspension of different garbage was inoculated into the well with the help of micropipette. The plates were then left for half an hour and incubated at 37^{o}c overnight. After incubation, the plates were viewed for the zone of inhibition (clear zone) without the growth around the well. The zones of inhibition were measured using a scale and mean was recorded. For the quality control of antimicrobial activity, ATCC culture of *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were used.

RESULTS

Table 1: Enzymatic activity of crude garbage enzyme in particular agar medium

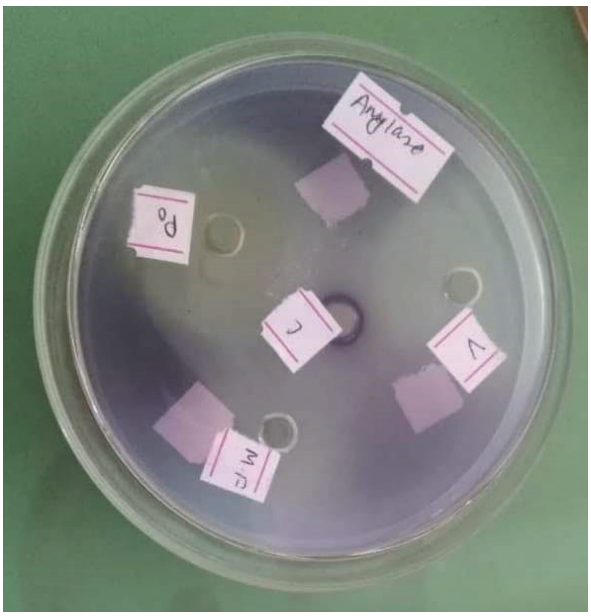
Name of the sample	Zone of inhibition (mm) in different media				
	Starch hydrolysis agar	Gelatin hydrolysisagar	Skimmed milk agar	Tween-20 hydrolysis agar	Cellulose hydrolysis agar
Mosambi *(Citrus limetta)*	29	0	10	0	0
Pomegranate *(Punica granatum)*	35	13	18	28	0
Pineapple *(Ananas comosus)*	28	15	12	0	0
Papaya *(Carica papaya)*	21	10	10	0	0
Mixed Fruits	23	8	12	0	0
Vegetables	25	0	9	0	0

Table 2: Antimicrobial activity of crude garbage enzyme on Gram positive bacteria

Name of the sample	Zone of inhibition (mm) on Gram positive bacteria		
	S. aureus	*S. aureus* (ATCC 25923)	*Bacillus* spp
Mosambi *(Citrus limetta)*	16	16	18
Pomegranate *(Punica granatum)*	30	25	18
Pineapple *(Ananas comosus)*	23	24	22
Papaya *(Carica papaya)*	18	0	13
Mixed Fruits	12	14	16
Vegetables	0	19	0

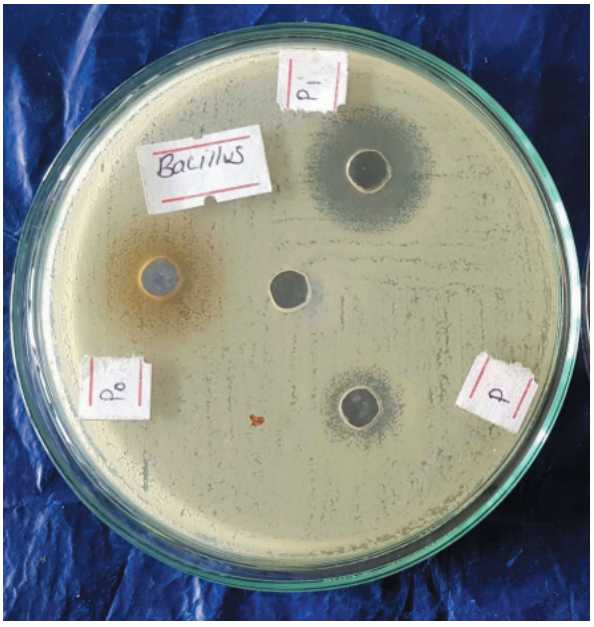
Table 3: Antimicrobial activity of crude garbage enzyme on Gram negative bacteria.

Name of the sample	Zone of inhibition (mm) on Gram negative bacteria				
	Shigella spp	*Pseudomonas aeruginosa*	*Salmonella Typhi*	*E. coli*	*E. coli* (ATCC 25922)
Mosambi *(Citrus limetta)*	20	17	20	18	24
Pomegranate *(Punica granatum)*	19	13	18	24	21
Pineapple *(Ananas comosus)*	28	25	20	18	27
Papaya *(Carica papaya)*	17	21	15	14	14
Mixed Fruits	20	18	0	17	19
Vegetables	0	0	0	0	23



V= Vegetable
M.F= Mixed fruit
Po= Pomegranate
C= Control

Photograph 1: Amylase enzyme activity of crude garbage enzyme



Pi= Pineapple
P= Papaya
Po= Pomegranate
Center = control

Photograph 2: Antimicrobial efficacy test of crude garbage enzyme in Bacilus species

DISCUSSION

In gelatin agar plate, only Pineapple(15mm), Papaya(10mm) and Mixed fruit (8mm) showed protease enzyme activity. Analysis of protease enzyme activity in gelatin agar plate by Thirumurugan (2016) taking Orange, Pomegranate, Mosambi and Watermelon as sample analyzed that in the pomegranate sample, the activity was slightly higher than other samples. In Thirumurugan study, pomegranate showed the zone of inhibition which was 34mm. However, Pomegranate didn't show the protease enzyme activity, reasons could be the difference in fermentation time of samples. In the study conducted by Madhumithah et al. (2011) using five vegetable wastes samples such as Potato, Brinjal, Pumpkin, Cauliflower and Cabbage, protease enzyme produced by solid state fermentation using *Aspergillus niger* showed maximum enzyme production in case of cauliflower substrate with an activity of 1.082 U g-1 and minimum production of 0.43 U g-1 of potato substrate. Protease enzyme was produced in both studies but the difference was based on whether the protease enzyme produced or not whereas total amount of protease produced in each sample per gram of substrate was calculated in the study of Madhumithah et al. (2011).

In starch hydrolysis agar, all the six samples showed the amylase enzyme activity. Pomegranate showed maximum amylase enzyme activity (35mm) whereas Papaya showed the minimum amylase enzyme activity (21mm). However, in the study conducted by Thirumurugan (2016) in case of amylase enzyme activity in caesine agar plate, Orange, Mosambi, Watermelon and Pomegranate were included as the sample, only Watermelon (15mm) and lime (19mm) showed the amylase activity. This difference is may due to the difference in the fermentation period and difference in the agar plate used.

In this study, all the six samples of fruits and vegetables waste showed casein hydrolysis. Among all the sample Pomegranate showed the maximum casein hydrolysis (18mm) whereas vegetable sample showed the minimum casein hydrolysis (9mm). This may conclude that all the sample produced caseinase enzyme during fermentation.

None of the fruits and vegetable sample showed cellulase enzyme activity which means that there was no production of cellulase during fermentation in all sample. But in the study conducted by Thirumurugan (2016) taking Orange, Watermelon, Mosambi and Pomegranate as the sample, only Water melon (18mm) and Mosambi (12mm) showed the cellulase enzyme activity however pomegranate didn't show the cellulase enzyme activity which means that Pomegranate may not produce cellulase enzyme. Mosambi did not show the cellulase enzyme activity which could be the difference factors like pH of the garbage enzyme, temperature etc. Duration of the fermentation of sample may also affected the cellulase enzyme activity. Among the six different fruits and vegetable samples, only Pomegranate showed lipase enzyme activity. This may also that only Pomegranate sample produced lipase enzyme activity during fermentation.

In this study the antimicrobial activity of enzyme from garbage on Gram positive and Gram-negative bacteria showed different zone of inhibition. Enzyme garbage produced from Papaya (*Carica papaya*) and mixed fruit showed no antimicrobial activity with *S. aureus* (ATCC 25923) and *Salmonella* Typhi respectively whereas garbage enzyme produced from vegetable wastes showed antimicrobial activity only with *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). Garbage enzyme produced from other samples showed antimicrobial activity with the Gram positive and Gram-negative bacteria used in the test. However, in the study conducted by Saramanda and Kaparapu (2017), the antimicrobial activity of garbage enzyme from citrus fruit peels extract showed zone of inhibition higher. It was observed by using 150µl of garbage enzyme solution, the zone of inhibition for *E. coli, S. aureus, Streptococcus pyogens, Salmonella* Typhi and *Pseudomonasa eruginosa* were 11mm,10mm, 10mm, 13mm and 9mm respectively. These difference in zone of inhibition might be due to the difference in the type of sample producing garbage enzymes. Also, the concentration of garbage enzyme diffused in well was different in both studies.

CONCLUSION

Different fruits and vegetables wastes showed different enzyme activity and antimicrobial activity. Enzymes produced from garbage showed the antimicrobial activity with Gram positive and Gram-negative bacteria so the garbage enzyme should be utilized to kill/inhibit the pathogens in house as well as laboratory.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Balouiri M, Sadak M and Ibnsounda SK (2016). Methods for in vitro evaluating antimicrobial activity. *J Pharm Anal* **6**: 71-79.

Chelliah A and Palani S (2015). Investigation of biocatalytic potential of garbage enzymeand its influence on stabilization of industrial waste activated sludge. *Process Safety and Environmental Protection* **94:** 471-478.

Emimol A, Ganga G, Parvathy R, Radhika G and Nair GM (2012). Screening of microbes producing extracellular hydrolytic enzyme from corporation waste dumping site and house hold waste for the enhancement of bioremediation methods. *IOSR-JPBS* **4**(1): 54–60.

Madhumithah CG, Krithiga R, Sundharam S, Changam SS, Guthakurta S and Cherian KM (2011). Utilization of vegetables wastes for production of protease by solid state fermentation by using *Aspergillus niger*. *World J Agric Sci* **7**(5): 550-555.

Palanisamy S and Palani (2017). Optimization of lipase production from organic solid waste by anaerobic digestion and its application in biodiesel production. *Fuel Process Technol* **165**: 1-8.

Pinang PS (2012). Change climate. http://www.enzymesos.com accessed on August 16, 2019.

Saramanda G and Kaparapu J (2017). Antimicrobial activity of fermented citrus fruit peel extract. *Int Journal of Engineering Research and Application* **7**: 25-28.

Sarkar P, Meghvanshi M and Singh R (2011). Microbial consortinum: Approach in effective degradation of organic kitchen wastes. *IJEST* **2(**3): 170-174.

Sazci A, Erenler K and Radford A (1986). Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. *J Appl Bacteriol* **61**(6): 559–562.

Selvakumar P and Sivashanmugam P (2017). Optimization of lipase production from organic solid waste by anaerobic digestion and its application in biodiesel production. *Fuel Process Technol* 165: 1-8.

Thirumurugan P (2016). Production and analysis of enzyme bio-cleaners from fruit and vegetable wastes by using yeast and bacteria. Student project Report (D.O.Rc.No.1082/2015A; Project No: 28) submitted to Tamil Nadu State Council for Higher Education (TANSCHE), India pp: 4-6.

Viturtia A, Alvarez JM, Cecchi F and Fazzini G (1989). Two-phase anaerobic digestion of a mixture of fruit and vegetable waste. *Biol Wastes* **29**: 189-99.

Voet V (2012). Ways to save energy (2012). http://www.waystosaveenergy.net accessed on August 16, 2019.

Antibiotic Susceptibility Pattern of Bacterial Isolates from Soft Tissues Infection among Patients Visiting Birendra Military Hospital, Chhauni, Kathmandu

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ABSTRACT

Objectives: To determine the rate of soft tissues infection and perform antibiotic pattern susceptibility test of bacterial pathogens isolated from soft tissue infected patients visiting Shree Birendra Hospital, Kathmandu, Nepal.

Methods: A total of 380 wound specimens (open and closed) including pus and wound swabs were processed in the laboratory of Birendra Military Hospital, Chhauni from August to November 2018. The specimens were cultured on Blood Agar blood agar and Mac-Conkey agar and incubated at 37°C for 24 hrs. Antibiotic Susceptibility Test was performed by using modified Kirby-Bauer disc diffusion method. Thus, multidrug resistant (MDR) bacteria and methicillin resistant *Staphylococcus aureus* (MRSA) were differentiated.

Results: Out of 380 bacterial isolates, 86(43.21%) were Gram positive and 113(56.78%) were Gram negative bacteria. Among all the Gram-positive isolates 43(53.09%) were found to be MRSA. Similarly, 62(54.86%) were found to be MDR among the Gram-negative bacteria. Gentamicin and Amikacin were found to be the most effective drug though the resistance pattern is not homogenous against all isolates.

Conclusion: Antibiotic susceptibility pattern of all bacterial isolates showed that, Gentamycin, Amikacin, Levofloxacin, Piperacillin/ Tazobactam, Doxycycline were the effective drug for Gram-negative bacteria and Amikacin, Teicoplanin, Linezolid, Doxycycline, Gentamycin and Azithromycin were the most effective drug for Gram-positive organisms. Thus it can be concluded that these antibiotics may be used for the empirical treatment of soft tissues infection.

Key words: Antibiotic susceptibility, bacteria, soft tissue, MRSA

INTRODUCTION

Soft tissues infections are infection of the skin and soft tissue and are usually caused by bacteria. The infection develops when there is a break in the skin, such as a wound or athlete's foot, which may be minor or even unnoticed. This allows bacteria to enter through the skin and grow, causing infection and swelling. People suffering from cut, scarps or other abrasion can get any of this infection. The symptoms of skin and soft tissue infections are all very similar and usually include swelling and redness of the skin as well as warmth radiating from the area. Other symptoms include smooth and shiny skin, small blisters and pimples that get formed in the area (Baddour 2019).

The most common pathogens in these infections are *Staphylococcus aureus* (including MRSA), *P. aeruginosa*, *Enterococcus* spp, *Escherichia coli* and other antibiotics resistant Enterobacteriaceae (Rosser et al. 2005).

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents or to detect resistance in individual bacterial isolates (Edelsberg et al. 2009). Multidrug resistant

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bacteria, bacteria that resist to more than three classes of antibiotics, are more problematic as compared to normal bacteria because infections with multidrug-resistant bacteria are hard to treat since few or even no treatment options remain (Magiorakos et al. 2012). In some cases, health care providers have to use antibiotics that are more toxic for the patient. Multidrug-resistance facilitates spread of antibiotic resistance. When multidrug-resistance plasmids are transferred to other bacteria, these become resistant to many antibiotics at once. In environments where bacteria are continuously exposed to antibiotics, like in hospitals or some large production animal farms, multidrug-resistance may be favorable and are therefore selected and spread further (Bessa et al. 2013). Multidrug-resistance complicates efforts to reduce resistance. When many different antibiotics are selected for the same resistant bacteria or plasmids, reducing use of one type of antibiotic is not enough to reduce resistance to that antibiotic. Thus, there is an increasing prevalence of pathogenic multidrug-resistant bacteria globally. An example is ESBL (extended spectrum beta lactamase)-producing Gram-negative bacteria like *E. coli* and *Klebsiella pneumoniae* (Woerther et al. 2013).

Methicillin resistant *Staphylococcus aureus* (MRSA) emerged as a cause of infection among patients exposed to the bacteria in health care centers. It is a common cause of hospital and community acquired infections worldwide (Barret et al. 1968). Treatment of *S. aureus* infections which has now become more challenging with the emergence of MRSA, are often multidrug resistant (Ciccarone et al. 2001).

MATERIALS AND METHODS

Study site and population

A hospital based descriptive cross-sectional study was conducted during August– November 2018 at Shree Birendra Hospital Chhauni, Kathmandu, Nepal. A total of 380 specimens (pus and swab) were processed from soft tissues infection during study period. The study populations were the patients irrespective of age and sex with soft tissue infection as referred by the physicians for routine clinical care.

Isolation and identification: Wound Swabs were collected and inoculated on Blood agar plates and Mac-Conkey agar plates. The blood agar plates were incubated at 37°C for 24 hrs enriched with CO_2 while Mac-Conkey agar plates were aerobically incubated in ordinary incubation at 37°C for 24 hrs. Blood agar was examined for haemolysis of the medium, colonial characteristic and gram staining was carried out. Mac-Conkey agar plates were examined for Gram's negative organism and lactose fermenter and non-lactose fermenter and colonial character of the organism (WHO 2003).

Isolates were identified using standard microbiological techniques as described by Cheesbrough (2006), comprising of colony morphology, Gram staining and various other biochemical tests such as catalase production test, coagulase production test, oxidase test, IMViC tests, Triple sugar iron agar tests, etc. and reported accordingly.

Antibiotic susceptibility testing: The antibiotic susceptibility testing of individual isolate was carried out by modified Kirby-Bauer disc diffusion method as per CLSI guidelines (2014) using Muller Hinton Agar (MHA). In this study antibiotics used were Ampicillin (10µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Cloxacillin (5µg), Cotrimoxazole (µ), Erythromycin (15µg), Gentamicin (10µg), Aztreonam (30µg), Amoxicillin (30µg), Ofloxacin (5µg), Cefepime (30µg), Amikacin (30µg), Amoxyclav (20/10µg), Clindamycin(2µg), Levofloxacin (5µg), Cefotaxime (30µg), Ceftazidime (30µg), Doxycycline (30µg), Azithromycin (15µg), Piperacillin (100µg), Piperacillin+Tazobactum (PTZ/100/10µg), Teicoplanin (30µg), Polymyxin B (300unit) and Linezolid (30µg). The organism's showing resistant to more than three different class of antibiotics was taken as Multi-drug resistant isolates (Magiorakos et al. 2012). Screening for methicillin resistance was performed by cefoxitin disc diffusion method and interpreted according to CLSI (2018).

RESULTS

Out of 380 samples collected, 199 (52.36%) sample showed growth and 181(47.63%) showed no growth. (Table 1).

Table 1: Growth pattern of the specimen

Growth	Number	Percentage
Growth	199	52.36
No growth	181	47.63
Total	**380**	**100**

Out of 380 patients, the rate of infection was found to be higher among the males (36.05%) in comparison to females (16.31%). (Table 2)

Table 2: Sex-wise distribution of the patients

Sex	Growth (%)	Total (%)
Male	137 (36.05)	247 (65)
Female	62 (16.31)	133 (35)
Total	**199 (52.36)**	**380 (100)**

As far as the age wise distribution is concerned, the highest rate of infection was observed in the age group 45 to 59 years as shown in table 3.

Table 3: Age-wise distribution of the patients

Age (Years)	Growth n (%)	Total (%)
≤ 14	15 (3.94)	23 (6.05)
15-29	42 (11.05)	82 (21.57)
30-44	45 (11.84)	94 (24.73)
45-59	58 (15.26)	88 (23.16)
60-74	31 (8.15)	78 (20.52)
75-89	7 (1.84)	14 (3.68)
90 above	1 (0.26)	1 (0.26)
Total	**199 (52.36)**	**380 (100)**

Out of total 199 bacterial isolates, 113 were Gram negative and 86-Gram positive bacterial isolates. The most predominant isolate was *Staphylococcus aureus* 81(40.70%), *Escherichia coli* accounting for 37 (18.59%) followed by *Pseudomonas* spp 30(15.07%), *Klebsiella pneumoniae* 18(9.04%), *Acinetobacter* spp 13(6.53%) and *Enterobacter* spp 6(3.01%). The least frequently isolated ones were CoNS 3(1.50%), *Proteus mirabilis* 2(1.005%), *Citrobacter freundii* 2(1.005%), *Serratia marcescens* 2(1.005%), *Citrobacter koserii* 2(1.005%), *Klebsiella oxytoca* 1(0.50%), *Enterococcus* spp 1(0.50%) and *Streptococcus* spp 1(0.50%). (Table 4)

Table 4: Distribution patterns of Gram positive and Gram-negative bacteria among growth

Organism	Number	Percentage
Staphylococcus aureus	81	40.70
Escherichia coli	37	18.59
Pseudomonas aeruginosa	30	15.07
Klebsiella pneumonia	18	9.04
Acinetobacter spp	13	6.53
Enterobacter spp	6	3.01
CoNS	3	1.50
Proteus mirabilis	2	1.01
Citrobacter freundii	2	1.01
Citrobacter koserii	2	1.01
Serratia marcescens	2	1.01
Klebsiella oxytoca	1	0.50
Enterococcus spp	1	0.50
Streptococcus spp	1	0.50
Total	199	100

Among all the antibiotics used, the highest number of *E. coli* (n=37) were found to be sensitive to gentamicin 29 (78.37%) followed by doxycycline 18(48.64%), amikacin 17(45.94%), levofloxacin 15(40.54%), Cotrimoxazole 13(35.13%), Piperacillin+Tazobactum 12(32.43%) as shown in table 5.

Out of 19 isolates of *Klebsiella* spp, 18 isolates were *Klebsiella pneumoniae* and 1 isolate were *Klebsiella oxytoca.* Among which the highest number of isolates were most sensitive to doxycycline 9(47.36%) followed by amikacin 8(42.105%), and others as shown in table 5

Among 13 isolates of *Acinetobacter* spp, was subjected

to AST against 14 antibiotics. Among which the highest isolate was found to be most sensitive to co-trimoxazole 3(23.07%), levofloxacin 1(7.69%), gentamicin 1(7.69%). All the isolates 13(100%) were resistant to Amoxycilin, Amoxyclav, Ceftriaxone, Cefotaxime, Amikacin, Ciprofloxacin, Ofloxacin, Piperacillin and PTZ.

Six isolates of *Enterobacter* spp, was subjected to AST against 14 antibiotics among which the isolate was found to be most sensitive to levofloxacin 5(83.33%), gentamicin 5(83.33%) and ofloxacin 4(66.66%).

Table 5: Antibiotic susceptibility pattern of *E. coli*, *Klebsiella* spp, *Acinetobacter* spp and *Enterobacter* spp.

Isolates	*E. coli* (n=37)		*Klebsiella* spp (n=19)		*Acinetobacter* spp (n=13)		*Enterobacter* spp (n=6)	
Antibiotics	Sensitive N (%)	Resistant N (%)	S ensitive N (%)	Resistant N (%)	Sensitive N (%)	Resistant N (%)	Sensitive N (%)	Resistant N (%)
Amoxycilin	3(8.10)	34(91.89)	0(0)	19(100)	0(0)	13(100)	0(0)	6(100)
Amoxyclav	8(21.62)	29(78.37)	1(5.26)	18(94.73)	0(0)	13(100)	0(0)	6(100)
Ceftriaxone	6(16.21)	31(83.78)	4(21.05)	15(78.94)	0(0)	13(100)	2(33.33)	4(66.66)
Cefotaxime	5(13.51)	32(86.48)	3(15.78)	16(84.21)	0(0)	13(100)	1(16.66)	5(83.33)
Cotrimoxazole	13(35.13)	24(64.86)	4(21.05)	15(78.94)	3(23.07)	10(76.92)	3(50)	3(50)
Gentamycin	29(78.37)	8(21.62)	6(31.57)	13(68.42)	1(7.69)	12(92.30)	5(83.33)	1(16.66)
Amikacin	17(45.94)	20(54.05)	8(42.11)	11(57.89)	0(0)	13(100)	2(33.33)	4(66.66)
Ciprofloxacin	7(18.91)	30(81.08)	4(21.05)	15(78.94)	0(0)	13(100)	1(16.66)	5(83.33)
Ofloxacin	9(24.32)	28(75.67)	7(36.84)	12(63.15)	0(0)	13(100)	4(66.66)	2(33.33)
Levofloxacin	15(40.54)	22(59.45)	7(36.84)	12(63.15)	1(7.69)	12(92.30)	5(83.33)	1(16.66)
Piperacillin	4(10.81)	33(89.18)	0(0)	19(100)	0(0)	13(100)	1(16.66)	5(83.33)
PTZ	12(32.43)	25(67.56)	3(15.78)	16(84.21)	0 (0)	13 (100)	3(50)	3(50)
Ampicilin	7(18.91)	30(81.08)	0(0)	19(100)	0(0)	13(100)	0(0)	6(100)
Doxycycline	18(48.64)	19(51.35)	9(47.36)	12(63.15)	0(0)	13(100)	0(0)	6(100)

Among 2 isolates of *Proteus mirabilis*, was subjected to AST against 14 antibiotics among which all isolates were found to be resistant to Amoxycilin 2(100%) and Cefotaxime 2(100%). Among four isolates of *Citrobacter* spp, two isolates were *Citrobacter freundii* and two were *Citrobacter freundii*. These bacterial isolates were subjected to AST against 14 antibiotics among which all isolates 4(100%) were found to be resistant to Amoxycilin, Amoxyclav, Ceftriaxone, Piperacilin, and Cefotaxime. Among 2 isolates of *Serratia marcescens*, was subjected to AST against 14 antibiotics among which both 2 isolates was found to be resistant to Amoxyclav and Doxycycline.

Out of 30 isolates of *Pseudomonas* spp, all were subjected to AST against 9 antibiotics. Among which the highest number of isolates were most sensitive to Polymyxin B 27(90%), followed by Gentamicin 24(80%), Amikacin 22(73.33%), PTZ 22(73.33%), Aztreonam 22(73.33%). The lowest sensitivity was towards Cefepime 12(40%).

Among 199 positive isolates, 113 were Gram negative organisms. Out of total Gram-negative organism isolates 62(54.86%) were multi drug resistant (MDR) and 51(45.13%) were not MDR.

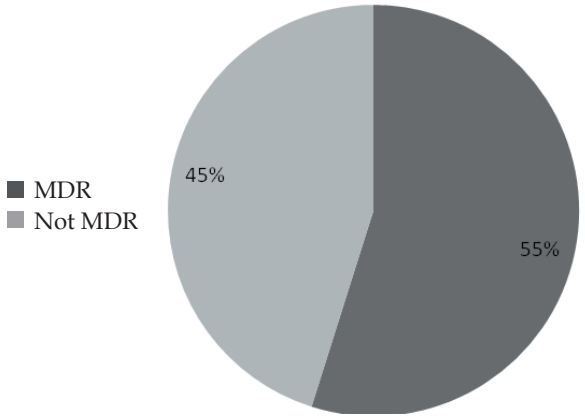

Figure 3: Distribution of MDR among Gram negative isolates

Altogether 81(94.17%) *Staphylococcus aureus* were isolated among 86 Gram positive cocci (GPC)GPC. Among *S. aureus*, 43 were MRSA and 38 were MSSA. These all *S. aureus* were subjected towards 12 antibiotics and highest sensitive towards Amikacin was found 79(97.53%) followed by Teicoplanin 75(92.59%) and Linezolid 73(90.12%) and the lowest sensitive to Ampicillin 5(6.17%).

Three CoNS were isolated among 86 GPC. Only one isolate was Coagulase Negative *Staphylococcus aureus* but other 2 were Methicillin resistant Coagulase Negative *Staphylococcus aureus* and subjected towards 12 antibiotics and found highest sensitivity towards Ampicillin 3(100%) followed by Cotrimoxazole 3(100%), Erythromycin 3(100%), and Azithromycin 3(100%).

Single *Enterococcus* spp was found and was subjected AST pattern against 12 antibiotics. It was sensitive against Cotrimozazole, Gentamicin, Amikacin, Ofloxacin, Cloxacillin, Erythromycin, Linezolid and resistant towards others.

Only 2 *Streptococcus* spp were isolated from 86 GPC isolates and subjected against 12 antibiotics and it was found highest sensitive towards Amikacin 2(100%), Ofloxacin 2(100%), Teicoplanin 2(100%), Linezolid 2(100%) followed by Gentamycin 1(50%), Clindamycin 1(50%), Doxycycline 1(50%). (Table 6)

Table 6: Antibiotic susceptibility pattern of Gram-positive cocci

Isolates	*Staphylococcus aureus* (n=81)		Coagulase Negative *Staphylococcus aureus* (n=3)		*Enterococcus* spp. (n=1)		*Streptococcus* spp.(n=2)	
Antibiotics	**S n(%)**	**R n(%)**	**S n(%)**	**R n(%)**	**S n(%)**	**R n(%)**	**S n(%)**	**R n(%)**
Cotrimoxazole	29(35.80)	52(64.197)	3(100)	0(0)	0(0)	1(100)	2(100)	0(0)
Gentamicin	63(77.78)	18(22.22)	1(33.33)	2(66.67)	0(0)	1(100)	1(50)	1(50)
Amikacin	79(97.53)	2(2.47)	0(0)	3(100)	0(0)	1(100)	0(0)	2(100)
Ofloxacin	31(38.27)	50(61.73)	0(0)	3(100)	0(0)	1(100)	0(0)	2(100)
Cloxacillin	41(50.62)	40(49.38)	3(100)	0(0)	0(0)	1(100)	2(100)	0(0)
Erythromycin	12(14.81)	69(85.19)	3(100)	0(0)	0(0)	1(100)	2(100)	0(0)
Azithromycin	49(60.49)	32(39.51)	3(100)	0(0)	1(100)	0(0)	2(100)	0(0)
Clindamycin	47(58.02)	34(41.98)	2(66.67)	1(33.33)	1(100)	0(0)	1(50)	1(50)
Teicoplanin	75(92.59)	6(7.41)	0(0)	3(100)	1(100)	0(0)	0(100)	2(100)
Doxycyclin	68(83.95)	13(16.05)	1(33.33)	2(66.67)	1(100)	0(0)	1(50)	1(50)
Linezolid	73(90.12)	8(9.88)	0(0)	3(s100)	0(0)	1(100)	0(0)	2(100)
Ampicillin	5(6.17)	76(93.83)	3(100)	0(0)	1(100)	0(0)	2(100)	0(0)

Antibiotic susceptibility pattern of *S. aureus*, CoNS, *Enterococcus* spp and *Streptococcus* spp.

Among 119 isolates, 81 were *S. aureus*. Out of total *S. aureus* isolates Methicillin sensitive *Staphylococcus aureus* were 38(46.91%) and Methicillin Resistant *Staphylococcus aureus* were 43(53.09%). (Figure: 4)

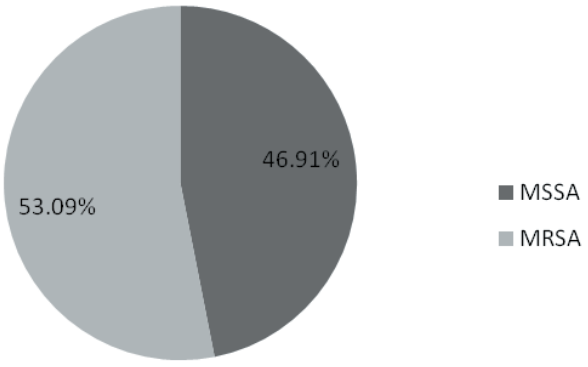

Figure 4: Distribution of MRSA among *S. aureus*

DISCUSSION

In this study the overall rate of bacterial Soft tissues infection among the study population was found to be 199 (52.36%). The result was in agreement with the study carried out by Sah et al. (2013) that reported 62% growth rate and close to the result reported by Acharya et al. (2008), accounting 50.7%. The predominance of male patients was seen in this study with male: female ratio of 65/35 and this finding was similar to the other studies where a much higher number of male patients have been reported Sharma et al. (2013) and Gurung et al. (2018). The patients with age >30 years had a much higher incidence of STIs (42.09%) in comparison to an incidence of 14.99% among the patients who were ≤29 years of age. Similarly, the study carried out by Murphy et al. (2001) also had a much higher incidence of STIs (89.41%) at age group >30 years. Advancing age is an important factor for the development of STIs, as in old age patients there is low healing rate, low immunity, increased catabolic processes and presence of co-morbid illness like diabetes, hypertension, etc. (Sharma et al. 2015).

In this study, the frequency of Gram-negative bacteria was higher than Gram positive bacteria. However, a similar study carried out by Surucuoglu et al. (2005) showed the higher prevalence of Gram-positive bacteria (69%) than Gram negative bacteria (29%). The higher prevalence of Gram-positive bacteria was also depicted in researches carried out by Kaftandzieva et al. (2012). Practically, *S. aureus* was the major pathogenic Gram positive organism and *E. coli* was the major pathogenic Gram negative organisms for STIs, as in the study carried out by Fazii et al. (2013), and Ranabhat et al. (2013) shows the most common bacterial species detected was *Staphylococcus aureus* (37.50%) and *E. coli* (25%). In the study carried out by Karkee (2008) reported similar results that the most common bacteria (46.58%) were *S. aureus, E. coli* (12.38%) emerged as the next common organism causing wound infection in this study as in the other previously reported studies which is followed by, CoNS (11.40%) and *P. aeruginosa* (7.49%). The least common bacteria isolated were *C. freundii* (0.65%). In Saudi Arabia, Abussaud (1996) isolated *S. aureus* (35%), *P. aeruginosa* (25%) and *Klebsiella* spp (10%) as the major causative agents.

However, different studies showed that *P. aeruginosa* was the leading cause of wound infections. In a study conducted by Mousa (1997) to assess the rate of wound infection by aerobic bacteria and found that 19.1% of the wound infection was caused by *P. aeruginosa*. Similar study on wound infection by Nasser et al. (2003) showed *P. aeruginosa* (21.6%) as the most common isolate which in compare to our result was similar as the rate of infection by *P. aeruginosa* was fond to be 15.07%.

In antibiotic susceptibility pattern of Gram negative organism, gentamycin was most sensitive (62.83%) followed by amikacin (47.78%), Levofloxacin (39.76%), PTZ (38.05%), Doxycycline (34.94%),Cotrimoxazole (32.53%), Ofloxacin (30.12%), Ciprofloxacin (28.32%), Piperacillin (22.12%), Ceftriaxone (18.07%), Cefotaxime (13.25%), Ampicillin (12.05%), Amoxyclav (12.04%) and Amoxycillin (4.81%). However, the study carried out by Timalsina et al. (2015) for Gram negative isolates, Amikacin (45, 93.75%) was found to be the most sensitive antibiotic followed by Gentamycin (42, 89.36%), Ciprofloxacin (27, 56.25%) while Amoxycillin (13, 32.5%) and Cotrimoxazole (14, 29.16%) being the least sensitive antibiotic respectively. In our study, among Gram positive isolates, the most effective antibiotic was Amikacin (91.86%) followed by Teicoplanin (88.37%), Linezolid (84.88%), Doxycycline (82.56%), Gentamycin (75.58%), Azithromycin (63.95%), Clindamycin (59.30%), Cloxacillin (53,49%), Cotrimoxazole (39.53%), Ofloxacin (36.04%), Erythromycin (19.77%) and Ampicillin (12.79%). However, Tuladhar (1999) reported that Gentamicin was found to be most effective (89.53%) drug followed by Ciprofloxacin (83.72%) while only 16.27% of Gram-positive cocci were sensitive to Ampicillin.

The patterns of MDR among Gram negative bacterial isolates were 100% in *Acinetobacter* spp, 83.33% in *Enterobacter* spp, 77.77% in *Klebsiella pneumoniae,* 64.86% in *Escherichia coli,* 50% in *Proteus mirabilis,* 50% in *Citrobacter freundii,* 50% in *Citrobacter koserii,* 10% in *Pseudomonas* spp and no any MDR isolates in *Klebsiella oxytoca* and *Serratia marcescens* which was in contrast to results shown by Bhandari (2014) that reported that higher number of *E. coli* isolates, 64 (72.7%) were multi drug resistant followed by *Pseudomonas aeruginosa* 11 (91.7%) and *K. pneumoniae* 6 (75%). Out of all GPC, 81(94.17%) were *S. aureus* in which 43(53.06%) were MRSA and 38(46.94%) were MSSA which was similar to the study performed by Khanal and Jha (2010) which showed 68% MRSA and 32% MSSA. The study performed by Edelsberg et al. (2009) also showed 35.9%

MRSA which is also contrast to our study. Though a great array of bacteria is involved in wound infections, we were able to trace limited pathogens due to lack of adequate laboratory facilities and time boundary.

CONCLUSION

The rate of wound infection is higher among the patients visiting the tertiary care hospital in Kathmandu. The antibiotic susceptibility pattern of the pathogens causing wound infections in the study population revealed higher rate of multidrug resistant, indicating the limited therapeutic alternatives for the management of wound infected patients.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Baddour ML (2019). Patient's education on skin and soft tissue infection (cellulitis). Available at https://www.uptodate.com/contents/skin-and-soft-tissue-infection-cellulitis-beyond-the-basics#!

Rosser WW, Pennie RA and Pila NJ (2005). The Anti-Infective Review Panel. Anti-Infective Guidelines for Community-Acquired Infections Toronto: MUMS Guideline Clearinghouse.

Woerther PL, Burdet C, Chachaty E and Andremont A (2013). Trends in human fecal carriage of extended-spectrum β-lactamases in the community toward the globalization of CTX-M: *Clin Microbiol Rev* **26**: 744–758.

Barrett PF, McGehee RF and Finland M (1968). Methicillin Resistant *Staphylococcus aureus* at Batten city Hospital: Bacteriological and epidemiological observations. *New Engl J Med* **279**: 441-448.

Ciccarone D, Bamberger J and Kral A (2001). Soft tissue infections among injection drug users- San Fransisco, California, 1996-2000. *J Amer Med Assoc* **285**: 2707-2709.

Hersh AL, Chambers HF, Maselli JH and Gonzales R (2008). National trends in Ambulatory visits and Antibiotic Prescribing for skin and soft-tissue infections: *Arch Intern Med* **168**: 1585–1591.

Edelsberg J, Taneja C, Zervos M, Haque N, Moore C, Reyes K, Spalding J, Jiang J and Oster G (2009). Trends in US hospital admissions for skin and soft tissue infections: *Emerg Infect Dis* **15**: 1516–1518.

WHO (2003). The World Health Report. Geneva, Switzerland.

Cheesbrough M (2006). District laboratory practice in tropical countries **2.**

CLSI (2014). Performance standards for antimicrobial susceptibility testing; twenty-second Informational supplement **33**. CLSI Document M100–S20. Wayne, PA.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT and Monnet DL (2012). Multidrug resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**: 268-281.

Sah P, Khanal R and Upadhaya S (2013). Skin and soft tissue infections: Bacteriological profile and antibiotic resistance pattern of isolates. *J Universal College Medical Science* **3**: 18-21.

Acharya J, Mishra SK, Kattel HP, Rijal B and Pokharel BM, (2008). Bacteriology of wound infections among patients attending Tribhuwan University Teaching Hospital, Kathmandu, Nepal: *Journal of Nepal Association for Medical Laboratory Sciences* **9**: 76-80.

Murphy EL, Vita DD, Liu H, Vittinghoff E, Leung P, Ciccarone DH and Edlin BR (2001). Risk Factors for Skin and Soft-Tissue Abscesses among Injection Drug Users: A Case-Control Study. *Clin Infect Dis* **33**: 35–40.

Kaftandzieva A, Cekovska Z, Kaftandzieva I, Panovski N and Petrovska M (2012). Bacteriology of wound-clinical utility of gram stain microscopy and the correlation with culture. *Macedonian*

Journal of Medical Sciences **5**: 72-77.

Bessa LJ, Fazii P, Giulio DM and Cellini L (2013). Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection: *The International Wound Journal* **12**: 47-52.

Surucuoglu S, Gazi H, Kurutepe S, Ozkutuk N and Ozbakkaloglu B (2005). Bacteriology of surgical wound infections in a tertiary care hospital in Turkey. *East Afr Med J* **82**: 331-336.

Karkee P (2008). Bacterial Isolates and their Antibiogram from Wounds and Abscesses of Surgical Outpatients Visiting Bir Hospital: M. Sc. Dissertation Submitted to the Department of Microbiology, Tribhuvan University: 13-29.

KC R, Shrestha A and Sharma V (2014). Bacteriological study of wound infection and antibiotic susceptibility pattern of the isolates. *Nepal Journal of Science and Technology* **14**: 143-150.

Abbussaud MJ (1996). Incidence of wound infection in three different departments and the antibiotic sensitivity pattern of the isolates in a Saudi Arabian hospital: *Acta Microbiol Immunol Hunga* **43**: 301-305.

Mousa HA (1997). Aerobic, anaerobic and fungal burn wound infections: *J Hosp Infect* **37**: 317-323.

Tuladhar P (1999). A prospective study on bacteriology of wound infection at TUTH: A dissertation presented to the Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal.

Khanal LK and Jha BK (2010). Prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) among skin infection cases at hospital in Chitwan, Nepal: *Nepal Med Coll J* **12**: 224-228.

Edelsberg J, Taneja C, Zervos M, Haque N, Moore C, Reyes K, Spalding J, Jiang J and Oster G (2009). Trends in US hospital admissions for skin and soft tissue infections: *Emerg Infect Dis* **15**: 1516–1518.

Comparison of Led Fluorescent Microscopy and the Gene Xpert MTB/ RIF Assay in Diagnosis of Pulmonary and Extrapulmonary Tuberculosis

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ABSTRACT

Objectives: The objective of this study was to evaluate Gene Xpert MTB/RIF Assay and anid fast staining (AFB) for rapid detection of *Mycobacterium tuberculosis* in specimen of patients suspected of pulmonary tuberculosis (PTB) and extra pulmonary tuberculosis (EPTB).

Methods: A comparative cross-sectional study of 400 samples (PTB-365 and EPTB-35) of patients visiting National Tuberculosis Centre (NTC) was conducted from July 2018 to December 2018. Gene Xpert MTB/ RIF Assay, smear microscopy were performed under standard guideline inside biosafety cabinet class II. The result obtained from both the tests were analyzed using SPSS 20.0 software and Excel 2019.

Results: Of the total samples, 18% (72/400) and 39% (156/400) were positive by AFB smear microscopy and Xpert MTB/RIF assay respectively. Prevalence of MTB positive was highest in the age group 35-44 years, 33cases (17.74%) were detected in total, with a male to female ratio of 2.3:1. Pleural fluid, pus, and CSF fluid also yielded positive results with the Gene Xpert MTB/RIF assay accounting 1.28%, 0.64% and 1.28% of MTB positive case respectively. Rifampicin resistance was observed in 1.28% of the cases.

Conclusion: The key findings of this study suggest that Gene Xpert test should be implemented as primary diagnostic test for PTB and EPTB.

Key words: Gene Xpert MTB/RIF Assay, *Mycobacterium tuberculosis*, Pulmonary tuberculosis.

INTRODUCTION

Tuberculosis (TB) is considered one of the most important infectious diseases through the course of human history which can affect nearly any organ in the body, but it mostly causes lung infections (Azadi et al. 2018). Tuberculosis is a communicable disease resulting from infection with *Mycobacterium tuberculosis* whose principal reservoir is man and also, but infrequently, with other mycobacterium belonging to the Mycobacterium tuberculosis complex (Ayieko 2015).

According to the Global TB Report 2017, tuberculosis mortality rate was 23 per 100,000 populations, which includes both HIV positive and HIV negative people. It causes ill-health in millions of people each year and in 2015 was one of the top 10 causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease. As per Global TB report 2017, 6000 to 7000 people were dying per year from TB disease in Nepal. NTP registered 31764 all forms of TB cases, which includes 30,957 incident TB cases (new and relapse) (NTP 2072/73 (2016). Most cases were reported in the productive age group (highest of 50 % in 15-44 year of age). The proportion of new cases with multidrug-resistant TB (MDR-TB) was 2.2% among new cases and 15.4% among retreatment cases based on DRS survey carried out in 2011/12 (NTP 2018). The majority of TB cases and deaths occur among men, the burden of disease among women seems significantly lower (2:1) (WHO 2015).

The standard WHO recommendation for TB diagnosis in the DOTS program is the use of direct sputum

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microscopy on 3 stained sputum specimens Sputum microscopy, the most widely available test for active TB (Matee et al. 2008). Although smear microscopy for acid-fast bacilli (AFB) is rapid and inexpensive, it has poor sensitivity and a poor positive predictive value (PPV). In HIV infected patients with pulmonary TB, 24–61% have acid-fast negative sputum smear (Pinyopornpanish et al. 2015). Mycobacterial culture is the gold standard and the most sensitive method for TB diagnosis; however, the use in clinical practice is limited due to a slow turnaround time (2 to 8 weeks), biosafety requirements, and high cost. Several studies have demonstrated that Xpert assay is highly sensitive and specific in diagnosis of both pulmonary and extrapulmonary TB (Pinyopornpanish et al. 2015). Thus, rapid identification, which is essential for earlier treatment initiation, improved patient outcomes, and more effective public health interventions, relies on nucleic acid amplification techniques. The GeneXpert MTB/RIF assay is a novel integrated diagnostic device that performs sample processing and heminested real-time PCR analysis in a single hands-free step for the diagnosis of tuberculosis and rapid detection of RIF resistance in clinical specimens (Zeka et al. 2011). The assay has been endorsed by the World Health Organization (WHO) since 2010 and its 2015 policy statement recommends that the Xpert®MTB/RIF should be available to all who need it and prioritized for persons at risk of multidrug-resistant TB (MDR-TB) and HIV- associated TB. Very importantly, in the same policy statement WHO emphasizes that DST for anti-TB medicines other than rifampicin should also be offered (Zaragoza and Laborin 2017). The MTB/RIF assay detects *M. tuberculosis* and RIF-resistance by PCR amplification of the 81-bp fragment of the *M. tuberculosis* rpoB gene and subsequent probing of this region for mutations that are associated with RIF-resistance. The assay can generally be completed in less than 2 hr (Zeka et al. 2011). It is technically simple to conduct and is safe as it produces no culturable aerosols (Bajrami et al. 2018).

The currently recommended treatment for new cases of drug-susceptible TB is a six-month regimen (fixed dose combination) of four first-line drugs: isoniazid, rifampicin, Ethambutol and pyrazinamide. Cured rates was 84% and Treatment Success Rates was 91% for new cases reported in 2016/17. The lack of availability and access to an early screening of presumptive TB cases with rapid DST may still be the main reasons for this stagnation of DR-TB cases (NTP 2018). Therefore, this study has significant importance in rapid diagnosis of tuberculosis of patients suspected of Pulmonary and Extra Pulmonary tuberculosis. Additional to diagnosis, this study has importance in direct detection of RIF-resistance which therefore, can provide the basis for prompt treatment of DR tuberculosis. Therefore, this study about rapid and effective diagnosis of pulmonary tuberculosis may evolve insight on use of Gene Xpert MTB/RIF assay throughout the country in achieving the target of treating all the infected patients of tuberculosis and therefore might helps in curbing rapturous distribution of tuberculosis.

MATERIALS AND METHODS

This hospital based cross-sectional study was carried out in National Tuberculosis center, Thimi, Bhaktapur, in collaboration with Department of Microbiology, Tri-Chandra Multiple Campus, Ghantaghar, Kathmandu, Nepal from July, 2018 to December 2018. A total of 400 different specimens from patients suspected of tuberculosis (pulmonary and extra pulmonary) were taken. The inclusion criteria were male or female patients with age ≥ 15 yrs, clinically suspected patients with characteristics symptoms and with or without abnormal chest radiography. While inappropriately labeled and collected specimens (sputum containing saliva only, food particles or other solid particulates) and Patients with previous history of receiving antituberculous drug within 3 months before enrollment were excluded.

For this study, they were requested for two consecutive sputum from 357 PTB suspects i.e. first day for Gene Xpert MTB/RIF Assay, considered as sample II and second day for LED fluorescent microscopy, considered as sample I (spot sample). Then the same specimen-II was processed for LED fluorescent microscopy and for Xpert MTB/RIF test. While a single specimen each from 35-EPTB suspects and 8-BAL (PTB suspects) were collected as per the collection and transportation policy of the laboratory and proceeded for AFB microscopy. Patients were instructed on the difference between sputum, saliva or nasopharyngeal secretions and the necessity for a deep, productive cough. And EPTB suspected body specimens (Pus, CSF fluid, Ascitic fluid, Pleural fluid and BAL) were brought by the patients in a container supplied by any other hospitals where they took service at.

Direct smear microscopy was performed to investigate presence of AFB with the sample-II using Fluorochrome staining technique. And the AFB results were reported using the criteria of WHO/International Union of Tuberculosis and Lung Diseases (IUTALD).

Gene Xpert testing was performed according to the manufacturer's instructions. Sample reagent was added to untreated specimen at a ratio of 2:1, vortexed thoroughly until clear solution was seen and it was left incubated at room temperature for 15 min on upright position. Between 5 and 10 minutes of incubation, the specimen was shaken vigorously again 10-20 times. The tube was again incubated at RT for another 5 min. 2ml of the liquefied and liquefied material was transferred to the test cartridge and inserted into the platform. The result interpretation was made by the GeneXpert DX System from measured fluorescent signals and embedded calculation algorithms and was displayed in the "View Results" window of the GeneXpert machine. The test was repeated using a new cartridge or initiated alternate procedures if test results exhibit error and invalid.

The data were collected, structured and analysis was done using SPSS version 20.0 System. Statistical analysis (i.e. Chi-Square) was employed on determining the association between fluorescent microscopy and Gene Xpert MTB/RIF Assay at 95% confidence interval. A p-value less than 0.05 was considered statistically significant.

RESULTS

A total of 400 specimens from TB suspected subjects were recruited in the study of which 283(70.75%) were male and 117(29.25%) were female with a male to female ratio of 2.42:1. Most of the subjects 98 (28.50%) were in the age 65 yrs and above (Table 1).

Table 1: Gender and age-wise distribution of patients

Characteristics		N = 400	%
Gender	Female	117	29.25
	Male	283	70.75
Age range (years)	15-24	34	8.50
	25-34	40	10
	35-44	71	17.75
	45-54	73	18.25
	55-64	84	21
	65+	98	24.50

Highest proportion was covered by pulmonary specimen (365) consists of 357(89.25%) sputum and 8(2%) BAL. 35 specimens were categorized as extrapulmonary that includes Pus-9, CSF-8, Pleural fluid-12 and Ascitic fluid-6. A total of 400 (365 pulmonary and 35 extra pulmonary) specimen were stained using LED Fluorescent staining technique. Of them 72 (18%) were AFB positive and 328 (82%) were AFB negative. Gene-Xpert positivity for MTB remained 156(39%) (Table 2).

Table 2: Result of specimen on AFB smear and Gene-Xpert MTB/RIF assay

Characteristics		N=400	%
Specimen	Sputum	357	89.25
	BAL	8	2
	Pus	9	2.25
	CSF	8	2
	Pleural fluid	12	3
	Ascitic fluid	6	1.5
AFB smear	Scanty	1	0.25
	1+	8	2
	2+	28	7
	3+	35	8.75
	N	328	82

Characteristics		N=400	%
Gene-Xpert assay	VL	25	6.25
	L	37	9.25
	M	68	17
	H	26	6.5
	N	244	61

Note: VL: Very low, L: Low, M: Medium. H: High and N: Negative

Eighty-four AFB negative samples gave MTB detected Gene-Xpert MTB/RIF assay result and one MTB not-detected case gave AFB positive result (Table 3).

Table 3: Distribution and findings on AFB smear and Gene-Xpert assay

AFB smear result	Gen-Xpert test					Total
	VL	L	M	H	N	
Scanty	-	1	-	-	-	1
1+	-	1	6	-	1	8
2+	-	2	5	21	-	28
3+		-	16	19	-	35
Negative	41	32	9	3	243	328
Total	**25**	**37**	**68**	**26**	**244**	**400**

Note: VL: Very low, L: Low, M: Medium. H: High and N: Negative

104 males and 45 females (149; 95.51 %) were sensitive and 2 males counting 100% were resistant to rifampicin among 156 MTB detected cases by Gene Xpert MTB/RIF assay (Table 4).

Table 4: Gene Xpert MTB/RIF assay rifampicin sensitivity results

Rifampicin sensitivity	Gender		Total
	Male	Female	
Sensitive	104(69.8%)	45(30.2%)	149
Resistance	2(100%)	-	2
Indeterminate	4(80%)	1(20%)	5
Total	**110**	**46**	**156**

DISCUSSION

Although AFB smear positive patients are considered highly infectious and being focused by most of clinicians, smear negative patients are also reported to responsible for approximately 17% of transmission and its impact on public health could not be neglected (Behr et al. 1999). Early diagnosis of TB is necessary to disrupt the disease transmission chain.

In this study, smear positivity was found to be 72(18%) and Gene-Xpert positivity for MTB remained 156 (39%), are not in agreement with a study, which showed higher smear positivity of 67.5% by Auramine fluorochrome staining and MTB positivity of 77.4% by Gene-Xpert (Munir et al. 2015). This variation may be explained by differences in physiological and medical conditions of the subjects, or inclusion of highly suspicious subjects.

On age wise distribution, 33(21.15%) out of 71 (male positive; 14.74% & female positive; 6.41%) being highest number of patients in age group 35-44 were diagnosed MTB positive respectively. The result suggests that TB infection case is most likely common among the economically active group that directly impacts to the family and the national economy (Bhatt 2009).

In this study, among 156 Gene Xpert Positive isolates, RIF resistance was detected in 2 male cases (1.28%), RIF sensitive in 104 male cases (66.67%) and 45 (28.85%) female cases. A wide range of 0-6.1% resistance for MTB positivity by Gene Xpert has been reported in earlier studies (Green et al. 2010 (6.1%); Khunjeli et al. 2014 (4.8%); Atashi et al. 2017 (3.1%); Pradhan et al. 2014 (4.2%). Low frequency of RIF-resistance TB in our study may be due to the exclusion criteria i.e excluding

retreatment cases or failure cases in our study design. The reasons for the marked resistance rate might be due to delay in treatment and unavailability of drugs among the subjects under study.

Sixty-nine (19.33%) spot sputum samples (sample I) and 72 (20.17%) morning samples (sample II) were smear positive (LED FM). In high-burden settings, the elimination of the third specimen and the resultant reduction in workload may actually improve case detection by improving the quality of examination of the first two specimens (Islam 2013).

Ironically, areas of high prevalence such as Nepal, the majority of suspected TB cases are assessed by sputum smear microscopy and, where available, by tuberculin test, ESR, ADA, CXR. Patients are often placed to pragmatic empirical treatment practices on the basis of symptomatic analysis or abnormal CXR alone (Shrestha et al. 2015). In these perspectives, Gene Xpert excludes "false cases" to "true" smear-negative TB cases, with enhanced accuracy of treatment, cost-effectiveness reducing the burden of toxicity and opportunity cost of treatment in patients suspected TB (Shrestha et al. 2015; Steingart et al. 2006; Dowdy et al. 2011).

Positive predictive value and Negative predictive value of Gene Xpert MTB/ RIF Assay with reference to culture in the diagnosis of PTB was 76.74%, 95.79%, 89.19% and 90.09% respectively (Thapa et al. 2016). With regard to this study, use of Gene Xpert MTB/ RIF assay could significantly reduce false negative AFB staining results and the delay in treatment initiation, reducing premature death and transmission. Rapid detection of RIF resistance is considered crucial for the control of MDR-TB.

CONCLUSION

This study reveals, an appliance of Xpert MTB/RIF assay as a diagnostic tool improved the additional case detection of smear negative Pulmonary and Extra pulmonary tuberculosis that often missed with smear microscopy. Therefore, implementation of molecular approaches for direct diagnosis of MDR TB, as a part of routine analysis in the laboratories of health care institutions, would be of great benefit in adapting treatment regimens, limiting dissemination of MDR TB strains.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Atashi S, Izadi B, Jalilian S, Madani SH, Farahani A and Mohajeri P (2017). Evaluation of GeneXpert MTB/RIF for determination of rifampicin resistance among new tuberculosis cases in west and northwest Iran. *New Microbes and New Infections* **19**: 117-20.

Ayieko YS (2015). Epidemiology of tuberculosis and human immunodeficiency virus co-infection, clinical presentations and impact on immunohaematological parameters in Mombasa county, Kenya (Doctoral dissertation, Kenyatta).

Azadi D, Motallebirad T, Ghaffari K and Shojaei H (2018). Mycobacteriosis and Tuberculosis: Laboratory Diagnosis. *The Open Microbiology Journal* **12**: 41.

Bajrami R, Mulliqi G, Kurti A, Lila G and Raka L (2018). Assessment of diagnostic accuracy of GeneXpert Mycobacterium tuberculosis/rifampicin in diagnosis of pulmonary tuberculosis in Kosovo. *Biomedical and Biotechnology Research Journal (BBRJ)* **2**(3): 191.

Bhatt CP, Bhatt AB and Shrestha B (2009). Nepalese People's knowledge about Tuberculosis. *SAARC Journal of Tuberculosis, Lung Diseases and HIV/ AIDS* **6**(2):31-7.

Dowdy DW, Cattamanchi A, Steingart KR and Pai M (2011). Is Scale-Up Worth It? Challenges in Economic Analysis of Diagnostic Tests for Tuberculosis. *PLoS Med* **8**(7): e1001063.

Green E, Obi CL, Nchabeleng M, De Villiers BE, Sein PP, Letsoalo T, Hoosen AA, Bessong PO and Ndip RN (2010). Drug-susceptibility patterns of Mycobacterium tuberculosis in Mpumalanga province, South Africa: possible guiding design of retreatment regimen. *Journal of Health, Population, and Nutrition* **28**(1): 7.

Islam MR, Khatun R, Uddin MK, Khan MS, Rahman MT, Ahmed T and Banu S (2013). Yield of two consecutive sputum specimens for the effective

diagnosis of pulmonary tuberculosis. *PLoS One* **8**(7): e67678.

Khunjeli R, Mohsin UR, Shrestha SK, Adhikari S, Srivastava B and Shrestha B (2014). Prevalence of Primary Drug Resistant Tuberculosis in a Tertiary Care Hospital, Nepal. *J Chitwan Med Coll* **4**(10): 36–38.

Matee M, Mtei L, Lounasvaara T, Wieland-Alter W, Waddell R, Lyimo J, Bakari M, Pallangyo K and Von Reyn CF (2008). Sputum microscopy for the diagnosis of HIV-associated pulmonary tuberculosis in Tanzania. *BMC Public Health* **8**(1): 68.

Munir MK, Rehman S, Aasim M, Iqbal R and Saeed S (2015). Comparison of Ziehl Neelsen microscopy with GeneXpert for detection of Mycobacterium tuberculosis. *IOSR Journal of Dental and Medical Sciences* **14**(11): 56-60.

NTP (2072/73 (2016)). Annual report. Government of Nepal Ministry of Health and Population, Department of Health services, Thimi, Bhaktapur.

NTP Nepal. (2018). NTP Nepal Annual Report. Ministry of Health & Population, Nepal, Department of Health Services. National Tuberculosis Center.

Pinyopornpanish K, Chaiwarith R, Pantip C, Keawvichit R, Wongworapat K, Khamnoi P, Supparatpinyo K and Sirisanthana T (2015). Comparison of Xpert MTB/RIF assay and the conventional sputum microscopy in detecting *Mycobacterium tuberculosis* in Northern Thailand. *Tuberc Res Treat* **2015**: 571782.

Pradhan P, Poudyal N, Gurung R, Acharya A and Bhattacharya SK (2014). Drug resistance pattern of Mycobacteria isolated from smear positive cases of pulmonary tuberculosis in eastern part of Nepal. *JGPEMN* **3**(4): 7-12.

Shrestha P, Arjyal A, Caws M, Prajapati KG, Karkey A, Dongol S, Pathak S, Prajapati S and Basnyat B (2015). The application of GeneXpert MTB/ RIF for smear-negative TB diagnosis as a fee paying service at a South Asian General Hospital. *Tuberc Res Treat* **2015**:102430.

Thapa G, Pant ND, Khatiwada S 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. *Antimicrobial Resistance & Infection Control* **5**(1): 30.

World Health Organization (2018). Global tuberculosis report 2018. Geneva, Switzerland: World Health Organization ISBN 978-92-4-156564-6

Zaragoza B and Laniado-Laborín R (2017). Diagnosing Drug-Resistant Tuberculosis with the Xpert® MTB/RIF. The Risk for Rifampin Susceptible Cases. *Journal of Tuberculosis Research* **5**(03): 155.

Zeka AN, Tasbakan S and Cavusoglu C (2011). Evaluation of the GeneXpert MTB/RIF assay for rapid diagnosis of tuberculosis and detection of rifampin resistance in pulmonary and extrapulmonary specimens. *Journal of Clinical Microbiology* **49**(12): 4138-4141.