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

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

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

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

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

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

(f) CONCLUSION:

(g) ACKNOWLEDGEMENTS:

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

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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.

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Tables must be prepared using the same word

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

Figures

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

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

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

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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.

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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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EDITORIAL

Antimicrobial Resistance in Nepal

Antimicrobials are the most effective treatment for infectious diseases. Antimicrobial resistance (AMR) has increased and spread due to overuse and misuse of antimicrobials. Antimicrobial resistance especially multidrug resistance threatens the effective prevention and treatment of infections resulting in prolonged illness and increased mortality.

Studies revealed that major antimicrobial resistant pathogens in Nepal included *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus* spp., *Salmonella* spp., *Shigella* spp., *Mycobacterium tuberculosis* bacteria and *Plasmodium falciparum, Leishmania donovani* parasites.

In 2011, the South East Asia Region's health ministers adopted the Jaipur Declaration on Antimicrobial Resistance, which states that combating antimicrobial resistance must be a priority for national governments. The countries are introducing legislation and policies to govern the use of antimicrobial medicines; establishing laboratory-based networks for surveillance of antimicrobial resistance; ensuring rational use of antimicrobial medicines in all health care settings; and promoting community awareness about antimicrobial resistance.

Antimicrobial resistance surveillance was started in 1998 in Nepal with 9 laboratories and gradually included other laboratories selected on convenient criteria: size and place of the hospitals in the health system (regional, zonal, district hospitals), geographical access and coverage. Nepal has developed the National Antimicrobial Resistance Containment Action Plan, in continuation to its ongoing laboratory based AMR surveillance including 10 priority bacterial pathogens recorded and reported through existing 22 AMR sentinel sites.

One health approach involving coordination among numerous international sectors and actors, including human and veterinary medicine, agriculture, finance, environment, and well informed consumers is needed to prevent further emergence and spread of antimicrobial resistance. For preventing AMR, biosafety and biosecurity policies should be in place and AMR surveillance capacities should be strengthened and expanded in human and animal health sectors.

Dr. Megha Raj Banjara
Editor in chief
Tribhuvan University Journal of Microbiology (TUJM)

Extended Spectrum β-Lactamase (ESBL) Producing Multidrug Resistant Gram-Negative Bacteria from Various Clinical Specimens of Patients Visiting a Tertiary Care Hospital

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ABSTRACT

Objectives: The purpose of this study was to assess multidrug resistance and Extended Spectrum β-Lactamase (ESBL) production in Gram negative bacterial pathogens.

Methods: The study included clinical specimens sent for routine culture and antibiotic susceptibility testing. A total of 469 different clinical specimens were processed according to the standard methodology. The isolates were identified by standard microbiological procedures and subjected to antimicrobial susceptibility testing by modified Kirby-Bauer disk diffusion method. Production of ESBL was determined by combined disk method.

Results: Of the total sample processed, 80 (17.0%) Gram negative bacteria were isolated and 82.5% of them were multidrug resistant (MDR). From the total MDR isolates, 47% were ESBL positive. The higher rate of growth among Intensive Care Units (ICUs) patients was found statistically significant. Higher prevalence of MDR isolates was observed in blood and pus specimens. The majority of the ESBL producers were *Escherichia coli* (38.7%). Higher rate of ESBL producers was detected from blood (55.6%). Polymyxin B, imipenem and amikacin were the most effective antibiotics against *Acinetobacter* spp. and *Pseudomonas aeruginosa* whereas imipenem, amikacin, meropenem were the most effective antibiotics against Enterobacteriaceae.

Conclusion: Higher prevalence of ESBL producing MDR Gram negative pathogens in hospitalized patients indicates these bacteria are important health care associated pathogens and requires proper infection control measures that check the transfer of MDR and β-lactamase producing bacterial pathogens among the hospitalized patients.

Key words: Gram negative bacteria, Antibiotic susceptibility testing, MDR, ESBL, Combined disk assay

INTRODUCTION

Multidrug resistant bacterial infections are spreading worldwide where Extended Spectrum β-Lactamases (ESBLs) are the major MDR (multidrug resistant) related bacterial enzymes in addition to metallo β-lactamases, carbapenemases and AmpC β-lactamases (Chakraborty et al. 2011). The increasing ability to make altered receptors for antimicrobial agents, enzymes to destroy antibiotics and resistant metabolic pathways have significantly increased drug resistances in Gram negative pathogens (Okonko et al. 2009).

Antimicrobial resistance (AMR) is defined as resistance of a microorganism to an antimicrobial medicine to which it was originally sensitive. AMR is a natural phenomenon, which is amplified by continuous and unnecessary exposure to antimicrobials (WHO 2014). According to European Center for Disease Prevention and Control (ECDC), for the Gram negative bacteria such as *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*, multidrug

resistance is defined as non-susceptible to at least one agent in at least three different antimicrobial categories. The antimicrobial categories are exclusive for the different organisms (Magiorakos et al. 2012).

ESBLs represent a major group of β-lactamases which have the ability to hydrolyze and cause resistant to various type of newer β-lactam antibiotics including the extended-spectrum (or third-generation) cephalosporins and monobactams (aztreonam) but not the cephamycins (cefoxitin and cefotetan) and carbapenems. They are also inhibited by clavulanate, sulbactam and tazobactam alone or in combination with β-lactams called β-lactam/β-lactamase inhibitors (Vinodhini et al. 2014). In addition to *E. coli* and *Klebsiella*spp, the production of ESBL has become more common in enteric bacilli e. g. *Enterobacter aerogenes, E. cloacae, Serratia marcescens, Morganella morganii, Providentia* spp., *Citrobacter freundii and C. koseri*as well as in non-enteric bacilli like *P. aeruginosa*. The ESBLs have also been reported in *Acinetobacter* spp., *Burkholderia cepacia* and *Alcaligenes fecalis* (Stürenburg and Mack 2003; Al-Jasser 2006).

As developed by CLSI, the phenotypic method of ESBL detection involves two steps. The first is a screening test with an indicator cephalosporin which looks for resistance or diminished susceptibility, thus identifying isolates likely to be harboring ESBLs. The second one tests forsynergy between oxyimino cephalosporin and clavulanate, distinguishing isolates with ESBLs from those that are resistant for other reasons (Paterson and Bonomo 2005).

The emergence of drug resistant organisms in both hospitals and community is a major concern. Surveillance studies have provided important information about changes in the spectrum of microbial pathogens and trends in the antimicrobial resistance patterns in nosocomial and community acquired infections and continued monitoring. of antimicrobial resistance patterns in hospitals is essential to guide effective empirical therapy. As the incidence of antimicrobial resistance rises, the costs associated with consequences also rises and hence can be considered an economic burden to society of developing country like Nepal. Antibiotic susceptibility profile and reporting of drug resistant strain especially ESBL producing strains would enlighten the appropriate antibiotic therapy and would help in awareness towards misuse and overuse of antibiotics (Paterson and Bonomo 2005). Thus this study was performed to screen and confirm ESBL producing organism.

MATERIALS AND METHODS

A prospective study was carried out in Shahid Gangalal National Heart Centre, Bansbari, Kathmandu at the Department of Pathology from 19 November 2014 to 18 May 2015. All the clinical specimens received in microbiology laboratory for routine culture and antibiotic susceptibility testing from all the units of hospital was included in this study.

Culture of different clinical specimens was performed using standard microbiological procedures (Forbes et al. 2007). Isolated colonies from the pure culture were identified by performing Gram staining and the standard conventional biochemical tests. Susceptibility tests of the different clinical isolates towards various antibiotics were performed by modified Kirby-Bauer disk diffusion method for the commonly isolated pathogens using Mueller Hinton Agar (MHA). The isolates resistant to at least one antibiotic in at least three different antimicrobial categories were considered as MDR. MDR isolates in pure culture were preserved in 20% glycerol containing trypticsoy broth and kept at -4^{o}C until subsequent tests for the presence of ESBL was performed.

The MDR isolates were screened for possible ESBL production using ceftazidime (30µg), cefotaxime (30µg) (CLSI 2014). The screen positive isolates, i.e. showing ceftazidime <22 mm, cefotaxime <27 mm zone of inhibition, were subjected to Combined Disk (CD) test using cefotaxime (30µg) andcefotaxime (30µg) plus clavulanate (10µg) for confirmation of ESBL production. An increase in zone of diameter of ≥5mm in the presence of clavulanate was concluded as confirmed ESBL producer.

The data obtained were analyzed using Statistical Package for Social Sciences (IBM SPSS) software (Version 21.0). Chi-square (χ^2) test was performed to test the significance of distribution of Gram negative bacteria in OPD, wards and ICU. The p value less than 0.05 was considered to be significant.

RESULTS

A total of 469 clinical specimens, of which 70 (14.9%) specimens from OPD, 186 (39.7%) specimens from various wards and 213 (45.4%) specimens from ICUs (intensive care units) were included in this study.

Among the 469 clinical specimens analyzed, 159 (33.9%) were urine, 121 (25.8%) were blood, 59 (12.6%) were Endotracheal tube (ET) tip and secretion, 55 (11.7%) were sputum, 33 (7%) were body fluids, 14 (3%) were Central venous pressure (CVP) tip, 12 (2.6%) were tissue, 11 (2.3%) were pus and 5 (1.1%) were suction tip specimens (Table 1).

Table 1: Distribution of total clinical specimens under investigation

Types of specimens	No. of specimens received	Percentage
Urine	159	33.9
Blood	121	25.8
ET tip and secretion	59	12.6
Sputum	55	11.7
Body fluids	33	7
CVP tip	14	3
Tissue	12	2.6
Pus	11	2.3
Suction tip	5	1.1
Total	469	100

A total of 80 (17.0%) isolates of eight different type of Gram negative bacteria were isolated from a total of 469 clinical specimens processed, of which 52 isolates belong to family Enterobacteriaceae, 15 isolates were *Acinetobacter* spp. and 13 isolates were *P. aeruginosa*. Among the total isolates of Gram negative bacteria *E. coli* was the predominant isolate with 35% followed by *K. pneumoniae* (22.5%), *Acinetobacter* spp. (18.8%), *P. aeruginosa* (16.3%), *Klebsiella oxytoca* (2.5%), *Citrobacter freundii* (2.5%) and 1.2% each of *Proteus mirabilis* and *Morganella morganii* (Figure1).

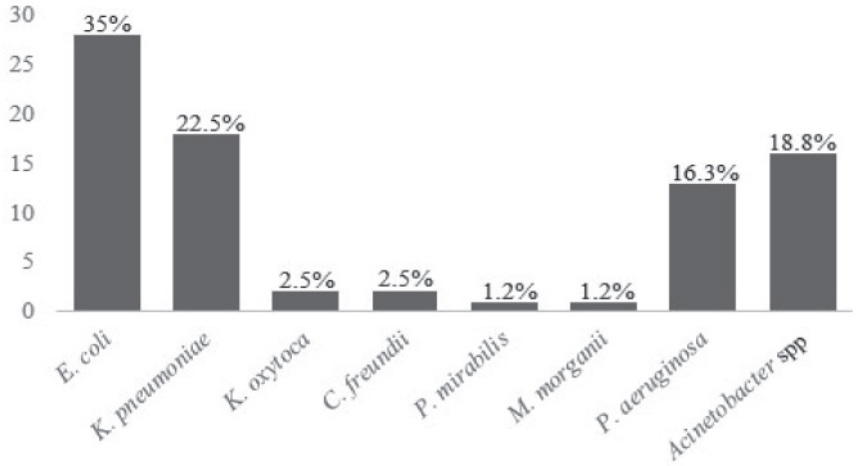

Figure 1: Frequency of different bacteria isolated

Of the total 80 Gram negative bacterial isolates, 52 (65%) from ICUs, 14 (17.5%) from wards and 14 (17.5%) from OPD were isolated. From the χ^2 test, it was found that the Gram negative bacteria isolated from OPD, wards and ICUs was statistically significant ($p<0.05$) (Table 2).

All the isolated strains of Gram negative bacteria from different clinical specimens were tested with specific antibiotics by using modified Kirby-Bauer disk diffusion method. According to CLSI 2014, 3 different sets of antibiotics were used to determine antibiotic susceptibility pattern of *P. aeruginosa*, *Acinetobacter* spp. and member of Enterobacteriaceae family. Amikacin and polymyxin B were the most effective drugs with 100% sensitivity against all the isolates of *P. aeruginosa* followed by imipenem (84.6%), piperacillin-tazobactam (76.9%) and piperacillin (61.5%) while ceftazidime,

Table 2: Distribution of Gram negative bacteriain OPD, wards and ICUs

Organisms	No. of isolates (%)			Total (%)	P-value
	OPD	Wards	ICUs		
E. coli	11(39.3)	7(25)	10(35.7)	28	<0.05
K. pneumonia	2(11.1)	4(22.2)	12(66.7)	18	
K. oxytoca	0(0)	0(0)	2(100)	2	
C. freundii	0(0)	1(50)	1(50)	2	
P. mirabilis	0(0)	1(100)	0(0)	1	
M. morgani	1(100)	0(0)	0(0)	1	
P. aeruginosa	0(0)	1(7.7)	12(92.3)	13	
Acinetobacter spp.	0(0)	0(0)	15(100)	15	
Total	**14(17.5)**	**14(17.5)**	**53(65)**	**80**	

cefotaxime, aztreonam, meropenem, gentamicin and ciprofloxacin were least effective antibiotics. Similarly, *Acinetobacter* spp. showed 100% sensitivity towards polymyxin B while among other antibiotics imipenem andamikacin were found effective against them and 100% isolates of *Acinetobacter* spp. were resistant to tetracycline. Imipenem with 100% susceptibility followed by amikacin, meropenem, gentamicin, chloramphenicol and piperacillin-tazobactam were most effective antibiotics while ampicillin was least effective antibiotic against members of Enterobacteriaceae. Nitrofurantoin was found 80% effective towards urinary isolates of Enterobacteriaceae.

Of the total 80 isolates of Gram negative bacteria, 66 (82.5%) isolates were MDR. Though *E. coli* was predominant bacteria among total MDR isolates, the highest percentage of MDR strains among each bacterial isolates were *K. oxytoca, P. mirabilis and C. freundii* with 100% multidrug resistance each followed by *K. pneumoniae* (94.4%) and *Acinetobacter* spp. (93.3%). A single isolate of *M. morganii* was non MDR. Of the total 66 MDR isolates, 58 (87.9%) MDR isolates were suspected of being producer of ESBL. Of the total 58 screen positive isolates for ESBL production, 31 (53.4%) isolates were found to be ESBL producer. The prevalence of ESBL producer among total isolates was 38.8% (31/80) whereas the prevalence of ESBL producers among MDR isolates was 47%. Both the isolates of *K. oxytoca* were ESBL producer while a single isolate of MDR *P. mirabilis* was non ESBL producer. Among the total ESBL positive isolates *E. coli* was the most predominant isolate with 38.7% (12/31) followed by *K. pneumoniae* 25.8% (8/31), *P. aeruginosa* 16.1% (5/31), *Acinetobacter* spp. 9.7% (3/31), *K. oxytoca* 6.5% (2/31) and *C. freundii* 3.2% (1/31) (Table 3).

High prevalence of MDR isolates was observed in blood and pus with 100% multidrug resistance which were followed by ET tip and secretion (95.7%), sputum

Table 3: Multidrug resistance and ESBL production profile of Gram negative isolates

Organisms isolated	Total isolates	No. of MDR strain (%)	No. of suspected ESBL producer	Confirmed cases of ESBL		
				No.	% among total isolates	% among MDR strains
E. coli	28	21 (75)	15	12	42.9	57.1
K. pneumonia	18	17 (94.4)	15	8	44.4	47.1
K. oxytoca	2	2 (100)	2	2	100	100
C. freundii	2	2 (100)	2	1	50	50
P. mirabilis	1	1 (100)	1	0	0	0
M. morganii	1	0 (0)	0	0	0	0
P. aeruginosa	13	9 (69.2)	9	5	38.5	55.6
Acinetobacter spp.	15	14 (93.3)	14	3	20	21.4
Total	**80(100)**	**66 (82.5)**	**58**	**31**	**38.8**	**47**

(76.9%), urine (74.2%) and suction tip (50%). A single isolate from CVP tip was non MDR. High prevalence of ESBL producer was observed in blood (55.6%) followed by suction tip (50%), sputum (46.2%), ET tip and secretion (39.1%) and urine (32.3%). No ESBL producer was detected in CVP tip and pus specimens (Table 4).

Table 4: MDR Gram negative isolates in different clinical specimens and their ESBL production profile.

Specimens	Total isolates	No. of MDR strains (%)	No. of ESBL positive isolates (%)
Urine	31	23 (74.2)	10 (32.3)
Blood	9	9 (100)	5 (55.6)
ET tip and secretion	23	22 (95.7)	9 (39.1)
Sputum	13	10 (76.9)	6 (46.2)
CVP tip	1	0 (0)	0 (0)
Pus	1	1 (100)	0 (0)
Suction tip	2	1 (50)	1 (50)
Total	**80**	**66 (82.5)**	**31 (38.8)**

DISCUSSION

The emergence of Gram negative bacterial species with acquired resistance to various broad spectrum β-lactams and other classes of antimicrobials is becoming a worldwide clinical problem. Furthermore, bacteria responsible for causing nosocomial infections are MDR strains, complicating the treatment process (Guthrie 2001).

The prevalence of Gram negative bacteria in various clinical specimens was found to be 17.0% while that of multidrug resistance was 82.5%. Similar study conducted in National Kidney Center by Panta (2013) showed 19.92% growth and 85.83% of them were MDR. However in a study by Upadhyaya (2015) high growth positivity of 27.45% was observed but the multidrug resistance among the isolates was 77.55%. Another study conducted by Poudyal (2010) showed 19.61% growth and 61.27% MDR among isolates.

Of the total 80 Gram negative isolates, *E. coli* (35%) was the predominant pathogen followed by *K. pneumoniae* (22.5%). Similar results was observed by Bomjan (2005), Maharjan (2010) and Upadhyaya (2015). In comparison of 8.1% of *Acinetobacter* spp. isolated by Upadhyaya (2015), 18.8% of *Acinetobacter* spp. was isolated in our study. But higher prevalence of *P. aeruginosa* (29.5%) was found in Upadhyaya (2015) in comparison to our study (16.3%).

The highest percentage of MDR strains among each bacterial isolates were *K. oxytoca*, *P. mirabilis* and *C. freundii* with 100% multidrug resistance each followed by *K. pnuemoniae* (94.4%), *Acinetobacter* spp. (93.3%), *E. coli* (75%) and *P. aeruginosa* (69.2%). These results resembled with the outcomes of previous studies by Poudyal (2010), Mishra et al. (2012), Thakur (2012), Koirala (2014) and Upadhyaya (2015).

High drug resistance in Enterobacteriaceae is attributed to mutations in chromosomal genes, ability to share genetic material and mobile resistant genes. The mobile genetic elements are responsible for capturing resistant genes from the chromosomes of a variety of bacterial species and moving them between DNA molecules horizontally and vertically (Partridge 2015).

The high level of drug resistance seen among *E. coli* is mediated by β-lactamases, which hydrolyze the β-lactam ring inactivating the antibiotic. The classical TEM-1, TEM-2, and SHV-1 enzymes are the predominant plasmid-mediated β-lactamases of Gram negative rods (Livermore 1995). Higher level of drug resistance seen among *K. pneumoniae* and *Acinetobacter* spp. is mediated by the production of different kind of β-lactamases primarily ESBL, AmpC and MBLs. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for β-lactamases have had a great impact on the drug resistance character shown by these pathogenic bacteria (Thomson and Moland 2000; Picao et al. 2003; Walsh et al. 2005 Lee et al. 2008). The multidrug efflux systems, inactivation and modification of antibiotics and changes in target sites for antibiotics are the major mechanisms for antibiotic resistance in *P. aeruginosa* (Lambert 2002).

The prevalence of ESBL producers among the total isolates in our study was 38.8% (31/80) whereas the prevalence of ESBL producers among total MDR

isolates was 47% (31/67). In similar studies Batchoun et al. (2009), Balan (2013), Thenmozhi and Sureshkumar (2013) and Vinodhini et al. (2014) reported 22.9%, 23%, 17.7% and 54.31% ESBL producer respectively from total Gram negative bacterial isolates. Among the total 31 ESBL positive isolates, majority of them were *E. coli* with 38.7% followed by *K. pneumoniae* 25.8%, *P. aeruginosa* 16.1%, *Acinetobacter* spp. 9.7%, *K. oxytoca* 6.5% and *C. freundii* 3.2%.

In this study higher prevalence of MDR isolates was observed in blood and pus with 100% multidrug resistance each. Similar to this study 100% blood isolates were MDR (Upadhyaya 2015) however only 33.6% (Dantas et al. 2014) and 18.6% (Tsai et al. 2014) bacteremia was caused by MDR Gram negative bacteria. In comparison to this study (76.9%) only 60% and 37% MDR isolates from sputum were reported by Poudyal (2010). Panta (2013) and Upadhyaya (2015) reported 88.9% and 83.3% MDR isolates respectively of the total urinary isolates while that of only 64.6% and 38.6% of the total urinary isolates were reported by Poudyal (2010) and Poudel (2013) respectively.

Higher prevalence of ESBL producer was observed in blood (55.6%) followed by suction tip (50%), sputum (46.2%), ET tip and secretion (39.1%) and urine (32.3%). Jagdeesh et al. (2014) reported among screen positive isolates for ESBL, 45.1%, 46.7% and 29.4% ESBL producers from urine, exudates/pus and sputum respectively while 100% ESBL producers were detected in stool. However, among total ESBL positive isolates Sharma et al. (2013) reported high prevalence of ESBL producer from respiratory tract specimens (63.83%) followed by stool (59.29%), urine (57.2%), body fluid (52.17%), pus (48.03%) and blood (31.07%). Similarly 75%, 66.7% and 25% of the isolates from urine, exudates and blood were ESBL positive (Umadevi et al. 2011).

The positive ESBL screening result may be more often due to AmpC β-lactamases than ESBL. It is difficult to detect ESBLs in those isolates that typically have inducible AmpC chromosomal enzyme which may be induced by clavulanate and attack the indicator cephalosporin, thus masking any synergy arising from ESBL production (Livermore and Brown 2001).

In this study the prevalence of MDR Gram negative isolates among the total isolates was high (82.5%). Among the total MDR isolates 47% were ESBL positive. Although most of the isolates in the present study were susceptible to carbapenem antibiotics, the resistance shown by some isolates towards this group of antibiotic indicates presence of carbapenemase β-lactamases in them which requires further characterization.

In addition to this study, observation of higher number of Gram negative bacteria, multidrug resistance and ESBL producing isolates among hospitalized patients in different studies conducted in Nepal indicate MDR Gram negative bacteria are emerging as important health care associated pathogens (Panta et al. 2013; Mishra et al. 2014; Parajuli et al. 2017). Thus it is essential for tertiary care hospitals of Nepal to perform routine detection of ESBLs and other β-lactamases.

Most of the ESBL producing bacteria show multi drug resistance pattern creating a therapeutic dilemma for the clinicians. It is very important to determine the preferable antibiotics for the treatment. Infection control measures, hygiene guidelines, appropriate antibiotic policies that control the widespread use of advanced cephalosporins are immediately required to prevent and to ameliorate the ever increasing problem of the emergence of MDR ESBL producing Gram negative bacteria (Giamarellou 2005).

CONCLUSION

Presence of ESBL producing MDR Gram negative pathogens in patients of different department of tertiary care hospital of Nepal indicates these bacteria are important health care associated pathogens which can pose threat to the treatment. Thus, a proper infection control measure is required to check the transfer of MDR and β-lactamase producing bacterial pathogens among the hospitalized patients.

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Liver Function Test on HBsAg Positive Blood Donors

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ABSTRACT

Objectives: The study was done to assess liver function test among hepatitis B surface antigen (HBsAg) positive blood donors.

Methods: Liver function test (LFT) were studied in 71 HBsAg positive serum samples from healthy blood donors.

Results: In the study, 14(19.7Ü) serum samples showed elevated alanine aminotransferase (ALT) level above the normal range (5_35 IU/I) with mean 66.3±27.6; 16(22.5%) showed aspartate aminotransferase (AST) level above the normal range (5-40 IU/I) with mean 87.5±35.7; 4(5.6%) serum samples showed alkaline phosphatase (ALP) level above the normal range (306 IU/I) with mean 376.5±31.5; 49(69%) samples were found to be below the normal albumin level (38-51gm/I) with mean 23.9±5.76.

Conclusion: Deviations in the serum enzymes (ALT, AST and ALP) as well as total protein and albumin level showed the silent infection of hepatitis B virus in healthy blood donors.

Key words: HBsAg, LFT, blood donors, Nepal

INTRODUCTION

Hepatitis B is one of the major diseases of mankind and is a serious global public health problem causing variety of liver diseases such as chronic hepatitis, and hepatocellular carcinoma. Of the 2 billion people who have been infected with the Hepatitis B virus (HBV), more than 350 million have chronic infections (WHO, 2000). The burden of HBV infection is heavy in most developing countries, particularly in rural areas; this burden is compounded by the high cost of prevention, management, and treatment (Rosa et al. 2015). It is estimated that about 1% of total population of Nepal is infected by hepatitis B virus (Park and Park, 1997). Transmission is mainly due to artificial inoculation of infected blood and blood products. Hepatitis infection often leaves no visible symptoms such as jaundice and liver disease because liver is a non-complaining organ. The disease is often gets severe before the symptoms occur (WHO 2000). Patients must follow periodic liver function tests for early detection of acute exacerbation of chronic hepatitis B and to avoid its progression into a severe illness (Ohta 1989).

MATERIALS AND METHODS

Blood samples were collected from blood donors by medical professionals, lab technicians and nurses using aseptic standard techniques. While drawing 350ml blood in blood bag, 5ml blood was dispensed in a small clean test tube and labeled with corresponding sample number. Serum was separated from the collected blood samples in a test tube by centrifuging at 2000 rpm for 2 minutes. The separated blood samples were serologically investigated for viral infection of hepatitis B by third generation ELISA (Enzygnost HBsAg 5.0, Dade Behring, Marburg, Germany). The 71 hepatitis B positive (HBsAg positive) serum samples were studied for liver function tests, estimation of levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein and albumin by using test kits (RANDOX company, UK and Human Germany).

RESULTS

Table 1: LFT profile of HBsAg positive samples

Test	normal	Deviated	Elevated/Lowered
ALT	57 (80.3%)	14 (19.7%)	Elevated
AST	55 (77.5%)	16 (22.5%)	Elevated
ALP	67 (94.4%)	4 (5.6%)	Elevated
Total Protein	22 (31%)	49 (69.0%)	Lowered
Albumin	40 (56.3%)	31 (43.7%)	Lowered

In the study, all the serum samples were studied for LFT which includes estimation of enzymes ALT, AST and ALP and determination of total protein and albumin level. In total number of 71 hepatitis B positive serum samples, 14 (19.7%) serum samples showed elevated alanine aminotrasferase (ALT) level above the normal range (5-35 IU/I) with mean 66.3±27.6 and 16(22.5%) showed aspartate aminotransferase (AST) level above the normal range (5-40 IU/I) with mean 87.5±35.7.

DISCUSSION

The samples showing the elevations in ALT and AST are lesser elevation than ALP. Lesser elevations are encountered in mild acute viral hepatitis as well as in both diffuse and focal chronic liver diseases e.g. chronic active hepatitis, cirrhosis, and hepatic metastases (Burtis and Bruns 2007). Hence, the cases in the study with lesser elevations may be of mild acute hepatitis due to Hepatitis B virus infection. Tsai et al. (1997) reported that 30 of 76 (39.5%) donors with raised ALT level were positive for HBsAg in a study in China. In this study only 14 of 71 (19.7%) showed ALT level. As shown in the table, 4 (5.6%) serum samples showed alkaline phosphatase (ALP) level above the normal range (306 IU/I) with mean 376.5±31.5. ALP is next indicator of hepatocellular damage. Elevated levels of alkaline phosphatase activity usually reflect impaired biliary tract function (Burtis and Bruns 2007). In this case, it may be due to recent attack of hepatitis B.

In total number of 71 HBsAg positive serum samples, 49 (69.0%) samples were found to be below the normal range (60-80 gm/L) of total protein with mean 44.8±6.38 and 31 (43.7%) samples were found to be below the normal albumin level (38-51gm/I) with mean 23.9±5.76. Total protein and albumin levels in serum are two important measurements of liver function tests. Extensive liver injury may lead to decreased blood levels of albumin, prothrombin, fibrinogen, and other proteins synthesized exclusively by hepatocytes. Extensive damage of liver tissue will result in the low serum levels of total protein (Burtis and Bruns 2007). As seen in the table, most of the cases showed the decrease in the total protein and albumin level. It may be due to the damage of hepatocytes which were not able to produce the serum proteins like albumin and other proteins synthesized by them.

CONCLUSION

There is potentially a substantial risk of HBV transmission despite HBsAg testing. This is an important message for clinicians deciding to transfuse blood. The result of the study showed that the asymptomatic impairment of liver is associated with hepatitis B infection. Therefore, blood donors, who are at the risk of getting infection, should be well- informed about the mode of transmission of hepatitis B and monitoring of liver function tests. Although the incidence of transfusion-transmitted HBV has steadily reduced over the last four decades, HBV still remains the most frequent transfusion-transmitted viral infection (Niederhauser et al. 2008; Calderon et al. 2009; Kafi et al. 2009; Gulia et al. 2010; Liu et al. 2010). HBsAg serological marker in blood detection is presently the only diagnostic screening test for HBV infection identification in blood transfusion centres in Nepal.

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Antibiotic Susceptibility Pattern of Nalidixic Acid Resistant *Salmonella* Isolates in Shree Birendra Hospital Chhauni

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ABSTRACT

Objectives: This study was aimed to know the prevalence of Nalidixic acid resistant *Salmonella* isolates and their antibiotic susceptibility pattern.

Methods: A total of 4619 febrile patients suspecting the cases of typhoid fever by clinician, attending at Shree Birendra hospital during May- November 2013 were subjected to culture. Blood sample (5ml)was collected from the suspected cases and inoculated immediately into 45ml of Brain heart infusion broth (BHI) and further processed for the identification of *Salmonella* Typhi and *S*. Paratyphi. Antimicrobial susceptibility pattern of *S*. Typhi and *S*. Paratyphi isolates were determined by the modified Kirby-Bauer disc diffusion method.

Results: Out of 4619 blood sample, 8.7%(n= 403) sample were culture positive. Among culture positive, 66.3%(n=267) cases were *S*. Typhi, 26.1% (n=105) cases were *S*. Paratyphi and 7.7% (n=31) were other than *Salmonella* isolates respectively. Out of 372 *Salmonella* isolates, most of the *S*. Typhi isolates i.e. 95.51%(n=255) and *S*. Paratyphi isolates i.e. 97.14%(n=102) are highly resistant to nalidixic acid. Most of these isolates were also found resistant to ciprofloxacin and ofloxacin.

Conclusion: Therefore, screening of nalidixic acid susceptibility might be done prior to prescribe the drug for the treatment of enteric fever.

Key words: Blood culture, Nalixidic acid, *Salmonella*, enteric fever

INTRODUCTION

The term enteric fever consists of both typhoid and paratyphoid fevers (Lesser and Miller 2003). Typhoid and paratyphoid fever remain important public health problems globally and major causes of morbidity in the developing world including Nepal (Bukle et al. 2010; Acharya et al. 2012). Enteric fever caused by *S*. Typhi and *S*. Paratyphi A is the most common clinical diagnosis among febrile patients presenting to hospital in Nepal (Acharya et al. 2012). Although a wide range of *Salmonella* serotypes may cause human disease, broadly grouped into several typhoidal species that are specific human pathogens and includes serotypes *S*. Typhi and *S*. Paratyphi, and other serotypes that are primarily spread to humans from animal sources are non-typhoidal (Laupland et al. 2010). However, non-typhoidal *Salmonella* can also cause a variety of life-threatening extra-intestinal infections. Typhoid is unique to human, characterized by malaise, fever, abdominal discomfort, transient rash, splenomegaly, hepatomegaly, bradycardia, and leucopenia, the most prominent major complications are intestinal hemorrhage, and perforation. The real impact of typhoid fever is difficult to estimate because the clinical picture is confused with other febrile infections (Saleh 2013). Therefore, this study was aimed to know the prevalence of Nalidixic acid resistant *Salmonella* isolates and their antibiotic susceptibility pattern.

MATERIALS AND METHODS

This study was conducted at Shree Birendra Hospital, Chauni, Kathmandu during May to November, 2013. A total of 4619 blood samples were collected from the patients suspected of enteric fever. The blood samples were collected by veni-puncture under aseptic condition and then collected sample was transferred in BHI broth (3ml or 5 ml blood in 45 ml of brain heart infusion broth). It was then subjected to culture for *Salmonella* at 37^{o}C and sub-cultured on MacConkey agar (MA) after every 24 hours of incubation. On the next day, tiny non-lactose fermenting colonies on MA was then processed for identification according to standard microbiological methods (microscopic examination, biochemical tests) (Cheesbrough, 2000). The isolates were then subjected to

antimicrobial susceptibility testing by modified Kirby-Bauer disk diffusion method following clinical and laboratory standard institute (CLSI) guideline on Muller-Hinton agar plates (Cheesbrough, 2000; CLSI 2011). The antibiotics used were: nalidixic acid, amoxycilin (10µg), ceftriaxone (30µg), cephotaxime (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), co-trimoxazole (25µg), and ofloxacin (5µg) (Hi Media., Mumbai, India) (CLSI 2011).

RESULTS

Out of 4619 blood specimens cultured, only 403 (8.7%) samples had shown bacterial growth. Out of 403, 372 (92.31%) were identified as *Salmonella* isolates. Among the *Salmonella* isolates, 267(66.2%) were *Salmonella* Typhi and 105(26.1%) were *Salmonella* Paratyphi A (Table 1).

Table 1: Month wise distribution of *Salmonella* isolates

Month	*S.*Typhi		*S.* ParatyphiA		Total *Salmonella* isolates	
	Number	%	Number	%	Number	%
May	22	8.2	13	12.4	35	9.4
June	98	36.7	23	21.9	121	32.5
July	52	19.5	22	21.0	74	19.9
August	54	20.2	12	11.4	66	17.7
September	20	7.5	22	21.0	42	11.3
November	21	7.9	13	12.3	34	9.2
Total	267	100	105	100	372	100

Out of total *Salmonella* Typhi isolated, nalidixic acid 255 (95.5%), ciprofloxacin 257 (96.3%), and ofloxacin 257 (96.3%) were found to be resistant to respective drugs whereas commonly used drug chloramphenicol 265 (99.3%) still found to be effective (Table 2).

Table 2: Antibiotic susceptibility pattern of *S.* Typhi

Antibiotics	Sensitive		Resistance		Total
	Number	%	Number	%	
Nalidixic Acid	12	4.5	255	95.5	
Amoxycilin	261	97.8	6	2.2	
Cotrimoxazole	264	98.9	3	1.1	
Ceftriaxone	261	97.8	6	2.2	
Cephotaxime	263	98.5	4	1.5	
Chloramphenicol	265	99.3	2	0.7	
Azithromycin	263	98.5	4	1.5	
Ciprofloxacin	10	3.7	257	96.3	**267**
Ofloxacin	10	3.7	257	96.3	

Out of 105 *Salmonella* Paratyphi A isolates, cotrimoxazole 104 (99%), ceftriaxone 104 (99%), and chloramphenicol 104 (99%) were found to be sensitive whereas nalidixic acid 102(97.1), ciprofloxacin 102 (97.1%) and ofloxacin102 (97.1%) were found to be highly resistant (Table 3).

Table 3: Antibiotic susceptibility pattern of *S.* ParatyphiA

Antibiotics	Sensitive		Resistance		Total
	Number	%	Number	%	
Nalidixic Acid	3	2.9	102	97.1	
Amoxycilin	97	92.4	8	7.6	
Cotrimoxazole	104	99	1	1	
Ceftriaxone	104	99	1	1	
Cephotaxime	102	97.1	3	2.9	105
Chloramphenicol	104	99	1	1	
Azithromycin	97	92.4	8	7.6	
Ciprofloxacin	3	2.9	102	97.1	
Ofloxacin	3	2.9	102	97.1	

Out of the total *Salmonella* isolates, 357(95.97 %) were NARS isolates which included both *S.* Typhi 255 isolates and *S.* Paratyphi A 102 isolates respectively. Out of total *S.* Typhi isolates 95.51% and *S.* Paratyphi A, 97.14% were NARS. (Table 4)

Table 4: Nalidixic acid susceptibility pattern *Salmonella* isolates

Bacterial isolates	Antibiotic susceptibility pattern of Nalidixic acid						Total
	Resistance			Sensitive			
	Male	Female	Total	Male	Female	Total	
S. Typhi	191	64	255(95.51%)	8	4	12(4.49%)	267
S. Paratyphi	78	24	102(97.14%)	3	0	3(2.86%)	105
Total	**269**	**88**	**357 (95.97%)**	**11**	**4**	**15 (4.03%)**	**372**

DISCUSSION

Enteric fever is a disease of concern in developing countries like Nepal and remains endemic in the capital city Kathmandu due to lack of supply of clean drinking water, poor sanitation, and cross-contamination of water supply with sewerage (Pokharel et al. 2009). Various researchers reported wide variation in the sensitivity patterns of various *Salmonella* strains circulating in different geographic regions of Nepal, so it is essential to assess the sensitivity of *Salmonella* serotypes to antibiotics before instituting empirical therapy (Arora et al. 2010). We attempted to evaluate antibiotic susceptibility patterns in blood isolates of *Salmonella* serotypes from Shree Birendra Army hospital in Kathmandu with a view to understanding current trends in antibiotic sensitivity patterns.

In this study, out of 4,619 specimens processed for culture, only 403 (8.7%) isolates had shown growth, i.e. 267(66.2%) were *Salmonella* Typhi and 105 (26.1%) were *Salmonella* Paratyphi. This is not in similar with the results of other studies in different parts of Nepal, where *S.* Paratyphi A had reported main causative organism for enteric fever. *S.* Typhi (8.96%) and *S.* Paratyphi A (13.17%) (Pokharel et al. 2006; Shirakawa et al. 2006; Pokharel et al. 2009). In this study, most of the febrile cases and diagnosed enteric fever cases were from month between June-July (121), July-August (74), and August-September (66). Similar results also have shown by Malla et al. (2005) and Acharya et al. (2012) i.e. the peak occurrence of enteric fever in summer and rainy season (Malla et al. 2005; Acharya et al. 2012). The reason behind such result may be at this time; temperature and rainfall are relatively high and higher chance of mixing sewage to water supply pipelines due to unmanaged water supply system in Kathmandu Valley. In this study, most of *S.* Typhi isolates were highly sensitive with amoxycillin, co-trimoxazole, ceftriaxone, cephotaxime, chloramphenicol and azithromycin but they are highly resistant to nalidixic acid, ciprofloxacin and ofloxacin. Amoxicillin, Chloramphenicol and Co-trimoxazole (ACCo) were found to be effective having efficacy rate of 97.8%, 98.9% and 99.3% respectively. Similarly, most of *Salmonella* Paratyphi A isolates were highly sensitive with Amoxycillin, Co-trimoxazole, Ceftriaxone, Cephotaxime, Chloramphenicol and Azithromycin. This study revealed a re-emergence of susceptibility to amoxicillin, chloramphenicol and co-trimoxazole in greater proportion than reported by other similar studies conducted in different parts of Nepal at different times (Sharma et al. 2003; Bhatta et al. 2005; Pokharel et al. 2006). In this study, the nalidixic acid resistance in *S.* Typhi was found to be 95.5% and in *S.* Paratyphi A was found to be 97.1% which is in agreement with the findings of Prajapati 2009 (Prajapati 2009). Furthermore, isolation of the higher frequency of nalidixic acid-resistant *Salmonella* isolates found in this study indicates the possibility of fluoroquinolone resistance occurring in near future as a consequence of the haphazard use of fluoroquinolones without antibiotic susceptibility test.

The major limitations of this study were limited sample size and short duration of time. Furthermore, the samples were collected from a tertiary care center, so cases that preferred to seek health care in local settings were missed. The inclusion of patients from different geographic areas would have been helpful for more specific results. Furthermore, minimum inhibitory concentration (MIC) value of the antibiotics was not calculated. Molecular identification and characterization of isolates werenot performed due to the unavailability of equipment and resources in this setting.

CONCLUSION

The higher sensitivity of third generation cephalosporins (ceftriaxone and cephotaxime) and macrolide (azithromycin) indicates that these drugs along with chloramphenicol and cotrimoxazole may

still be considered as better options for the treatment of enteric fever. Hence cephalosporins remain the alternative drugs against infections with ciprofloxacin resistant *Salmonella* isolates. Therefore, the use of cephalosporins in the empirical therapy, misuse and over use should be discouraged. Resistance to nalidixic acid as a screening test for detecting reduced susceptibility to the fluoroquinolones helps in early diagnosis and substitution of appropriate antibiotic therapy which is very important in the management of enteric fever.

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Drug Resistance Pattern of Bacterial Pathogens of Enterobacteriaceae Family

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ABSTRACT

Objectives: This study was done to determine the drug resistance pattern and Extended Spectrum β-Lactamase (ESBL) in bacterial isolates of Enterobacteriaceae family from different clinical specimens.

Methods: The isolates were identified by conventional culture techniques and subjected to antimicrobial susceptibility testing by modified Kirby Bauer disk diffusion methods and ESBL detection by combined disk method.

Results: Of the total 1602 sample processed 200 (12.5%) bacteria of Enterobacteriaceae family were isolated and 85.5% of them were multidrug resistant. Of the total Enterobacteriaceae isolates 27% were ESBL producers. Single isolate of stool was MDR and ESBL producer. Higher prevalence of MDR isolates (100%) and ESBL producer (41.2%) was observed in sputum specimen. Higher multidrug resistance (92.1%) and ESBL production (35%) was detected in *Klebsiella pneumoniae*.

Conclusion: The most effective antibiotics towards the isolates of Enterobacteriaceae were imipenem, amikacin, chloramphenicol and tetracycline. Emergence of MDR and ESBL producing Enterobacteriaceae requires proper infection control measures and routine and reliable detection of ESBL with rationale use of antibiotics.

Key words: Enterobacteriaceae, Multidrug resistance, ESBL, Antibiotic susceptibility testing.

INTRODUCTION

The prevalence of multidrug resistance (MDR) bacterial species has increased considerably since the introduction followed by arbitrary use of new generation extended spectrum antibiotics like third and fourth generation cephalosporins, carbapenems, monobactams, broad and extended spectrum penicillins and other antibiotics (Fang et al. 2008).

Multidrug resistance bacterial isolates have been frequently reported from different parts of the world as an emergence of treatment problem. The World Health Organization (WHO), the European Commission (EU), and the U.S. Centers for Disease Control and Prevention (CDC) have recognized the importance of studying the emergence and determinants of multidrug resistance as well as the need for control (Alekshum 2007). *Citrobacter, Enterobacter, Escherichia, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Yersinia,* etc. are the medically important genera of the family Enterobacteriaceae (Gray 1995).

Extended spectrum β-lactamases (ESBLs) in Enterobacteriaceae is defined as β-lactamases capable of hydrolyzing penicillins, broad and extended-spectrum cephalosporins and monobactams and are inhibited by clavulanic acid. They are generally derived from TEM and SHV-type enzymes. ESBLs are often located on plasmids that are transferable from strain to strain and between bacterial species. ESBLs-producing Enterobacteriaceae have been responsible for numerous outbreaks of infection throughout the world and pose challenging infection control issues (Rupp and Fey 2003).

Antibiotic resistance is one of the alarming issues, affecting human health. There are various factors responsible to the emergence of resistance such as misuse and overuse of antibiotics, patients related factors, inappropriate prescriptions by physicians, self-medications especially young adults, use of broad

spectrum antibiotics and synergistic combinations, unnecessary promotion by pharmaceutical industry, and lack of awareness with the new guidelines recommended for antimicrobial testing, etc. Multidrug resistance is getting common phenomenon and are being reported worldwide (Khan et al. 2014).

Multidrug Resistance among bacteria is one of the greatest challenges in the field of medicine. Resistance mechanism to different classes of antibiotics such as tetracyclines, aminoglycosides and cotrimoxazole is of bigger issue. However, broad spectrum resistance to β-lactam and fluoroquinolones are of utmost significance. In the early 1950s, enteric bacteria that mediated resistance to first penicillin attracted attention of the researchers. The introduction of 3rd generation cephalosporins was milestone in antimicrobial chemotherapy but after few years resistance to these drugs was observed in different species (Sanders and Sanders 1987).

The outbreaks of infectious disease caused by known commensal and pathogenic bacteria by acquisition of new resistance determinants have eluded the action of multiple antibiotics. The development of resistance in the responsible pathogens has worsened the situation and very little resources to investigate and provide reliable data. The emergence of multiple drug resistance in gastrointestinal tract infection and several other infections has had its greatest toll in developing countries. This condition has large population around the globe at great risk of numerous infections and even greatest risk of acquiring nosocomial infection caused by MDR isolates of Gram negative bacteria (Walsh 2003; Byarugaba 2004).

Extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. Incidence of multidrug resistant and extended spectrum β-lactamases producing Enterobacteriaceae has been rising in several parts of the world and has been associated with high morbidity and mortality. Through this research an effort was made to find out the prevalence of bacterial pathogens of Enterobacteriaceae family in different clinical specimens. This research also conducted the antimicrobial susceptibility test of isolated Enterobacteriaceae and assessed the burden of multidrug resistant and extended spectrum β-lactamases producers among the isolates. Bacterial pathogens of Enterobacteriaceae family have received much more attention in recent years because of their involvement in number of severe infections and multiple drug resistance patterns. Much of the study regarding such bacterial infections is limited to Kathmandu valley. So an effort was made to assess the burden of infection caused by Enterobacteriaceae and their antibiotic susceptibility pattern around Birgunj city. It is aimed that this study will aid in treatment procedures to patient suffering from infections of Enterobacteriaceae from that region.

MATERIALS AND METHODS

Study population: A prospective cross-sectional study was performed for 6 months (14/10/2015 to 13/04/2016) at National Medical College and Teaching Hospital, Birgunj. This study included patients of all age group and both sexes visiting this hospital from whom samples were sent for routine culture and antibiotic susceptibility testing.

Conventional microbiological tests: The identification of various gram negative isolates was done using standard microbiological techniques which comprises of studying of colonial morphology, staining reactions and various biochemical properties. Isolated colonies from the pure culture were identified by standard conventional biochemical tests. Antibiotic susceptibility testing was performed by using Kirby-Bauer disk diffusion method on Mueller-Hinton agar. Screening tests for ESBL detection was done according to the CLSI guidelines and confirmed by combination disk method.

RESULTS

A total of 1602 clinical specimen were investigated during a six-month study period, of which 877 (54.7%) and 725 (45.3%) were from female and male patients respectively. Among 1602 specimens, 1192 (74.4%) were from indoor patients and 410 (25.6%) specimens from outdoor patients were included in the study.

Distribution of total clinical specimens under investigation

Among the total 1602 clinical specimens analyzed, 649 (40.5%) were urine, 360 (22.5%) were pus, 329 (20.5%) were blood, 169 (10.5%) were sputum, 84 (5.2%) were body fluids, 6 (0.4%) were stool and 5 (0.3%) were ET tip and secretion specimens.

A total of 403 (25.15%) bacteria was isolated from

1602 different clinical specimens among which 255 (63.3%) isolates were Gram negative while 148 (36.7%) were Gram positive bacteria. Among the total isolates of Gram negative 200 (75.5%) belong to the family Enterobacteriaceae, 45 (19.6%) were *P. aeruginosa* and 10 (3.9%) were *Acinetobacter* spp.

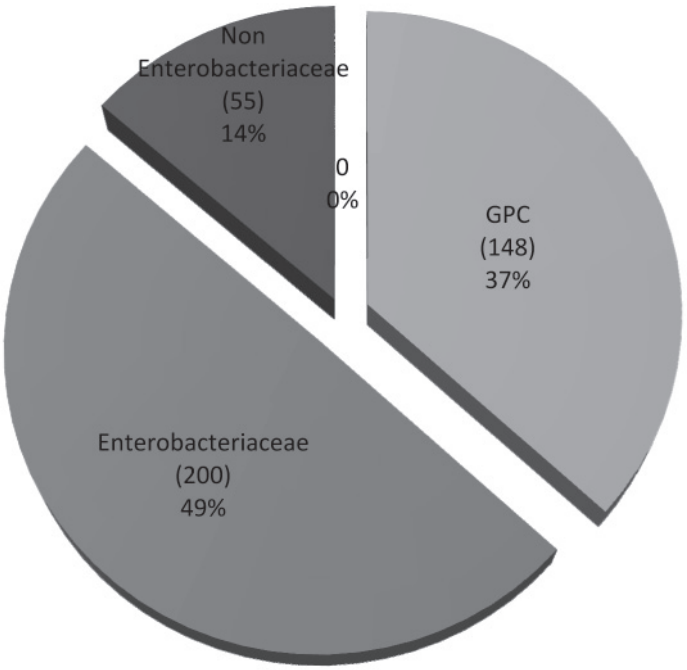

Figure 1: Prevalence of different types of bacteria

Among the total 200 isolates of Enterobacteriaceae *E. coli* was the most predominant isolate with 56.5% followed by *K. pneumoniae* (31.5%), *P. mirabilis* (5.5%), *P. vulgaris* (3.5%), *Enterobacter* spp. (1.5%), 0.5% *Citrobacter* spp., 0.5% S. Paratyphi A and 0.5% *Serratia* spp. (Figure 1).

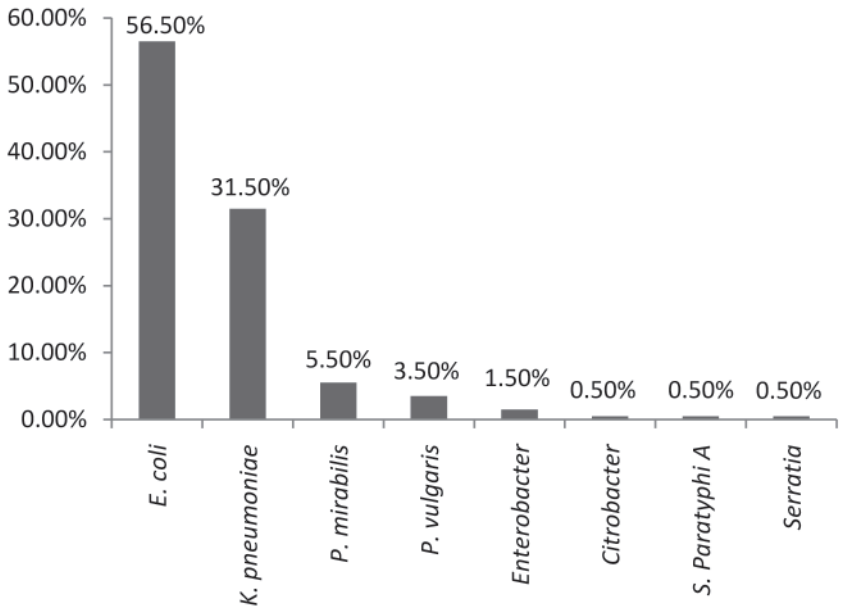

Figure 2: Members of Isolated Enterobacteriaceae

Antibiotic susceptibility pattern of members of Enterobacteriaceae from various specimens

All the isolated strains of Enterobacteriaceae from different clinical specimens were tested with specific antibiotics by using Kirby – Bauer disk diffusion method. According to CLSI 2014, a set of 12 antibiotics were used to determine antibiotic susceptibility pattern of members of Enterobacteriaceae family.

Imipenem was found most effective antibiotic against members of Enterobacteriaceae with sensitivity 89.5% (179/200). They showed 73% (146/200) sensitivity towards amikacin, followed by chloramphenicol 69.5% (139/200) and tetracycline 52.5% (105/200). Nitrofurantoin was found to be effective among 77.9% (88/113) of the urinary isolates. Ampicillin with 98.5% (197/200) resistance was the least effective drugs towards the isolates of Enterobacteriaceae followed by cefixime 80.5%(161/200), amoxyclav 77% (154/200), ceftriaxone 73.5% (147/200), cefotaxime 72% (144/200), cefoxitin 64.5% (129/200) and ciprofloxacin and cotrimoxazole with 58% (116/200) resistance each.

Table 5: Antibiotic susceptibility pattern of Enterobacteriaceae from various specimen

S.N	Antibiotic	Sensitive		Intermediate		Resistant		Total
		N	%	N	%	N	%	
1.	Imipenem	179	89.5%	7	3.5%	14	7%	200
2.	Amikacin	146	73%	5	2.5%	49	24.5%	200
3.	Chloramphenicol	139	69.5%	15	7.5%	46	23%	200
4.	Tetracycline	105	52.5%	26	13%	69	34.5%	200
5.	Nitrofurantoin	88	77.9%	13	11.5%	12	10.6%	113
6.	Ciprofloxacin	74	37%	10	5%	116	58%	200
7.	Cotrimoxazole	73	36.5%	11	5.5%	116	58%	200
8.	Cefoxitin	58	28%	12	6%	130	65%	200
9.	Cefotaxime	56	28%	0	0%	144	72%	200
10.	Ceftriaxone	40	20%	13	6.5%	147	73.5%	200
11.	Cefixime	33	16.5%	6	3%	161	80.5%	200
12.	Amoxyclav	26	13%	19	9.5%	155	77.5%	200
13.	Ampicillin	3	1.5%	0	0%	197	98.5%	200

MDR Enterobacteriaceae isolates in different clinical specimens

Out of total 200 isolates, 171 (85.5%) isolates were multidrug resistant among which all the isolates from sputum and stool specimens showed 100% multidrug resistance while blood specimens showed 93.3% MDR followed by urine (84.1%), and pus (81.5%).

Table 6: MDR Enterobacteriaceae isolates in different clinical specimens

		MDR	NON-MDR	TOTAL
Sample	Urine	95(84.1%)	18(15.9%)	113
	Pus	44(81.5%)	10(18.5%)	54
	Blood	14(93.3%)	1(6.7%)	15
	Sputum	17(100%)	0(0%)	17
	Stool	1(100%)	0(0%)	1
TOTAL	**171(85.5%)**	**29(14.5%)**	**200**	

ESBL production profile of Enterobacteriaceae in different clinical specimens

Out of 200 isolates of Enterobacteriaceae, 54 (27%) isolates were ESBL producer. Single isolate of stool (*S.* Paratyphi A) was an ESBL producer. High prevalence of ESBL producer was observed in sputum with 41.2% followed by pus (31.5%), blood (26.7%) and urine (22%).

Table 7: ESBL production profile of Enterobacteriaceae in different clinical specimens

Samples	No of ESBL		Total
	Positive (%)	Negative (%)	
Urine	25(22.1)	88(77.9)	113
Pus	17(31.5)	37(88.5)	54
Blood	4(26.7)	11(73.3)	15
Sputum	7(41.2)	10(58.8)	17
Stool	1(100)	0(0)	1
Total	**54(27)**	**146(73)**	**200**

Multidrug resistance and ESBL production profile of Enterobacteriaceae

MDR Enterobacteriaceae were identified by their antibiotic sensitivity pattern. Out of the total 200 Enterobacteriaceae 171 (85.5%) isolates were MDR. *E. coli* was the most predominant among the total MDR isolates. Single isolates of *Citrobacter* spp. and *S.* Paratyphi A were MDR strain. The highest percentage of MDR strains among each bacterial isolates were *K. pneumoniae* with 92.1% multidrug resistance followed by *P. vulgaris* (85.7%), *E. coli* (83.2%), *P. mirabilis* (81.9%) and *Enterobacter* spp. (66.7%). The MDR isolates of Enterobacteriaceae was suspected as ESBL producer on the basis of reduced susceptibility to at least one screening agent i.e, cefotaxime or ceftriaxone. Of the total Enterobacteriaceae (200) isolates, 147 isolates were suscpected of being ESBL producer. Of the total 147 screen positive isolates for ESBL production, 54 (36.7%) isolates were found to be ESBL producer. The prevalence of ESBL producer among total isolates was 27% (54/200) whereas the prevalence of ESBL producers among MDR isolates was 27.5% (47/171) and among non-MDR isolates was 24.1%(7/29). Among the total ESBL positive isolates *E. coli* was the most predominant isolate with 51.9% (28/54). Among each isolates higher prevalence of ESBL producer was detected in *K. pneumoniae* with 35% (22/63) followed by *P. mirabilis* 27% (3/11) and *E. coli* 24.%(2/113). Single isolate *S.* Paratyphi A 100% were ESBL producer (1/1). All isolates of *P. vulgaris*, *Enterobacter* spp., *Citrobacter* spp. and *Serratia* spp. were ESBL non-producer (Table 7).

Table 8: Multidrug resistance and ESBL production profile of Enterobacteriaceae

	ESBL		
	Positive	Negative	Total
MDR	47	124	171
NON-MDR	7	22	29
TOTAL	**54**	**146**	**200**

DISCUSSION

Bacterial pathogens of Enterobacteriaceae family are often associated with diffferent human infections like respiratory tract infections, blood stream infections, meningitis, endocarditis, urinary tract infections, skin, soft tissue and bone infections, etc.The emergence of ESBLs producing bacteria, particularly *K. pneumoniae* and *E. coli*, is now a critical concern for the development of therapies against bacterial infection (Canton and Coque 2006).

A total of 1602 different clinical specimens from the patients of OPD, various wards (medical, surgical, paediatric, emergency observations and ICUs of the hospital that were sent for the microbiological investigation were analyzed. Urine (40.5%) was the most frequently analyzed specimens followed by pus (22.5%), blood (20.5%) and sputum (10.5%). Other specimens analyzed were body fluids (5.2%), stool (0.4%) and ET Tip and secretion (0.3%). Presence of high number of urine sample indicated the high urinary tract infection among the patient visiting the hospital. Similar pattern of specimen distribution was observed in other similar studies (Bhandari 2011; Upadhyaya 2015 ; Ghimire 2016). In almost all hospital based studies urine was found to be the most frequently analysed specimen (Bomjan 2005; Poudyal 2010; Gautam 2015; Ghimire 2016).

Of the total 200 isolates of Enterobacteriaceae, *E.*

coli was the the predominant pathogen with 56.5% which was comparable with the result of Mulla (2012) i.e, 55.6%. Ghimire (2016) reported 53.8% but high prevalence i.e, 73.4% of *E. coli* reported by Panta (2012) in the similar study. Following *E. coli, K. pneumoniae* was found to be the most frequently isolated bacteria with 31.5% which was comparable with the result of Mulla (2012) i.e, 31.2%. Ghimire (2016) reported 34.6% but very low prevalence of *K. pneumoniae* was reported by Panta (2012) in the similar study. In comparison of 4% of *Proteus* species isolated by Mangaiarkkarasi et al. (2013), 5.5% of *P. mirabilis* and 3.5% *P. vulgaris* were isolated in our study. But higher prevalence of *Enterobacter* (7.9%) was found in the study of Mulla (2012) in comparison to our study (1.5%). Other bacteria isolated were *S.* Paratyphi, *Citrobacter* spp. and *Serratia* spp. 0.5% each. Sample from patients under probable treatment, sample transport delay, sampling errors or nosocomial transmission could be the possible reasons for some variable results in our study

In this study all the isolated strains of Enterobacteriaceae isolates were tested with specific antibiotics according to CLSI 2014. Among the 12 different antibiotics used against all the isolates of Enterobacteriaceae , imipenem (89.5%) was found to be the most effective antibiotic followed by amikacin (73%) which was in accordance with the similar study conducted by Ghimire (2016). Nitrofurantoin was found to be 77.9% sensitive among urinary Enterobacteriaceae isolates. Similarly in a study antibiotic sensitivity pattern of the isolates revealed that 89.5% of the isolates were sensitive to imipenem, 73% were sensitive to amikacin followed by chloramphenicol (69.5%), and tetracycline (52.5%) whereas high resistance was seen for ampicillin (98.5%), cefixime (80.5%), amoxyclav (77.5%), ceftriaxone (73.55%), cefotaxime (72%), cefoxitin (65%), cotrimoxazole (58%) and ciprofloxacin (58%) (Metri et al. 2011).

In this study a total of 200 isolates of Enterobacteriaceae were isolated from 1602 different clinical specimens processed. Thus, the prevalence of Enterobacteriaceae in various clinical specimens was found to be 12.5%. Of the total isolates of Enterobacteriaceae, 171 (85.5%) were found to be multidrug resistant. Similar study conducted in Sahid Gangalal Memorial Hospital by Ghimire (2016) showed 11.8% growth and 82.6% of them were MDR. In a study by Yadav (2015) showed high growth positivity of 31.3% and 96.8% multidrug resistance among them. Another study conducted by Panta (2012) at Tertiary Hospital showed 13.9% growth positivity of Enterobacteriaceae with 59.7% MDR among them. However, in a study by Baral (2008) rather high growth positivity of 22.4% was observed but the MDR among the isolates was only 40.7%.

High drug resistance in Enterobacteriaceae is attributed to mutations in chromosomal genes ability to share genetic material and mobile resistant genes. The mobile genetic elements are responsible for capturing resistant genes from the chromosomes of a variety of bacterial species and then DNA molecules horizontally and vertically (Patridge 2015).

The high level of drug resistance seen among *E. coli* is mediated by beta-lactamases, which hydrolyzes the beta-lactam ring inactivating the antibiotic. The classical TEM-1, TEM-2 and SHV-1 enzymes are the predominant plasmid-mediated beta-lactames of Gram negative rods (Livermore 1995). Mutations at the target site i.e, *gyrA*, which is a gyrase subunit gene, and *parC*, which encodes a topoisomerase subunit, confer resistance to fluoroquinolones (Ozeki et al. 1997). In addition to this mechanism, there are more than efflux systems in *E. coli* that can export structurally unrelated antibiotics; these multidrug resistance efflux pump (MDR pump) systems contribute to intrinsic resistance for toxic compounds such as antibiotics, antiseptics, detergents and dyes (Sulavik et al. 2001).

Higher level of drug resistance seen in *K. pneumoniae* is mediated by the production of different kind of beta-lactamases primarily ESBL, AmpC and Metallo beta-lactamases. The fact that the carriage of resistance trait for quinolones and aminoglycoside in the plasmid along with the gene for beta-lactamases have had a great impact on the drug resistance character shown by this pathogenic bacteria (Thomson 2001; Walsh et al. 2005; Picao et al. 2008). The *acrR* and *ramA* genes are involved in expression of the MDR phenotype in strains of *K. pneumoniae* (Paudel 2013).

There are many mechanisms whereby *Proteus* spp. confer resistance to the drugs including impermeability and acquired resistance ass plasmids, transposons and mutations (Paudel 2013). However, the production of plasmid or chromosomal encoded beta-lactamase enzyme is the most common mechanism of resistance in Gram negative bacteria causing clinical significant infection (Bush et al. 1995).

Of the total MDR Enterobacteriaceae isolates tested for screening of ESBL production, 132 (77.2%) isolates were screened as positive. Of the total 132 screened positive isolates, 47 (33.8%) isolates confirmed as ESBL producers. 24.1% (7/29) of Enterobacteriaceae isolates were NON-MDR but ESBL producers. The prevalence of ESBL producers among total MDR isolates was 27.5% (47/171). In similar studies Balan (2013), Batchoun et al. (2009) and Thenmozhi and Sureshkumar (2013) reported 23%, 22.9% and 17.7% ESBL producer respectively among total Gram negatve bacterial isolates. Among the total 54 ESBL positive isolates, majority of them were *E. coli* with 58.9% followed by *K. pneumoniae* 40.8%, *P. mirabilis* 5.6% and *Salmonella* Paratyphi 1.9%. In a similar study Khanal et al. (2013) ESBL detection was reported highest in *E. coli.*

Only 38.8% of the total 139 screen positive isolates for ESBL production were ESBL producer. The positive ESBL screening result may be due to AmpC beta-lactamases more often than to ESBL. It is difficult to detect ESBL in those isolates that typically have inducible AmpC chromosomal enzyme which may be induced by clavulanate and attack the indicator cephalosporin, thus masking any synergy arising from ESBL production. In case of *Citrobacter* spp, *Serratia* spp. *Enterobacter* spp. resistance to oxyimino-cephalosporin is due to mutational hyper production of chromosomal AmpC beta-lactamase production rather than ESBL (Beceiro et al. 2008; Livermore and Brown 2001).

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Phenotypic Assays for Detection of AmpC and MBL Producers among the Clinical Isolates of Multi Drug Resistant *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: In order to determine the prevalence of multi-drug resistance along with AmpC and metallo-β-lactamase producing *P. aeruginosa*, a six month cross-sectional study was carried out at Shahid Gangalal National Heart Center.

Methods: A total of 756 clinical specimens were analyzed for bacteriological profile. The bacterial isolates were identified by cultural and biochemical techniques. Antibiotic susceptibility testing of the isolates was performed by Kirby-Bauer disc diffusion method. MDR isolates were screened and tested for MBL and AmpC production. Ceftazidime resistant isolates were tested for MBL and Cefoxitin resistant isolates for AmpC.

Results: Among all the clinical samples analyzed, *P. aeruginosa* was detected in 75 samples (9.92%). Antibiotic susceptibility testing showed Imipenem as the most effective drug with susceptibility of 76% followed by Piperacillin-Tazobactam (74.7%) and Piperacillin (41.3%). Out of 75 *P. aeruginosa* isolates, 53 (70.6%) of them were found to be resistant to at least three out of four anti-pseudomonal agents, thus were considered as MDR. Out of 53 multi-drug resistant *P. aeruginosa* (MDRPA), all were resistant to ceftazidime whereas 85% (45/53) were resistant to cefoxitin. Out of 53 isolates, 11 (20.75%) showed positive result for MBL. Similarly, 7 out of 45 i.e. 13.2% were found to be AmpC producers.

Conclusion: This study signified the high prevalence of MDRPA which is an alarming rate. Also multiple β-lactamase producing *P. aeruginosa* were detected which can further complicate the treatment options. Regular monitoring of antibiotic susceptibility and rational use of antibiotics would be helpful in eliminating the outbreaks of multiple β-lactamase producing MDRPA.

Key words: *P. aeruginosa*, β-lactams, MDRPA, AmpC, MBL

INTRODUCTION

Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen and the most common gram-negative bacterium found in nosocomial infections (Kalaivani 2011). Most distinguishing feature of this organism is the capability to confer resistance to several classes of antibiotics using distinctive mechanisms (Kapoor et al. 2011; Tian et al. 2011). Though carbapenems remain one of the best drugs to treat infections caused by *P. aeruginosa*, increasing usage of these drugs and other expanded-spectrum antibiotics has resulted in the development of carbapenem resistant *P. aeruginosa* creating therapeutic problem (Yousefi et al. 2010; Martinez et al. 2011). Aztreonam is the only β-lactam antibiotic that is saved from the activity of β-lactamases (Tsakris et al. 2009). The common form of resistance is mediated by lack of drug penetration and/or carbapenem hyrolysing β-lactamase enzymes including the metallo-β-lactamases (MBL) (Shanthi and Sekar 2009; Ishii et al. 2010). Metallo-β-lactamases are a diverse set of enzymes having zinc ion at the active site that catalyze the hydrolysis of a broad range of β-lactam drugs (including carbapenems) with the exception of monobactams. An increasing prevalence of carbapenem

resistance mediated by acquired metallo-β-lactamases (MBLs) is being reported, particularly for *P. aeruginosa* clinical isolates in several countries. The resistance may spread rapidly to various species of gram-negative bacilli, as the MBL genes reside in mobile gene cassettes inserted in integrons. The production of metallo-β-lactamases by bacteria is becoming a serious threat to the clinical community because these enzymes are responsible for the development of antibiotic resistance to the commonly employed β-lactam antibiotics.

Ambler class C, confer resistance to cephamycins, narrow, expanded and broad-spectrum cephalosporins, Aztreonam and β-lactam/ β-lactamase inhibitor combination. The enzyme may be chromosomal or plasmid encoded. Although these two enzymes have similar substrate profile, the only difference is chromosomal AmpC are inducible whereas plasmid mediated AmpC are uninducible.

Despite the discovery of AmpC and Metallo-β-lactamases at least a decade ago, there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting AmpC and metallo-β-lactamase. Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures (Singhal et al. 2005).

MATERIALS AND METHODS

This hospital based cross sectional study was carried out fromApril 2014 to September 2014 in the Microbiology laboratory of Shahid Gangalal National Heart Center. A total of 756 different samples incuding blood, sputum, urine, pus, endo-tracheal (ET) secretion, wound swab, bed sore swab, Central Venous Pressure (CVP) tip, nasal secretion and oral secretion were collected from the patient and processed following standard laboratory techniques. The samples were inoculated on Blood agar and MacConkey agar and incubated at 37°C for 24 hours. For the identification of *Pseudomonas* spp., typical non-fermenting colonies from MacConkey agar were further processed via gram staining and other biochemical tests. *P. aeruginosa* was identified on the basis of various characteristics such as positive catalase and oxidase test, motile, mannitol non-fermenter, growth at 42°C, oxidative in Hugh and Leifson's medium, indole negative, methyl red/voges proskauer test negative, citrate positive, Alk/no change, H_2S negative, and gas negative in TSIA medium, urease negative and growth on cetrimide agar with production of pigments and fruity odour. Antibiotic susceptibility testing of isolates was assessed as per CLSI (2013) guidelines following modified Kirby-Bauer disc diffusion method. For differentiation of MDRPA isolates, 4 different antipseudomonal classes i.e. carbapenem (Imipenem), cephalosporin (Ceftazidime), fluoroquinolone (Ciprofloxacin) and aminoglycoside (Amikacin) were used.

Only those MDR isolates resistant to Ceftazidime were tested for metallo-β-lactamase enzyme by two methods. Combined disc testwere done by placing two 10µg Imipenem discs 25mm apart on the surface of Mueller Hinton Agar plates inoculated with a bacterial suspension equivalent to 0.5 McFarland standard. One of the disc was loaded with 10µl of 0.5M EDTA to obtain desired concentration of 750µg. After 24 hours of incubation at 37°C, an increase of ≥7 mm in the zone diameter of EDTA containing Imipenem disc compared to Imipenem disc alone was considered to be a positive test for the presence of an MBL. In double disc synergy test, a10µg Imipenem disc was placed 20mm centre to centre from a blank disc containing 10 µl of 0.5M EDTA on the surface of Mueller Hinton Agar inoculated with test organism. After incubation at 37°C overnight, an increase of ≥7 mm in the zone diameter of blank disc containing EDTA compared to Imipenem disc was considered to be a positive test for the presence of an MBL.

AmpC β-lactamase production was screened by Cefoxitin disc diffusion test. Isolates showing inhibition zone diameter <18mm (screening positive) were further subjected to disc antagonism test for inducible AmpC enzyme and AmpC disc test for the detection of plasmid AmpC βlactamases.

In disc antagonism test, 0.5 McFarland suspension of test isolate was swabbed on MuellerHinton agar plate and Cefotaxime (30µg) and Cefoxitin (30µg) discs were placed 20 mm apart from centre to centre. After incubation at 37°C overnight, isolates showing blunting of the Cefotaxime zone of inhibition adjacent to the Cefoxitin disc were considered positive for inducible AmpC β-lactamase.

In AmpC disc test, a lawn culture of 0.5 McFarland suspensions of *Escherichia coli* ATCC 25922 was inoculated on surface of MuellerHinton agar plate. Sterile disc (6mm) was moistened with sterile saline

(20µl) and inoculated with several colonies of test organism. The inoculated disc was then placed beside a 30 µg Cefoxitin disc, almost touching it, on the inoculated plate. After overnight incubation at 37°C, positive test was interpreted as a flattening or indentation of the Cefoxitin inhibition zone in the vicinity of the test disc and negative test as absence of distortion.

RESULTS

Out of 756 samples investigated, significant growth was observed in 242 of them, among which 75 clinical isolates were identified as *P. aeruginosa* by standard microbiological procedures showing prevalence of 9.92% (Figure 1).

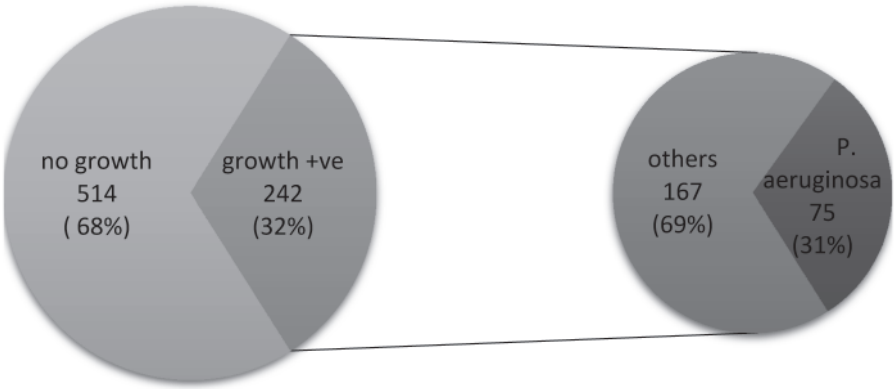

Figure 1: Prevalence of *P. aeruginosa* among clinical samples

Among different type of clinical samples analyzed, maximum number of *P. aeruginosa* was isolated from endo-tracheal (ET) secretions (69.3%) followed by the category others (swabs, oral and nasal secretions) (12%), sputum (8%), urine (5.3%), blood (4%) and pus (1.3%) (Figure 2).

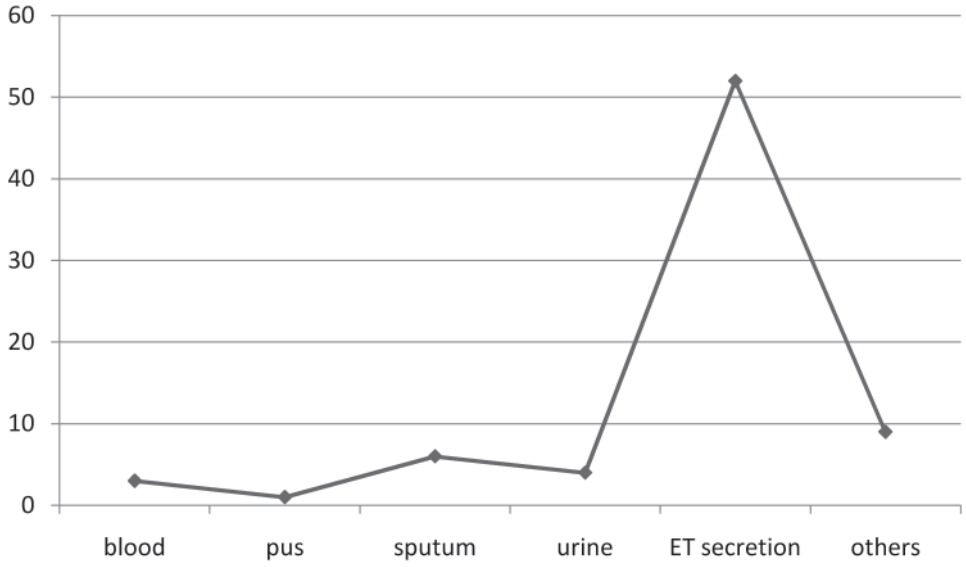

Figure 2: Distribution of *P. aeruginosa* among different specimens

Majority of the isolates were found to be resistant to most of the antibiotics tested. They showed highest resistance to Ceftazidime (88%) followed by Gentamicin (84%), Ofloxacin (82.7%) and Ciprofloxacin (78.7%). Imipenem was found to be the most effective drug with susceptibility of 76% closely followed by Piperacillin-Tazobactam to which 74.7% of the organisms were susceptible (Figure 3).

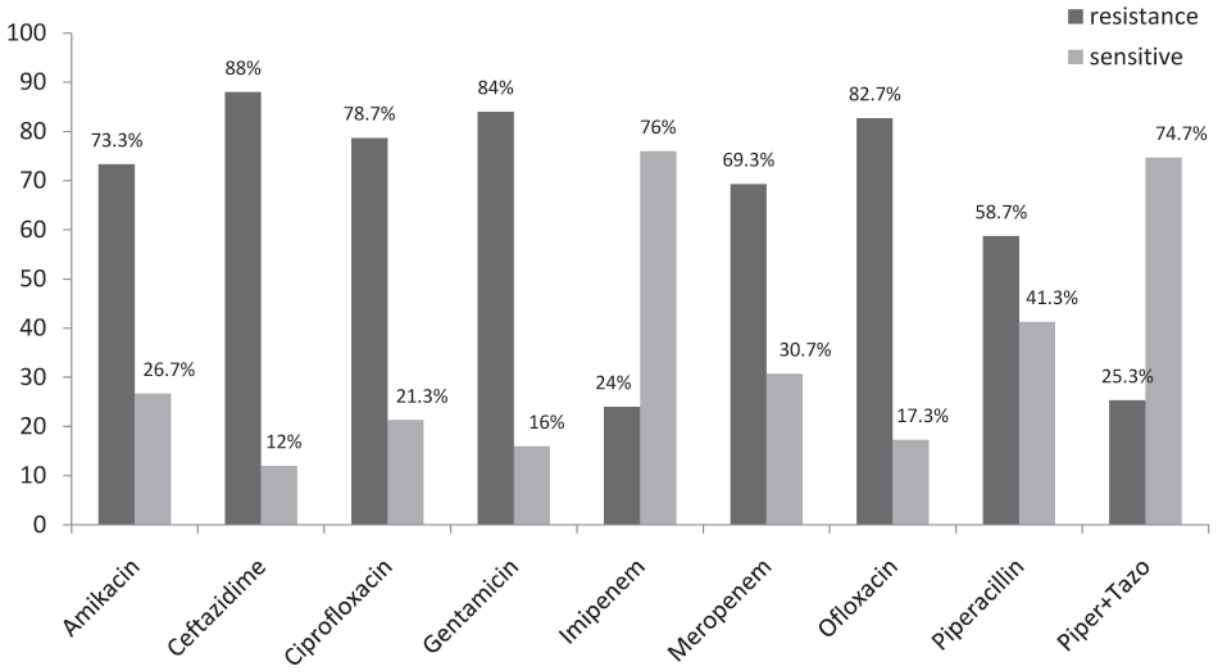

Figure 3: Antibiotic susceptibility pattern of *P. aeruginosa*

Out of 75 isolates, 70.6% of them were found to be multi-drug resistant *P. aeruginosa* (MDRPA). Out of 68 isolates from inpatient, 72.05% showed multi-drug resistance whereas in case of outpatient, out of 7 isolates, 4 of them were MDR. Altogether out of 53 MDRPA, 92.45% were from inpatient whereas 7.54% were from outpatient. There was no significant association between the MDR occurrence and the type of patient (p>0.05) (Table 1).

Table 1: Distribution of multi drug resistant *P. aeruginosa*

Type of strains	Type of patient		Total (%)	p-value
	Inpatient	Outpatient		
MDR	49(92.45%)	4 (7.54%)	53 (70.6%)	0.41
Non-MDR	19	3	22 (29.3%)	
Total	**68**	**7**	**75 (100%)**	

Among different types of clinical samples analyzed during the study, maximum no. of MDR isolates were observed in endotracheal (ET) secretion (73.58%) followed by the category others (9.43%), sputum (7.54%), and urine (5.66%). Only 1.88% of the total MDR isolates were isolated from both blood and pus individually. The association between the MDR occurrence and specimen was found to be statistically insignificant (p>0.05) (Table 2).

Table 2: Distribution of MDRPA among various samples

Type of Specimen	No. of *P. aeruginosa*	No. of MDR isolates	% of MDR isolates	p-value
Blood	3	1	1.88	0.55
Pus	1	1	1.88	
Sputum	6	4	7.54	
Urine	4	3	5.66	
ET secretion	52	39	73.58	
Others	9	5	9.43	
Total	**75**	**53**	**100**	

Ceftazidime resistant isolates were tested for MBL production. In this study, out of 53 MDR isolates, all were resistant to Ceftazidime. Altogether 11 (20.75%) of them gave positive results for MBL by combined disc test and double disc synergy test (Figure 4).

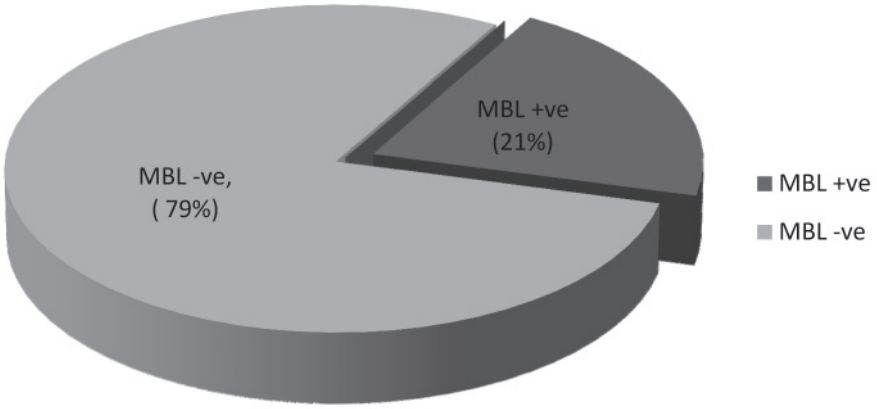

Figure 4: MBL production among MDR *P. aeruginosa*

Among two methods used for the detection of metallo-β-lactamase enzyme, double disc synergy test (DDST) showed only7 out of 53 isolates (13.2%)) to be positive for metallo-β-lactamase enzyme whereas combined disc test (CDT) revealed 9 isolates (16.98%) to be positive for MBL. Five isolates gave positive result by both CDT and DDST methods whereas 2 isolates showed positive result by DDST only and 4 by CDT only (Table 3).

Table 3: Comparison of combined disc test and double disc synergy test

Combined disc test (CDT)	Double disc synergy test (DDST)	No. of isolates
+	–	4
–	+	2
+	+	5
–	–	42
Total		**53**

When screening for AmpC-β-lactamase producing organism was done by Cefoxitin disc diffusion test, out of 53 MDR isolates, 45 isolates were found to be resistant which were further tested by Disc antagonism test for inducible AmpC and AmpC disc test for plasmid mediated AmpC (Figure 5).

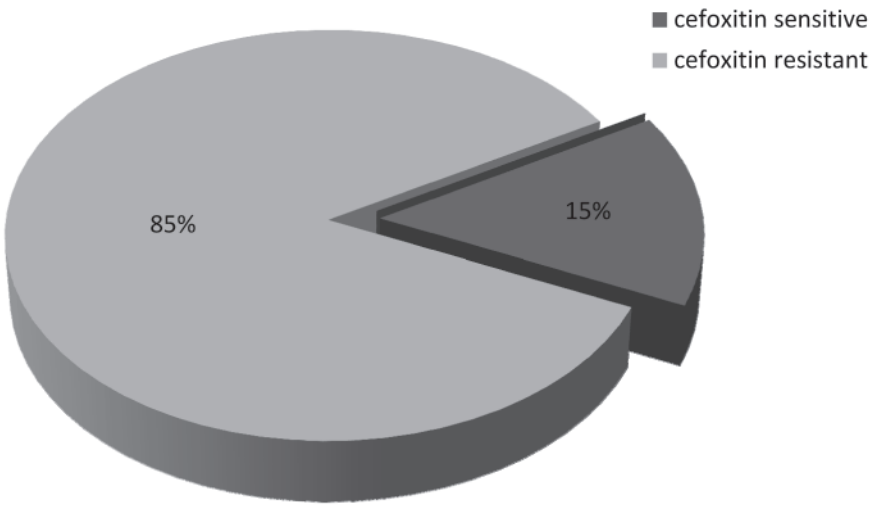

Figure 5: Cefoxitin susceptibility of the MDR isolates

Out of 45 positively screened MDR isolates, none of them were found to produce chromosomal (inducible) AmpC β-lactamase whereas 7(15.55%) were found to produce plasmid mediated (constitutive) AmpC β lactamase. Altogether out of 53 MDRPA, the prevalence of AmpC producing isolate was found to be 13.2%.

Among the 53 MDRPA strains which were screened phenotypically for various mechanisms of resistance, 20.75% were MBL producers, 13.2% AmpC producers, 3.77% produced both AmpC and MBL and 73.58% produced neither AmpC nor MBL (Figure 6).

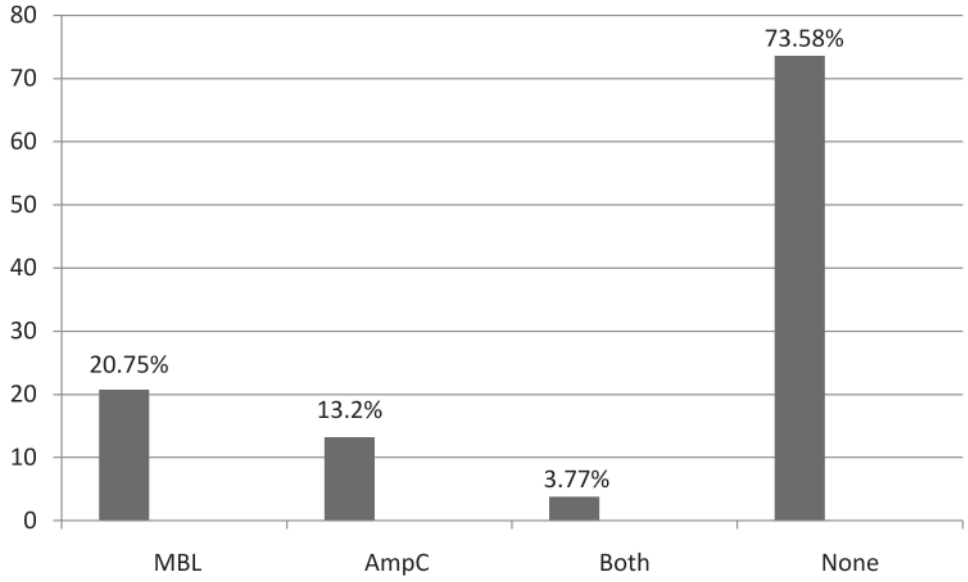

Figure 6: Various mechanisms of resistance among MDR isolates

DISCUSSION

A total of 756 clinical specimens from the patients of different age groups and wards were included in the study, out of which 75 (9.92%) of them were identified as *P. aeruginosa* by standard microbiological procedure. A recent study by Khan et al. (2014) reported similar prevalence of *P. aeruginosa* i.e. 6.67%. Another study by Mohanasoundaram (2011) showed the prevalence rate of *P. aeruginosa* to be 5%, 6.8% and 5% in three different years which is comparable with our studies. However a study done by Goel et al. (2009) reported a comparatively higher prevalence rate of *P. aeruginosa* (35%).

The distribution of specimens of *P. aeruginosa* may vary with each hospital as each hospital facility has a different environment associated with it. A study by Chander et al. (2013) showed the major source of *P. aeruginosa* pus/wound (27.6%) followed by sputum (24.1%), urine (20.7%), tracheal aspirate (10.35%), CVP catheter tube (3.45%), and 3.45% from each of BAL fluid, bile, catheter, and high vaginal swab. In another study by Basak et al. (2009), the clinical specimen following *P. aeruginosa* infections were most commonly from urinary tract infection followed by wound infections. However a study done by Mohanasoundaram (2011) showed the maximum no. of isolates from pus and endotracheal secretions and Khan et al. (2014) in his study reported the isolation of maximum no. of *P. aeruginosa* from tracheal secretion and pus. In our study, maximum no. of isolates were obtained from endotracheal secretions (69.3%) followed by the category others (12%), sputum (8%), urine (5.3%), blood (4%) and pus (1.3%).

Increasing resistance to different anti-pseudomonal drugs particularly among hospital strains has been reported world-wide. In this study, the isolates showed highest resistance to Ceftazidime (88%) followed by Gentamicin (84%). The isolates resistant to Ciprofloxacin were 78.6%. These strains also showed resistance to carbapenems like Imipenem (24%) and Meropenem (69.3%), the precious weapon against *P. aeruginosa* infections (even against MDR isolates) which is an alarming sign. Imipenem was found to be the most effective drug with susceptibility of 76% followed by Piperacillin/Tazobactam (74.7%), Piperacillin (41.3%) and Meropenem (30.7%). Piperacillin alone tested showed a resistance rate of 58.7% in this study

whereas beta-lactam/beta-lactamase inhibitor drug Pipercillin/Tazobactam showed a lower resistance of 25.3% only, indicating beta-lactamase inhibitor markedly expands the spectrum of activity of beta-lactams, which makes the combination drug the preferred choice against *P. aeruginosa* infections. A recent study by mohanasaundaram (2011) with 193 *P. aeruginosa* showed 79% resistance to Gentamicin followed by Ceftriaxone (75%), Ciprofloxacin (73%), Ceftazidime (63%), Piperacillin (44%) and Amikacin (41.5%) which correlates with our study. They also reported resistance to Imipenem (3.7%) which is less compared to the present study. A study done by Landman et al. (2007) reported resistance of 24% to imipenem which exactly correlates with our study. In another study by Mohanasoundaram (2011), the resistance rate to imipenem was 27% in 2009 and 16% in 2010 which is comparable with this study. Ceftriaxone and ceftazidime are commonest 3rd generation antibiotics in hospital protocols. In our study, the rate of aminoglycoside resistance was also found to be relatively high (Amikacin-73.3% and gentamicin 84%). This study shows that the clinical isolates of *P. aeruginosa* are becoming resistant to commonly used antibiotics and gaining more and more resistance to newer antibiotics.

A total of 53 out of 75 isolates were found to be multi-drug resistant in this study with the prevalence rate of MDRPA to be 70.6%. Moniri et al. (2005) reported a prevalence rate of 73.9% of MDRPA. A study by Mahmoud et al. (2012) and Ahmed et al. (2013) reported the prevalence of MDRPA to be 52% and 63.2% respectively which is also comparable to our study.

In this study, it was found that 92.45% of the total MDRPA was isolated from inpatients whereas only 7.54% belonged to outpatient. As observed in the study, the multidrug resistant strains of *P. aeruginosa* were mainly obtained from inpatients which might be due to increasing invasive procedures that are required for diagnosis and chemotherapy and predispose patients to acquire nosocomial infections with such pathogen. Ahmed et al. (2013) also mentioned the high prevalence of multi-drug resistant *P. aeruginosa* in inpatient than outpatient.

A study by Aggarwal et al. (2008) showed the major source of MDRPA were from sputum and tracheostomy specimen (28.57%), followed by pus (24.13%), urine (19.04%), cerebrospinal fluid and other sterile body fluids (15.38%) and blood (7.14%). In a study by Shashikala et al. (2006), 27.6% of carbapenem resistant *P. aeruginosa* were obtained from surgical wounds and 20.7% were from endotracheal aspirate showing indwelling devices as major risk factors for the development of resistance. Present study revealed the major source of MDRPA to be endotracheal secrtions (73.58%) followed by the category others(9.43%), sputum (7.54%), urine (5.66%) and 1.88% each from blood and pus.

The numerous β-lactamases are encoded either by the chromosomal genes or by the transferable genes which are located on the plasmids or the transposons. Among various mechanisms of resistance, AmpC and metallo-β-lactamase were detected in this study which is very effective in degrading the anti-pseudomonal agents these days. In 2002 from India, Navaneeth et al. reported MBL production in *P. aeruginosa* to be 12%. In the present study, we found 20.75% of MDRPA to be MBL producers. A recent study by Varaiya et al. (2008) showed 20.8% of MDRPA to be MBL producers. Ugargol (2012) in his study reported 18.8% of the isolates to be positive for MBL. Similarly, Navaneeth et al. (2002) and Willmann et al. (2013) respectively reported 18% and 15.9% of the MDRPA had the ability to produce MBL. Pandya (2011) showed MBL prevalence of 30%.

Since there is no standard guideline for detection of MBL production, different studies have reported to have used different methods. Two different phenotypic methods were used for the detection of metallo-β-lactamase in our study; Combined disc test (CDT) and Double disc synergy test (DDST). Combined disc test revealed 9 out of 53 i.e. 16.98% of the MDRPA isolates as MBL positive whereas Double disc synergy test showed only 7 out of 53 i.e. 13.2% as MBL producers. Five isolates gave positive result by both methods. In this study, both the combinations simultaneously detected 11 isolates i.e. 20.75% to be positive for MBL. In the evaluation of two selected MBL phenotypic assays, CDT was found to be more sensitive (16.98%), than DDST (13.2%). These results correlated with studies by Behera et al. (2008) (CDT- 88.8% and DDST-57.14%), and Pandya et al. (2011) (CDT-96.3%, DDST- 81.48%) both of which showed higher susceptibility of CDT than DDST although prevalence rate differs widely from our study. However, it differs from studies by Picao et al. at Brazil from January–December 2005 (CDT- 80%, DDST-82.6%) who showed DDST to be a

more sensitive method than CDT.

Out of 53 MDR isolates, 8 (15%) were found to be sensitive to cefoxitin whereas 45 of them i.e. 85% were resistant. A study done by Upadhyaya et al. (2010) reported 196 out of 202 isolates (97%) to be cefoxitin resistance which is comparable to our study. Hassan et al. (2011) in their study reported 57.33% (86/150) to be cefoxitin resistant. Similarly, another study by Behera et al. (2008) found 78.2% of the isolates to be cefoxitin resistant.

In our study, the overall prevalence of AmpC was found to be 13.2% which is comparable to study done by Basak et al. in 2009 (19.3%). Our study was also comparable with the reports from Aligarh by Shahid et al. in 2004 as 20% and from Kolkata by Arora et al. in 2005 as 17.3% and from Varanasi as 22%. Also, Ugargol (2012) in his study reported 12.8% of the isolates to be AmpC producer. However, Upadhyaya et al. (2010) reported a high prevalence of 59.4% of AmpC producing isolates out of which 7% were positive for inducible and 52.4% for non-inducible AmpC.

Co-production of AmpC and MBL was found in 3.77% of the MDRPA isolates in this study. Both of these isolates were from ICU and were resistant to imipenem. Singhal et al. (2005) showed the co-production of AmpC and MBL in 11% of the isolates. However, Upadhyaya et al. (2010) reported the co-production of AmpC and MBL in 46.6% of the isolates. Behera et al. (2008) showed the co-production of AmpC and MBL in 48.5% of the isolates. Both of these findings are comparatively much higher than our study. Production of multiple β-lactamases by *P. aeruginosa* has tremendous therapeutic consequences and there is a need for urgent action to control the spread of resistant strains. In our study, 39 MDRPA isolates were found to produce neither AmpC nor MBL. These strains may be harboring some other resistance mechanisms like biofilm formation or through virulence factors.

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Bacterial Analysis of Different Types of Milk (Pasteurized, Unpasteurized and Raw Milk) Consumed in Kathmandu Valley

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ABSTRACT

Objectives: The presence of pathogenic bacteria in milk is the major public health concern resulting in food borne illness. The aim of this study is to determine the microbial quality of three different types of milk consumed in Kathmandu Valley with respect to the acceptable standard guideline and measure the antibiotic susceptibility pattern of the *Escherichia coli* and *Staphylococcus aureus* isolates.

Methods: A total of 66 samples (16 pasteurized, 25 unpasteurized and 25 raw milk) were collected from various sites of Kathmandu Valley. Those samples were subjected for total plate count and total coliform count by pour plate method. Furthermore, identification was made for the presence of *E. coli* and *S. aureus* with biochemical tests.

Results: The mean total plate count (TPC) of pasteurized, unpasteurized and raw milk was $1.2X10^6$ cfu/ml, $2.3X10^7$cfu/ml and $2.0X10^7$cfu/ml respectively. And, the mean total coliform count (TCC) of pasteurized, unpasteurized and raw milk was 2.9 X 10^4cfu/ml, $6.3X10^5$cfu/ml and $1.6X10^5$cfu/ml respectively. Coliforms were detected in 50%, 84% and 56% of the pasteurized, unpasteurized and raw milk sample respectively. *E. coli* and *S. aureus* were isolated from 18.8% and 12.5% of pasteurized, 40% and 16% of unpasteurized and 20% and 24% of the raw milk samples respectively. Among total *E. coli* isolates *(n=18)*, 16.7% were susceptible to ampicillin whereas 100% isolates were susceptible to other tested antibiotics. Similarly, 33.3% and 66.7% of the isolated *S. aureus* were susceptible to penicillin and cefoxitin respectively, whereas all *S. aureus* isolates were sensitive to all other antibiotics.

Conclusion: The mean value of TPC and TCC of pasteurized and raw milk exceed the standard guideline by FDA. Higher total plate count and presence of coliforms (also *E. coli*) and *S. aureus* in this study necessitates the close monitoring of the pasteurization process and post pasteurization process (packaging, transportation, storage etc.).

Key words: milk, coliforms, pasteurization, hygienic

INTRODUCTION

Milk, a great source of nutrients including protein with all ten amino acids, essential fatty acids, immunoglobulin and other micronutrients has become important part of diet to all age group including expectant mothers (Wijesihha-Bettoni and Burligame 2013). Most of the people in the world consume pasteurized milk and few people prefer raw milk as they believe that raw milk is more beneficial, tastier and convenient than pasteurized one (Altalhi and Hassan 2009). There isalso abundancy in view that raw milk could reduce allergic reactions and cure other ailments.

Milk, from the synthesis in specialized cells of mammary gland to the secretion to the alveoli of udder, is virtually sterile (De Silva et al. 2016). The microbial contamination of milk then after occur within the udder, exterior of the udder or from the surface of milk handling and storage equipment (Bramley and McKinnon 1990). Milk can also be cross contaminated during food preparation and by infected workers

who don'tpractice good hygiene (Lore et al. 2006). Pathogens involved in causing food borne diseases due to the consumption of raw milk include *Escherichia coli, Listeria monocytogenes, Salmonella, Campylobacter, Brucella abortus, Staphylococcus aureus, Bacillus cereus, Mycobacterium* spp. *and Clostridium botulinum* (Chye et al. 2004). Post-treatment contamination of milk caused outbreaks of campylobacteriosis, salmonellosis, yersiniosis and staphylococcal enterotoxin "food poisoning" (Lecos 1985).

Presence of *E. coli* in milk and milk products reported to bring public health hazard. Enteropathogenic *E. coli*are potential to cause severe diarrhea and vomiting in infants and young children (Sousa 2005). *E. coli* in milk is also the indicator of fecal contamination of milk.Similarly, *S. aureus* is another important human pathogen that causes food borne infections including milk and milk products (Bergdoll et al. 1989). Although *S. aureus* is effectively killed by pasteurization, but the enterotoxins produced by the *S. aureus* retain their biological activity even after pasteurization, which is becoming a hazard for consumers (Asao et al. 2003). Therefore, this study aimed to determine the degree of bacterial load and occurrence of *S. aureus* and *E. coli* with their antimicrobial susceptibility pattern in three different types of milk (pasteurized, unpasteurized and raw) consumed in Kathmandu Valley.

MATERIALS AND METHODS

Study period and study site: This research was conducted from February 2016 to May 2016 at Microbiology Laboratory, Department of Microbiology, GoldenGate International College, Kathmandu, Nepal.

Sample and sampling method: Pasteurized packaged milk from local shops (n=16), unpasteurized milk from local dairy (n=25) and raw milk from cow farm (n=25) of Kathmandu valley were included in this study. All sixteen pasteurized milk samples were from different brands. For unpasteurized milk and raw milk, sample was placed separately in a sterile plastic bag and transported to the laboratory in an ice box within 2 hours of collection and promptly processed.

Sample preparation: During sample preparation, 10 ml of each sample was taken and added to 90 ml distilled water. Further, a serial 10-fold dilution was made until a dilution of 10^{-6} was obtained.

Enumeration of bacteria: After the sample was prepared, 1 ml of each dilution of every samples were transferred to the sterile petriplate and molten plate count agar (PCA) and violet red bile agar (VRBA) (at around 45°C) were poured into respective petriplates for the enumeration of total bacterial count and total coliform count respectively. The overlay plate method was used for the total coliform count. Then, the plates were incubated at 37°C for 24 hours for total plate (bacterial) count and total coliform count (Cheesebrough 2006). Since, one ml of sample was inoculated in each plate, the number of colonies in each plate indicated the cfu/ml of each dilution. However, different dilutions of each samples were inoculated, mean of all dilutions was calculated to get cfu/ml of the particular sample (Aneja 2003).

Isolation and identification of *S. aureus* and *E. coli*: One loopful each of the sample from 10^{-1} dilution was inoculated on to Mannitol Salt agar (MSA) and MacConkey Agar (MA). The plates were incubated at 37° C for 24 hours. *S. aureus* produce yellow colonies with yellow zone on MSA. The isolated colonies were taken and identified as Gram positive, catalase positive, oxidase positive, Coagulase positive and DNA-ase positive (Isenberg 2004). Lactose fermenting colonies on MacConkey agar were sub-cultured to obtain pure culture. Pure cultures were tested biochemically (catalase test, oxidase test, Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Triple sugar iron agar test, urease test, oxidative-fermentative test) for confirmation of *E. coli* (Isenberg 2004; Cheesbrough 2006).

Antimicrobial susceptibility testing (AST) of the identified microorganisms: In vitro antimicrobial susceptibility testing towards different antibiotics was performed by modified Kirby Bauer disc diffusion method on to Muller Hinton Agar (MHA) and zone size was interpreted by using CLSI guideline (2014).

Statistical analysis: All data obtained from the sample analysis were tabulated using SPSS v. 19 and Microsoft Excel.

RESULTS

Frequency Distribution of Total Plate Count of Different Milk Samples

Of 16 pasteurized milk samples, 7 (43.8%) were observed with total plate count in the range of 10^5 ($\times 10^5$) cfu/ml and other 7 (43.8%) samples with total plate count of $\times$ 10^6 cfu/ml respectively. Similarly, 13/25 (52%) unpasteurized milk samples were observed with

total plate count of × 10^7 cfu/ml and 9/25 with total plate count of × 10^6 cfu/ml respectively. And, 14/25 raw milk samples exhibited total plate count of × 10^7 cfu/ml and 5 each of 25 raw samples contained total plate count of × 10^5 cfu/ml and × 10^6 cfu/ml respectively.

Table 1: Frequency distribution of total plate count

Count/ml (in range)	Pasteurized n=16		Unpasteurized n=25		Raw n=25	
	No.	%	No.	%	No.	%
× 10^0	-	-	-	-	-	-
× 10^1	-	-	-	-	-	-
× 10^2	-	-	-	-	-	-
× 10^3	1	6.3	-	-	-	-
× 10^4	1	6.3	-	-	1	4
× 10^5	7	43.8	2	8	5	20
× 10^6	7	43.8	9	36	5	20
× 10^7	-	-	13	52	14	56
× 10^8	-	-	1	4	-	-
Total	**16**	**100**	**25**	**100**	**25**	**100**

Note: No. - number

Frequency Distribution of Total Coliform Count of Different Milk Samples

Of 16 pasteurized milk samples, 8 (50%) samples were obtained to contain less than 10 coliforms (i.e, in the range of × 10^0cfu/ml). Of remaining, 3 samples contained coliform in the range of × 10^3, and two samples each contained coliform in the range of × 10^4 and ×10^5 respectively. In unpasteurized samples (n=25), 9 samples exhibited coliform in the range of × 10^6, followed by 6 samples in the range of × 10^5 and 4 samples in the range of less than 10 coliforms (× 10^0cfu/ml). And, in raw samples, large proportion of the samples, i.e, 11/25 (44%) exhibited coliforms less than ten (× 10^0cfu/ml), followed by 5 samples in the range of × 10^5.

Table 2: Frequency distribution of total coliform count

Count/ml (in range)	Pasteurized n=16		Unpasteurized n=25		Raw n=25	
	No.	%	No.	%	No.	%
× 10^0	8	50	4	16	11	44
× 10^1	-	-	1	4	-	-
× 10^2	1	6.3	-	-	-	-
× 10^3	3	18.8	2	8	4	16
× 10^4	2	12.5	3	12	4	16
× 10^5	2	12.5	6	24	5	20
× 10^6	-	-	9	36	1	4
Total	16	100	25	100	25	100

Note: No. – number

Comparison of Mean Microbial Load among Three Different Types of Milk Samples

The mean total plate (bacterial) count was obtained higher in unpasteurized milk (2.3 × 10^7 ± 35.96) and raw milk (2.0 × 10^7 ± 19.18) in comparison to pasteurized milk (1.2 × 10^6 ± 1.17). Similarly, total coliform count was also higher in unpasteurized milk (6.3 × 10^5 ± 60.4) and raw milk (1.6 × 10^5 ± 36.44) in comparison to pasteurized milk (2.9 × 10^4 ± 5.51).

Table 3: Comparison of microbial quality of different type of milk samples

	Pasteurized cfu/ml	Unpasteurized cfu/ml	Raw cfu/ml
TPC	1.2 × 10^6 ± 1.17	2.3 × 10^7± 35.96	2.0. × 10^7±19.18
TCC	2.9 × 10^4 ± 5.51	6.3 × 10^5± 60.41	1.6 × 10^5±36.44

Note:TPC: Total Plate (Bacterial) Count; TCC: Total Coliform Count

Occurrence of *E. coli* and *S. aureus* in milk sample

Among all the milk samples, *E. coli* were isolated from 18 samples (3 from pasteurized, 10 from non-pasteurized and 5 from raw milk) and *S. aureus* were isolated from 12 samples (2 from pasteurized, 4 from non-pasteurized and 6 from raw milk).

Table 4: Occurrence of *E. coli* and *S. aureus* in different milk sample

Sample	*E. coli*	*S. aureus*
Pasteurized milk (n=16)	3	2
Non-pasteurized milk (n=25)	10	4
Raw cow milk (n=25)	5	6
Total number of samples (N=66)	**18**	**12**

Microbiological evaluation of three disfferent types of milk samples

Coliforms were found in 50% of the pasteurized milk, 84% of non-pasteurized milk and 52% of raw milk samples respectively. *E. coli* were isolated from 18.8% of pasteurized milk, 40% of non-pasteurized milk and 20% of raw milk samples respectively. *S. aureus* were isolated from 12.5% of pasteurized milk, 20% of unpasteurized milk and 24% of raw milk samples respectively.

Table 5: Microbial analysis of different type of milk samples

Sample	Pasteurized (%)	Non-pasteurized (%)	Raw(%)
Coliform	50	84	56
E. coli	18.8	40	20
S. aureus	12.5	16	24

Antimicrobial Susceptibility Testing of *E. coli* and *S. aureus*

All 18*E. coli* were susceptible to the tested antibiotics, namely amikacin, gentamycin, imipenem, chloramphenicol, cotrimoxazole, ceftazidime, piperacillin-tazobactam, cefotaxime and levofloxacin except ampicillins. However, only 16.7% of the *E. coli* isolates were susceptible to ampicillins.

Table 6: Antimicrobial susceptibility testing of *E. coli* (n=18)

Antibiotics	Susceptibility percentage
Ampicillin	16.7
Amikacin	100
Gentamicin	100
Imipenem	100
Chloramphenicol	100
Cotrimoxazole	100
Ceftazidime	100
Piperacillin-Tazobactam	100
Cefotaxime	100
Levofloxacin	100

All *S. aureus* (n=12) were susceptible to antibiotics, amikacin, gentamicin, erythromycin, levofloxacin, chloramphenicol, clindamycin and ofloxacin except penicillin and cefoxitin. In case of penicillin and cefoxitin 33.3% and 66.7% of the isolated *S. aureus* was susceptible respectively. Isolates resistant to cefoxitin are methicillin-resistant *S. aureus* (MRSA).

Table 7: Antimicrobial susceptibility pattern of *S. aureus* (n=12)

Antibiotics	Susceptibility percentage
Penicillin	33.3
Cefoxitin	66.7
Amikacin	100
Gentamicin	100
Erythromycin	100
Levofloxacin	100
Chloramphenicol	100
Clindamycin	100
Ofloxacin	100

DISCUSSION

In this study, the total plate (bacterial) count (TPC) results showed that none of the samples were free of bacterial contamination. The mean TPC of pasteurized, unpasteurized and raw cow milk was 1.2×10^6 cfu/ml, 2.3×10^7 cfu/ml and 2.0×10^7 cfu/ml respectively. The mean total plate count of pasteurized milk and raw milk obtained in this study exceeds the range as per FDA Pasteurized milk ordinance (FDA 2015). The mean TPC value was higher than the findings of Al-Mazeedi et al. (2013) where mean counts of the aerobic bacteria in the pasteurized milk from three different dairy companies were 3×10^4 cfu/ml, 9×10^1 cfu/ml and 5×10^3 cfu/ml respectively. High bacterial counts reflects poor production hygiene or ineffective pasteurization of milk. (Harding 1995). The mean TPC of raw milk obtained in this study is higher than the findings obtained by El-Diasty and El-Kaseh(2009), Tasci (2011) and Belbachir et al. (2015) who found mean Aerobic Plate Count of 6.1×10^5, 3.95×10^6 and 1.4×10^6 cfu/ml respectively but lower than those reported by Moustafa et al. (1988) and Mohamed and El Zubeir (2007) who found mean value of 1×10^9 and 5.63×10^9 cfu/ml respectively.

In this study, coliforms were present in 65.1% of total milk samples. The mean Total Coliform Count (TCC) of pasteurized, unpasteurized and raw milk was 2.9×10^4 cfu/ml, 6.3×10^5 cfu/ml and 1.6×10^5 cfu/ml respectively. The mean TCC of Pasteurized milk was found greater than the FDA Pasteurized Milk Ordinance (FDA 2015). The coliform was present in 50% of the pasteurized milk samples, which was in harmony with the research conducted by Silva et al. (2010) from Brazil. In contrary to this result, in similar research carried out in Kathmandu valley by Arjyal et al. (2004), out of 140 samples of 14 different brands, coliforms were detected in all sample except one. According to the annual report published by DFTQC (2011/2012) out of 65 milk and milk products analysed, 31 (47%) samples were found to be microbiologically unsafe.

The value of TCC of raw milk in this study (1.6×10^5) is less than the findings of Moustafa et al. (1988), Mohamed and El Zubeir (2007) and Hassan et al. (2015) where the mean TCC of raw milk sample was found to be 1×10^6, 3.3×10^6 and 1.8×10^6 cfu/ml respectively. But higher than the result reported by Belbachir et al. (2015) where mean TCC was 2.6×10^3 cfu/ml. The existence of coliform bacteria may not necessarily indicate a direct fecal contamination of milk but it is a precise indicator of poor hygiene and sanitary during milking and further handling processes (Hassan et al. 2015).

E. coli and *S. aureus* was isolated from 18.75% and 12.5% of the pasteurized milk sample respectively. In similar research carried out in Kathmandu valley by Arjyal et al. (2004); out of 140 pasteurized milk samples of 14 different brands, the presence of *S. aureus* (15%) was similar to this study. But the presence of *E. coli* was higher (i.e. 92%) than the current study. Out of 25 raw milk samples, *E. coli* and *S. aureus* was isolated from 20% and 24% of the samples respectively. Joshi et al. (2014) also reported the similar prevalence of *S. aureus*, i.e. 29.7%. Of 25 unpasteurized milk samples, *E. coli* and *S. aureus* were obtained from 40% and 16% samples respectively.

All the isolated *E. coli* were sensitive to all the administered antibiotics disc amikacin, gentamicin, imipenem, chloramphenicol, ctrimoxazole, ceftazidime, piperacillin-tazobactam, cefotaxime and levofloxacin except ampicillins. Among 18 *E. coli* isolates, 16.7%

were susceptible and remaining 83.3% were resistant to the antibiotic ampicillins. For *S. aureus*, all isolates were susceptible to the administered antibiotics amikacin, gentamicin, erythromycin, levofloxacin, chloramphenicol, clindamycin and ofloxacin except two antibiotics penicillin and cefoxitin. In case of penicillin only 33.3% isolates were susceptible and 66.7% isolates were resistant. And, 33.3% of S. *aureus* were resistant to cefoxitin, i.e. MRSA.

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Sodium Azide Induced Mutation in Actinomycetes

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ABSTRACT

Objectives: The study was done with an aim to determine the gain and loss of functions among the actinomycetes mutants induced by sodium azide.

Methods: The study was carried out in the laboratory of the Sainik Awasiya Mahavidhayala, Bhaktapur, Nepal from 2016 December to 2017 March. A total of 30 soil samples were collected from Tokha, Bhaktapur area and Godawari area. Actinomycetes were isolated from the soil sample using pour plate technique on selective media; starch casein agar. The isolates were identified by using standard microbiological methods and each isolate was exposed to different concentration of sodium azide to generate mutants. The wild type and mutants were compared in morphology, biochemical reactions and antibiotic susceptibility to test organism to determine the gain and loss of functions.

Results: Among 30 samples processed, 20(67%) actinomycetes were isolated, in which 6 (20%) were identified as the *Streptomyces* spp. A total of 28 mutants were isolated from 6 wild types by exposed at 10ppm, 20ppm, 40ppm, 50ppm, 100ppm concentration of sodium azide. Out of 28 mutants formed, only 10 mutants from sample showed same pigmentation as its wild type while other 18 mutants showed change in their pigmentation. In sugar utilization test, 8 different sugars for 28 mutants each, 56 cases showed Gain of Function (GOF), similarly 44 cases showed Loss of function (LOF). Antibiosis remained unaffected against *Pseudomonas* i.e. no GOF or LOF was seen. Only 2 cases of LOF against *Staphylococcus aureus* were seen while there were no cases of LOF in other pathogens. 3 cases of GOF against *E. coli*, 4 against *S* Typhi and 4 against *S. aureus* were observed.

Conclusion: The potential of mutant actinomycetes has been realized, and hence opens exciting avenues in the field of biotechnology and biomedical research.

Key words: *Streptomyces*, Sodium azide, Wild type, Mutants, GOF, LOF

INTRODUCTION

Actinomycetes are gram positive, filamentous bacteria, with high G+C content (69-78%) in DNA exhibiting highly differentiated developmental cycle (Williams et al. 1989), inhabiting a wide range of habitats. Unlike bacteria, actinomycetes are unique in their morphology with extensive branching substrate and aerial mycelium bearing chain of arthrospores. Of the 22,000 known microbial secondary metabolites, 70% are produced by actinomycetes, and two thirds of them are contributed by the genus *Streptomyces* (Subramani and Aalbersberg 2012). Actinomycetes contain about 40 families and over 170 genera and about 2000 species have been validly described and published (Harwani 2013).

Actinomycetes are numerous and widely distributed in soil, compost etc. and are next to bacteria in abundance. The most common genus of actinomycetes in soil is *Streptomyces* that produces straight chains or coils of spores or conidia. More than one-half of the antibiotics used in human medicine, including aureomycin, chloromycetin, kanamycin, neomycin, streptomycin, and terramycin, come from soil actinomycetes. The smell of freshly turned soil is due to metabolic end products called geosmins that are produced by these organisms and move through soil as unseen volatiles (Dindal 1990; Sylvia et al. 2005). This study will be useful for determination of novel strains of actinomycetes and check the potency by mutated by sodium azide

of antimicrobial agents against gram positive and gram negative pathogens. This novel strains will be application in pharmaceutical industry and cope the challenge of MDR. Actinomycetes are of enormous importance since they possess a capacity to produce and secrete a variety of extracellular hydrolytic enzymes (Saadoun et al. 2007; Tan et al. 2009) about 60% of the new insecticides and herbicides reported in the past 5 year originate from *Streptomyces* (Tanaka and Omura 1993). Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polypetides, B-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, anti-tumor and immunosuppressive activities (Behal 2000).

MATERIALS AND METHODS

The study was carried out in the laboratory of the Sainik Awasiya Mahavidhayala, Bhaktapur, Nepal from 2016 December to 2017 March. A total of 30 soil samples, 10 samples were collected from Tokha, 10 samples were collected from Bhaktapur area and others 10 were collected from Godawari area.First, the soil slurry was made by suspending 10 g of the collected dry soil in 90 ml distilled water.The pour plate technique was done in which 0.1 ml of sample and SCA broth was poured and incubated at 25^{O}C for 1 week. After growth of actinomycetes, the wild cultures were further streaked in other SCA plates for obtaining pure culture. Obtained pure culture was streaked in SCA incorporated with different concentration of Sodium Azide i.e. 10ppm, 20ppm, 40ppm, 50ppm and 100ppm. After obtaining the growth of actinomycetes in different concentration the mutants were compared with wild type by the help of different biochemical tests, gelatin hydrolysis test and antibiosis.

RESULTS

Out of 30 soil samples collected from different areas, 20 (67%) actinomycetes were isolated. Out of 20 (67%) actinomycetes, 6 (20%) isolates were identified as *Streptomyces* spp. which were further used in this study to generate mutagens using different concentration of sodium azide. A total of 28 mutants were isolated from 6 wild types exposed at 10ppm, 20ppm, 40ppm, 50ppm, 100ppm concentration of sodium azide.

Out of 28 mutants formed only 8 mutants from samples of Godawari showed same colony color (both dorsal and ventral) as its wild type. While other 20 mutants showed change in its colony color.

No changes in gelatin hydrolysis test of wild type and their respective mutants were observed. In sugar utilization test, different sugars i.e. arabinose, fructose, galactose, glucose, lactose, mannitol, maltose and sucrose were used where all the mutants showed color change from blue to either yellow or green indicating sugar utilization except in case of lactose where only one sample (i.e. Bhaktapur 1 mutants grown in concentration 50ppm) showed color change while others remained unchanged.

Among AST test performed against four organisms, *Pseudomonas* was not inhibited by the growth of actinomycetes. In case of *S. aureus* 18% (5 out of 28) developed resistance. 14% (4 out of 28) in case of *S.* Typhi and 11% (i.e. 3 out of 28) in case of *E. coli* developed resestance while rest of mutants were susceptible to those microorganisms.

In case of morphology, only three mutants showed GOF out of 28 mutants only 11%. In sugar utilization test, most mutants seem to metabolise glucose while in galactose and lactose, only one mutant out of 28 showed gain of function. In AST, mutants could not develop GOF. Out of 28 mutants, 2 mutants showed LOF on the basis of pigmentation. In case of glucose and lactose, no any mutants showed loss of function. While in AST, only two mutants failed to act against *S. aureus* while there is no LOF in other mutants.

Table 1: Comparison of AST between wild types and mutants

	Zone of inhibition given by antibiotics from/mm					
Test organism	**B1W**	**B1M10**	**B1M20**	**B1M40**	**B1M50**	
E.coli	6	6	6	6	6	
Pseudomonas	6	6	6	6	6	
S. Typhi	6	16	6	6	6	
S. aureus	16	16	16	16	16	
	B2W	**B2M10**	**B2M20**	**B2M40**	**B2M50**	**B2M100**
E.coli	6	6	6	6	16	6
Pseudomonas	6	6	6	6	6	6
S. Typhi	6	6	16	6	6	6
S. aureus	16	16	6	36	36	36

	Zone of inhibition given by antibiotics from/mm					
Test organism	B1W	B1M10	B1M20	B1M40	B1M50	
	G1W	G1M10	G1M20	G1M40	G1M50	G1M100
E.coli	6	6	6	6	6	6
Pseudomonas	6	6	6	6	6	6
S. Typhi	16	16	16	16	16	16
S. aureus	6	6	6	6	6	6
	G2W	G2M10	G2M20	G2M40	G2M50	G2M100
E.coli	6	6	6	6	26	6
Pseudomonas	6	6	6	6	6	6
S. Typhi	6	6	6	26	6	6
S. aureus	6	16	6	6	6	6
	T1W	T1M10	T1M20	T1M40	T1M50	T1M100
E.coli	6	6	6	6	16	6
Pseudomonas	6	6	6	6	6	6
S. Typhi	6	6	6	6	16	6
S. aureus	26	6	6	6	6	16
	T2W	T2M10	T2M20	T2M40		T2M100
E.coli	6	6	6	6		6
Pseudomonas	6	6	6	6		6
S. Typhi	6	6	6	6		6
S. aureus	6	6	6	6		6

Note: 6mm= diameter of well, 1 and 2 are the different samples where B is bhaktapur, G is Godawari, T is Tokha and 10, 20, 40, 50 and 100 are sodium azide concentration.

DISCUSSION

In this study, out of 30 samples processed 6 isolates were identified as *Streptomyces* spp on the basis of microscopy and sugar fermentation tests. Studies carried out by Iwami et al. (1986); Kavita and Vijayalakshmi (2007); Wijittra et al. (2006) also suggest that *Streptomyces* spp. exist as the major component of actinomycetes population isolated from soil.

In this case, out of 28 actinomycetes isolates were exposed to five different concentrations of sodium azide, i.e.10ppm, 20ppm, 40ppm, 50ppm, 100ppm. Out of which, 26 (92.9%) isolates were tolerant except B1M100 and T1M50 which account for 7.14%. These two mutants were found to be sensitive to sodium azide. However, in study done by Shrestha et al. (2015), out of total 38 actinomycetes isolates, 2 (5.3%) were highly sensitive, 23(60.5%) were moderately sensitive and 13 (34.2%) were tolerant to sodium azide. Out of total isolates two (2/38; 5.3%) were highly sensitive and couldn't grow even on 10 ppm.

On doing antibiosis, these mutants couldn't develop antagonistic activity against *Pseudomonas*. Out of 28 mutants only 12 mutants showed antagonistic activity against pathogens which accounts for 42%. While in case of Kumar et al. (2011) out of 56 strains, only ten strains showed higher antagonistic activity against all the tested human bacterial pathogens which counts for 17%.

All the mutants in our case have shown sugar utilization in arabinose, fructose, galactose, glucose, sucrose, maltose and mannitol except in lactose while in case of Subedi et al. (2015) all mutants had one or more positive mutations (GOF) of fructose, mannitol, arabinose and salicin utilization.

CONCLUSION

From the finding of this study carried out by collecting soil samples from Bhaktapur, Godawari, Tokha and their periphery, it can be concluded that species of *Streptomyces* were the major among actinomycetes present in the soil. Furthermore, mutants obtained by treating those *Streptomyces* with mutagen i.e. Sodium azide at different concentrations viz. 10, 20, 40, 50 and 100 ppm can affect their colony morphology, enzymatic activities, sugar utilization pattern and antibiosis.

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Rotavirus Infection among Diarrhoeal Children under 10 Years of Age Visiting a Children's Hospital in Kathmandu, Nepal

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ABSTRACT

Objectives: The present study was conducted to assess the rate of rotavirus causes of diarrhoea among children under 10 years of age visiting a children's hospital in Kathmandu, Nepal.

Methods: This study was carried out from October 2010 to July 2011. During the study, a total of 1068 diarrheal stool samples were collected from inpatient and outpatient department of children less than 10 years of age attending Kanti Children's Hospital and processed in Public Health Research Laboratory, Tribhuvan University Teaching Hospital (TUTH). About 5 ml of fresh diarrhoeal stool samples was collected and were transferred into vials which were preserved by adding buffer solution for detection of rotavirus. Samples for rotavirus antigen detection were detected by using Enzyme Immuno Assay. Data were entered into Microsoft excel and SPSS and analysis was done employing Chi-square test.

Results: Among 1068 total cases, 22.0% (235/1068)children were infected with rotavirus which was found to be highest in inpatient (26.2%) with severe case (37.1%)then outpatients (12.1%). Rotavirus were found to be highest in male children (22.9%)under 2 years (23.8%)of age group. Among all season, the rotavirus infection was highest in winter seasons (29.6%) followed by spring season (26.7%) and others. According to father occupation and mother education, the prevalence of rotavirus was found to be high in children with fathers having occupation labor (27.8%) and found to be higher in children with illiterate mother (38.9%).

Conclusion: The routine diagnosis of rotavirus in Nepal is not done. So it can be concluded that routine stool examination should include detection of Rotavirus antigen in diagnosis of acute pediatrics diarrhoeal illness which helps in manangement for diarrhoeal population.

Key words: Diarrhoea, Rotavirus, ELISA, Nepal

INTRODUCTION

Diarrhoea remains the second leading cause of death among children under five globally. Nearly one in five child deaths about 1.5 million each year is due to diarrhoea. Diarrhoea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria, viruses and protozoa (WHO, 2009).Cumulative data from epidemiological studies show that approximately 20-50 % of diarrhoeal cases are attributable to known bacterial or parasitic pathogens, which suggests that viruses may be responsible for the remainder (Black et al. 2001).

Five million children under the age of 2 years die from diarrhoeal disease in developing countries each year, rotavirus infections account for about 20.0% of these deaths (Desselberger et al. 2006).Rotavirus infections occur worldwide. Globally, each year, 2 million children are hospitalized and 700,000 children die due to rotavirus diarrhea (Uchida et al. 2006). Rotavirus is estimated to cause about 40.0% of all hospital admissions due to diarrhoea among children under five years of age worldwide leading to some 100 million episodes of acute diarrhoea each year that result in 350,000 to 600,000 child deaths (WHO, 2009).

Among three human Rotaviruses (A, B and C), group A Rotaviruses have been established as causing several diarrhoeal diseases in infants and young children worldwide (Estes 2001). By the age of five, nearly every child in the world has been infected with Rotavirus at least once (Velazquez et al. 1996). However, with each infection, immunity develops and subsequent infections are less severe. So adults are rarely affected (Bishop 1996).

Diarrhoeal disease occupied the second place among the top ten diseases in Nepal (NPR, 2007). It has been estimated that approximately 25% of child death are associated with diarrhoeal diseases, particularly acute diarrhea (Sherchand et al. 2011). Studies published on rotavirus infection from 1999 to 2007 showed rotavirus positivity rates ranged from 17.0-39.0% (median 31.8%) in among all hospitalized children less than 5 years (Shariff et al. 2004; Sherchand et al. 2004; Uchida et al. 2006; Pun 2007; Sherchand et al. 2009;).

In Nepal rotavirus is a leading cause of acute diarrhoea in children less than 5 years old, accounting for up to 38.0% of all diarrhoea cases (Black et al. 2001; Sherchand et al. 2009) however, the routine diagnosis of rotavirus is not done in Nepal. This study aims to explore the rate of Rotavirus infection among children's visiting the tertiary hospital of Nepal. This study will reveal the current status of Rotavirus infection in Nepal and helps in the managements of such population.

MATERIALS AND METHODS

This study was carried out from October 2010 to July 2011. During the study, a total of 1068 diarrheal stool sampleswere collected from inpatient (747) and outpatient (321) department of children less than 10 years of age attending Kanti Children's Hospital and processed in Public Health Research Laboratory, Tribhuvan University Teaching Hospital (TUTH). During the visit on Children's Hospital for collection of sample, questionnaires on different demographics were filled up. Verbal consent was obtained from patient parents. About 5 ml of fresh diarrhoeal stool samples was collected from babie's diapers or clean bed pans or direct in possible cases with a clean plastic spatula in Clean, leak proof, screw capped plastic container. Samples were transported to the laboratory as soon aspossibleand subjected to macroscopic examination in which the direct visualization of each sample was done for the color, consistency and presence of mucus, blood. Then samples were transferred into vials which were preserved by adding buffer solution for detection ofrotavirus. Samples for Rotavirus antigen detection were detected by using an antigen detection test (ELISA kit of Premier Rotaclone; Meridian Bioscience, USA), according to the instructions of the manufacturer.

Data were entered into Microsoft excel and SPSS and analysis was done employing Chi-square test. The Chi-square test was performed with a significance level of< 0.05 for statistical analysis.

RESULTS

Out of total stool samples, 22.0%of children were found to be infected with rotavirus in which inpatient children showed the highest positive cases (26.2%) than outpatient children which was 12.1%.

Table 1: Distribution of Rotavirus among patients depending on hospital admission

Patient	Total N (%)	Rotavirus	Negative	P-Value
		N (%)	N (%)	
Inpatient(ORT)	747(70.0)	196 (26.2)	551(73.8)	< 0.05
Outpatient(OPD)	321(30.0)	39 (12.1)	282(87.8)	
Total	**1068 (100)**	**235 (22.0)**	**833(78.1)**	

The diarrheal children with different symptoms cases and severe cases showed higher rotavirus infection which were 76.4% and 37.1% respectively.

Table 2: Distribution of Rotavirus according to clinical symptoms and clinical cases

Different clinical symptoms	Total Cases N (%)	Rotavirus N (%)	Negative N (%)
Yes	816 (76.4)	199 (24.4)	617 (75.6)
No	252 (23.6)	36 (14.3)	216 (85.7)
Total	1068(100)	235 (22.0)	833(78.0)
Different Clinical Cases			
Severe	321 (30.0)	119 (37.1)	202 (62.9)
Moderate	426 (40.0)	77 (18.1)	349 (82.0)
Mild	321 (30.0)	39 (12.1)	282 (87.8)
Total	**1068(100)**	**235 (22.0)**	**833(78.0)**

Among total rotavirus infected children, male children (22.9%) were more infected than female children (20.6%).

Table 3: Gender wise distribution of Rotavirus infection among study population

Gender	Total N (%)	Rotavirus	Negative	P-Value
		N (%)	N (%)	
Male	669(62.6)	153 (22.9)	516(77.1)	> 0.05
Female	399(37.4)	82 (20.6)	317(79.4)	
Total	**1068(100)**	**235 (22.0)**	**833(78.0)**	

According to age of children, rotavirus infection was found to be highest in 2 years and under 2 yearschildren (23.8%).

Table 4: Age wise distribution of Rotavirus among study population.

Age groups in years	Total enrolled cases N (%)	Rotavirus N (%)	Negative N (%)
≤2	835 (78.3)	199 (23.8)	636 (76.2)
3-4	149 (13.9)	26 (17.4)	123 (82.6)
5-6	40 (3.8)	6 (15.0)	34 (85.0)
7-8	23 (2.1)	3 (13.0)	20 (87.0)
9-10	21 (2.0)	1 (4.7)	20 (95.2)
Total	**1068 (100)**	**235 (22.0)**	**833 (78.0)**

Among all season, the rotavirus infection was highest in winter seasons (29.6%) followed by spring season (26.7%) and others.

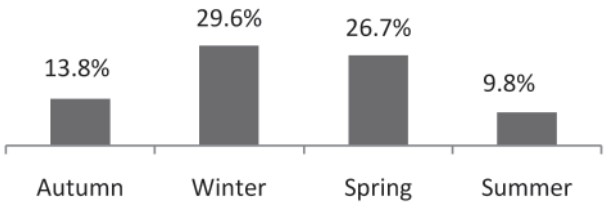

Figure 1: Season wise distribution of Rotavirus infection among study population

Among study population, the prevalence of rotavirus was found to be high in children with fathers having occupation labor (27.8%) and found to be higher in children with illiterate mother (38.9%).

Table 5: Characteristics of family background and Rotavirus among study population

Father's occupation	Rotavirus N (%)	Total N (%)
Business	29 (19.6)	148 (13.9)
Service	49 (25.9)	189 (17.7)
Agriculture	57 (20.8)	274 (25.7)
Labor	55 (27.8)	198 (18.5)
Driving	38 (18.9)	201 (18.8)
Other	7 (12.1)	58 (5.4)
Total	**235 (22.0)**	**1068 (100)**
Mother's education		
None	91 (38.9)	267 (25.0)
primary	58 (19.7)	364 (34.1)
Secondary	49 (18.9)	258 (24.2)
Higher level	37 (20.1)	179 (16.8)
Total	**235 (22.0)**	**1068 (100)**

DISCUSSION

As in other developing countries, diarrhoeal diseases are one of the primary health problems in Nepal. Rotavirus was one of leading diarrhoeal causing agent which was 22.0% positive from this study. This finding is in harmony with the study carried out by Saravanan (2004) in Chennai, South India in which the overall infection rate was to be 22.6% among children with acutediarrhoea.But some studies (Meqdam et al. 1997; Youssef et al. 2000 ; Rerksuppaphol et al. 2011) showed 33% to 40% of Rotavirus causing diarrhoea.

Rotavirus was higher in inpatient which was 196 (26.2%) than in outpatients which was 36 (11.2%) and the result was significant statistically (P <0.05). The proportion of rotavirus positive cases was always higher among hospitalized children than non-hospitalized children with acute gastroenteritis (Michiyo et al. 2004). From previous report, Rotavirus infection was found in 30.0-50.0% with acute gastroenteritis from hospitalized and none hospitalized infants and young children (Shariff et al. 2004; Sherchand et al. 2009). These all findings concur with this finding.

In this study, the clinical features of patients in relation to the microorganisms were isolated by a physician as having diarrhoea. Diarrhoea may be accompanied by cramping, abdominal pain, bloating, nausea, or an urgent need to use the bathroom. Among inpatient, 816 cases showed symptoms in which 663 cases has symptoms like nausea, vomiting, diarrhoea and fever whereas, 153 cases has other medical condition like pneumonia, enteric fever, typhoid etc. other medical condition children infected with Rotavirus is the important cause of hospital acquired infection (Chandran et al. 2006), Rotavirus were highest positive in systemic cases 24.4% (199/816). Though the result was significant statistically (P <0.05). On basis of patient's condition and physician reports, patients were categorized into three groups i.e. severe, moderate and mild. Most of the cases were moderate 426 (40.0%) followed severe 321 (30.0%) and mild 321(30.0%) in which highest Rotavirus seen in severe case (37.1%) with intravenous fluid treatment. Rotavirus is the most common cause of severe diarrhoea among infants and young children.

The diarrhoeal disease treatment include zinc supplementation, oral rehydration solution (ORS), antibiotic, IV etc. Rotavirus doesn't respond to antibiotic. ORS are only effective in the treatment of dehydration caused by Rotavirus but donot kill it. There are no antiviral agents available for the treatment of Rotavirus infection in Nepal.

The gender wise distribution of Rotavirus infection was found to be higher in male patients (22.9%) which was found statistically insignificant. At similar result was also found by Rerksuppaphol and Rerksuppaphol (2011) in which male were higher in proportion (68.6%) with Rotavirus. The study carried out in Nigeria also found the higher prevalence of 57.1% in male (Tinuade 2006). However, no association of rotavirus infection could be discerned between male (23.9%) and female (21.1%) children in a study carried out by Saravanan (2011).

According to the distribution of diarrhoea cases among the different age groups showed that the children less than 2 years (78.3%) were more infected than other age groups which was 23.8% i.e. 199 out of 835. Most of infected children in this study were under 2 years of age, with highest prevalence between 6 and 12 months. The greater risks of infants and young children in the period between 6 to 12 months with declined levels of maternal antibodies to rotavirus infection have been documented (Mata 1983 and Zheng 1992).

Under 2 years children, according to type of nutrition (breast feeding vs. bottle feeding) rotavirus found to be highest in breast feeding children than bottle feeding children. This may be due to the apparent lack of protection against rotavirus infection in this age group may be partly due to reduced total daily intake of breast milk or to the reduced amount of rotavirus antibody in mature breast milk or to both. However, study in Pakistan found that bottle feeding infants were about two folds higher rotavirus positive than in breast feeding infants which is not consistent with this finding. This may be different in sample sizes and types of nutrition intake along with breast milk (Kazemi 2001). Observations in studies suggested that active immunization through repeated exposure and prolonged breastfeeding may protect against the diarrhoeagenic effect of these agents (WHO 2009).

The highest positive cases of Rotavirus was observed in winter season constitute 29.6% followed by spring season 26.7%, autumn season 13.8% and summer season 9.8%. Similarly, Study conducted in Pakistan showed highest Rotavirus (41.4%) in winter with

highest prevalence in January (63.6%) (Kazemi 2001). The result was consistent with the finding of Sherchand et al. (2004) in which Rotavirus was predominant in winter particularly in December to February. In contrast a study by Naficy et al. (1999) found that 90% of Rotavirus diarrhoeal episodes occurred between July and November. Saudi Arabia reported a peak for Rotavirus isolate during dry, hot season (Al-Bwardy et al. 1988). Rotavirus is a disease of later warm and early cool season but seasonally may vary year to year.

The diarrhoeal disease also related with socio economic status of family. So, in this study occupation of father was also mentioned. The rotavirus was found to be high in children with fathers having occupation labor (27.8%). Due to low income in labour family, children may not get sufficient food, hygienic condition, treatment etc. The prevalence of rotavirus (38.9%) found to be higher in children with illiterate mother. This may suggests that it was the better hygiene knowledge and practice of literate mothers that reduces the risk of childhood diarrhoea than illiterate mother. Literate mothers were more likely to seek medical care for a child with diarrhoea.

CONCLUSION

The incidence of diarrhoea has been drastically decreased due to improved sanitation and drinking water. However, winter diarrhoea did not reduce significantly, which could be attributed to Rotavirus infection. The routine diagnosis of Rotavirus in Nepal is not done. So it can be concluded that routine stool examination should include detection of Rotavirus antigen in diagnosis of acute pediatrics diarrhoeal illness which helps in manangement for diarrhoeal population.

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Prevalence of Extended Spectrum Beta Lactamases (ESBL) and Metallo Beta Lactamases (MBL) Mediated Resistance inGram Negative Bacterial Pathogens

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ABSTRACT

Objectives: This study was conducted to determine the prevalence of multi-drug resistance (MDR) along with Extended Spectrum β-lactamase (ESBL) and Metallo β-lactamase (MBL) producing gram negative bacterial isolates among the patients attending Shahid Gangalal National Heart Centre, Kathmandu, Nepal.

Methods: This cross-sectional study was carried out from June to December; 2016. Altogether 977 clinical specimens were processed for analysis of bacteriological profile and the isolates were identified by culture, morphological and biochemical tests. Antibiotic susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion methods following Clinical and Laboratories Standard Institute guideline and the isolates were tested for ESBL and MBL by combined disk method.

Results: out of 977 clinical specimens, 254 **(**25.99%) were found to be gram negative bacterial isolates, among them *Klebsiella pneumoniae* 83 (32.67%) was the most predominant organism followed by *E. coli* 51 (20.07%), *Pseudomonas aeruginosa* 36 (14.17%), *K. oxytoca* 32 (12.59%), *Proteus mirabilis* 13 (5.11%) and *P. vulgaris* 13 (5.11%), *Acinetobacter* spp. 11 (4.33%), *Citrobacter* spp. 10 (3.93%) and *Enterobacter* spp. 5 (1.96%) respectively. 83 (32.67%) isolates were found to be MDR, 38(14.96%) were positive for ESBL while 19 (7.48%) were MBL producer.

Conclusion: The determent drug resistance among ESBL and MBL producers, reflect the extensive use of antibiotics possessing difficulties in therapeutic potions in hospital setting which might be overcome by proper microbiological analysis of pathogenic isolates and judicious use of antibiotics for emergence of resistance strains.

Key words: Clinical Specimen, Gram negative bacteria, MDR, ESBL, MBL

INTRODUCTION

Antibiotics have been critical in the fight against infections caused by bacteria and other microbes. The most common and important mechanism of resistance in Gram-negative bacteria is production of β-lactamases. Antibiotic resistance is particularly rising because of inappropriate use of antibiotics in human medicine (Tiersma 2013). Multidrug resistance has been increasing among Gram negative bacteria (Bush 2010). Beta- Lactamases are enzymes produced by some bacteria that hydrolyze the amide bond of the four-membered characteristic β-lactam ring (Peshattiwar and Peerapur 2011) of β-lactam antibiotics (penicillin, cephalosporin, monobactams, and carbapenems) (Noyal et al. 2009), result in an inactive product when the ring is broken (Simoens et al. 2006). In recent years, there has been an increase in incidence and prevalence of ESBL producing microbial diseases (Thokar et al. 2010). More than 200 types of extended spectrum β-lactamases (ESBLs) have been found worldwide, most belonging to the Enterobacteriaceae family (Yazdi et al. 2012). MBLs have been globally isolated from various bacteria and more than 80 types of MBLs have been identified worldwide, with over 75% occurring

as plasmid encoded enzymes (Bush and Jacoby 2010). Early detection of MBL and ESBL producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their interhospital and intrahospital dissemination (Pandey et al. 2011). Thus, the present study was conducted with an objective to find out the presence of ESBL and MBL producing gram negative bacterial isolates and multidrug resistant strains to formulate effective antibiotic strategy on the basis of the local epidemiological data to control infection and to prevent the spread of these strains.

MATERIALS AND METHODS

A total of 977 clinical specimens including blood, urine, sputum, pus, ET tip secretion, CVP tip, suction tube, Foley's tip, pericardial fluid, body fluid (peritoneal fluid), throat swab, pleural fluid, wound swab, mitral valve vegetation, mediastinal drain culture, aortic valve, tracheal aspirate wascollected in a clean, leak-proof sterile container during June, 2016 to December, 2016 from the cardiac patients attending Shahid Gangalal National Heart Center, Bansbari, Kathmandu, Nepal.

The specimens were subjected for routine culture and susceptibility testing in the microbiology department. For blood sample 3-5 ml of blood was inoculated into brain-heart infusion broth and incubated at 37°C and sub-cultures were made on blood agar (BA) and McConkey agar (MA) after 24, 48 and 72 hours. BHI broth was incubated aerobically for upto 7 days at 37 °C whereas others samples were inoculated onto BA and MA plates. The plates were incubated at 37 °C for 24 hrs. The significant bacterial isolates were identified on the basis of morphology, gram staining & various biochemical tests. Antibiotic susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method following Clinical and Laboratories Standard Institute (CLSI) guideline and they were tested for ESBL and MBL by combined disk method. *K. pneumoniae* ATCC 700603 positive controls for ESBL and *P. aeruginosa* PA 105663 positive control for MBL were used. Statistical analysis was done by using SPSS version 16. Frequency and percentages were calculated and Chi-square test was done whenever applicable with $P<0.05$ regarded as significant.

RESULTS

Among the total 977 clinical specimens for culture,295 (30.19%) showed significant bacterial growth of which 254 (25.99%) were gram negative bacteria while 41 (4.19%) were gram positive bacteria. Out of 254 gram negative bacterial isolates, 123 (48.42%) were from the samples of female and 131 (51.57%) were from male patients. Likewise, 52 (5.32%) were from outpatients and remaining 202 (20.67%) were from inpatients. However, there was significant difference in bacterial growth between the samples of outpatients and inpatients (P value = 27.16). The age of the patients ranged from 49 days to 87 years. The highest percentage of bacterial growth 7 (18.50%) was obtained in samples of age group <10yrs, followed by 44 (17.32%) in age group 51-60yrs, whereas least 6 (2.36%) was from age group 81-90 yrs. Highest percentage of growth was found to be in samples of male 11 (4.33%) in age group 51-60 yrs. whereas for Female, highest % of growth 14 (5.51%) was in age group 21-30 yrs.

Table 1: Distribution of clinical specimens

Clinical samples	Gender		In patients		Out patients		Significant growth		Total No.
	Female	Male	No.	%	No.	%	No.	%	
Blood	96	166	144	54.96	118	45.03	22	**8.39**	262
Body Fluid	1	8	7	77.78	2	22.22	2	22.22	9
CVP tip	8	19	27	100	0	0	10	37.03	27
ET Tip	26	55	62	76.54	19	23.45	40	49.38	81
Foley Tip	3	9	11	91.66	1	8.33	8	**66.67**	12
Others	19	35	43	79.62	11	20.38	11	20.37	54
Pericardial fluid	15	22	29	78.37	8	21.62	7	18.91	37
Pus	32	15	35	74.46	12	25.53	13	27.65	47
Sputum	44	40	48	57.14	36	42.85	37	44.04	84
Suction Tip	6	10	14	87.5	2	12.5	10	62.5	16
Throat Swab	5	6	8	72.73	3	27.27	2	18.18	11
Urine	189	138	211	64.52	116	35.47	92	28.13	327
Total	**447 (45.75)**	**530 (54.24)**	**647 (66.22)**		**330 (33.77)**		**254 (25.99)**		**977**

In this study, out of 7 different bacterial genera isolated, *Klebsiella pneumoniae* 83 (32.67%) was being the most predominant whereas *Enterobacter* spp. was being least 5 (1.96%). *Pseudomonas aeruginosa* was most predominant from indoor patients 34 (94.44%) while *Citrobacter* spp. was being least 4 (40%). Similarly, from outpatient, *Citrobacter* spp. was most predominant 6 (60%) whereas *P. aeruginosa* showed least growth 2 (5. 56%).

Table 2: distribution pattern of gram negative bacterial isolates from various clinical samples

Clinical samples	*Acinetobacter*	*Citrobacter*	*Enterobacter*	*E. coli*	*Klebsiella*		*Proteus*		*P. aeruginosa*	Total	
					oxytoca	*pneumoniae*	*mirabilis*	*vulgaris*		No.	%
Blood	0	0	3	5	3	6	0	1	4	22	8.66
Body fluid	0	0	0	0	1	0	0	0	1	2	0.78
CVP tip	1	0	1	2	1	4	0	0	1	10	3.93
ET tips	2	1	0	8	3	11	2	1	12	40	15.74
Foley's tip	0	0	0	1	2	2	0	0	3	8	3.14
Others	0	1	0	1	2	4	2	0	1	11	4.33
Pericardial fluid	1	0	0	1	0	1	0	0	4	7	2.75
Pus	0	1	0	1	5	4	1	1	0	13	5.11
Sputum	0	0	0	2	1	24	2	1	7	37	14.56
Suction tip	2	1	0	1	0	2	3	0	1	10	3.93
Throat swab	0	0	0	1	0	1	0	0	0	2	0.78
Urine	5	6	1	28	14	24	3	9	2	92	36.22
Total	**11**	**10**	**5**	**51**	**32**	**83**	**13**	**13**	**36**	**254**	**100**
	4.3	**3.9**	**1.9**	**20.1**	**12.6**	**32.7**	**5.1**	**5.1**	**14.2**		**100**

The most effective drug for gram negative pathogenic bacteria was nitrofurantoin followed by Gentamicin, ciprofloxacin and cotrimoxazole. The highest MDR strain was found to be *Acinetobacter* spp. 6 (54.54%) whereas *Enterobacter* spp. (0%) did not show any MDR pattern.

Table 3: Antibiotic resistance pattern of gram negative bacterial isolates

Bacterial isolates / total no.	Antibiotics (% Resistance)						
	Amp	Caz	Cipro	Cotri	Genta	Nali	Nitro
Acinetobacter (11)	7 (63.63)	8 (72.73)	2 (18.18)	4 (36.36)	3 (27.27)	7 (63.63)	0 (0)
Citrobacter (10)	7 (70.0)	6 (60.0)	3 (30.0)	4 (40.0)	3 (30.0)	6 (60.0)	0 (0)
Enterobacter (5)	4 (80.0)	1 (20.0)	2 (40.0)	0 (0)	0 (0)	3 (60.0)	0 (0)
E. coli (n=51)	43 (84.31)	36 (70.58)	21 (41.17)	21 (41.17)	17 (17.64)	31 (60.78)	4 (14.28)
K. oxytoca (32)	23 (71.87)	14 (63.85)	11 (34.37)	10 (31.25)	9 (28.12)	27 (84.37)	0 (0)
K. pneumonia (83)	68 (79.06)	53 (63.85)	17 (20.48)	30 (36.14)	13 (15.66)	48 (57.83)	2 (8.33)
P. mirabilis (13)	5 (38.46)	6 (46.15)	2 (15.38)	4 (30.76)	2 (15.38)	4 (30.76)	0 (0)
P. vulgaris (13)	4 (30.76)	8 (61.53)	5 (38.46)	5 (38.46)	8 (61.53)	8 (61.53)	1 (11.11)
P. aeruginosa (36)	29 (80.56)	26 (72.22)	14 (38.89)	9 (25.0)	10 (27.78)	27 (75.0)	0 (0)
Total (254)	**190 (74.48)**	**158 (62.2)**	**77 (30.31)**	**87 (34.25)**	**65 (25.59)**	**161 (63.38)**	**7 (2.75)**

Table 4: Distribution of MDR, ESBL and MBL producer among gram negative bacterial isolates

Bacterial isolates	No.	MDR strains No. (%)	ESBL producer No. (%)	MBL producers No. (%)
Acinetobacter	11	6 (54.54%)	0	1 (9.09)
Citrobacter	10	3 (30%)	2 (20%)	0
Enterobacter	5	0	0	0
E. coli	51	19 (37.25%)	6 (54.54%)	2 (3.92%)
K. oxytoca	32	7 (21.87%)	5 (15.62%)	0
K. pneumonia	83	29 (34.93%)	13 (15.66%)	7 (8.43%)
P. mirabilis	13	2 (15.38%)	1 (7.69%)	0
P. vulgaris	13	1 (7.69%)	1 (7.69%)	0
P. aeruginosa	36	16 (44.44%)	4 (11.11%)	9 (25%)
Total	**254**	**83 (32.67%)**	**38 (14.96%)**	**19 (7.48%)**

The highest no. of ESBL producer was isolated from ET Tip 10 (25%) and least from pus 1 (7.69%). Similarly, for MBL producers, it was throat swab 1 (50%) and least from urine 3 (3.26%). The Antibiotic susceptibility pattern of ESBL producers revealed that, they were 100% resistant against ampicillin and ceftazidime but sensitive towards nitrofurantoin (100%) followed by imipenem (81.58%), amikacin (73.7%), ofloxacin (63.2%) gentamicin (57.9%) and cotrimoxazole (50%). MBL producers showed resistance towards most of the drug used and were sensitive towards polymyxin.

DISCUSSION

This study was aimed to examine the status of MDR among different gram negative bacterial pathogens and underlying production of ESBLs and MBLs. The highest percentage of sample obtained was urine (33.46%) whereas body fluid i.e. peritoneal fluid (0.92%) was found in a least percentage which was correlated with the study done where higher percent of sample collected was urine (Sherchan et al. 2016). The frequency of growth pattern among individual specimens were highest from Foley tips (66.67%) similar to the study done by Sherchan et al. 2016 which shows 61.11% from catheter, whereas least growth obtained from blood sample (8.39%), a similar study conducted in western Nepal in 2007 showed the isolation rate from blood was 10.28% (Easow et al. 2010) and a study by (Vanitha et al. 2012) also showed 8.39%.This least growth from blood sample might be due to patient may have already taken antibiotics or he or she may not have systemic infection so far. and high % of urinary catheter use is a risk factor for inoculation of bacteria into bladder.No significant difference in growth number between male and female patients revealed that both of them have equal chance of having heart diseases. *Enterobacter* spp. were least isolates to cause infection predominated from blood sample whereas *E. coli* (20.07%) was second predominant organism from urine after *Klebsiella pneumonia* (32.67%) from urine and sputum sample contrast to a study (Chander and Shrestha 2013), reported low prevalence of *K. pneumoniae* from urine sample. More isolation rate of *K. pneumoniae* due to contamination of invasive devices, weakened immune system, nosocomial infection. Whereas *E. coli* was predominant in urine sample, resembled the study done by various others workers viz: (Shrestha et al. 2012), (Mishra et al. 2012) and (Manandhar et al. 2006) in Nepal. This high incidence of the *E. coli* is a commensal of the bowl and infection due to poor hygiene and anatomy proximity to the genito-urinary area. Highest percentage of resistance towards first line antibiotic ampicillin, Nalidixic acid, and ceftazidime with considerable resistivity to ampicillin (74.48%) which was found to be congruous with the study done by (Bhatt et al. 2012).

In this study, no MDR strains were observed in *Enterobacter* spp. while highest number of MDR was found in *Acinetobacter* spp. (54.54%) whereas lowest from *P. vulgaris* (7.69%). These results were less than outcomes of previous studies (Karn et al. 2016) which showed 42.91% were MDR, and *Acinetobacter* spp. account for 60%. This is due to drugs are easily available without doctor's prescription from pharmacy and in developing countries like Nepal self-medication is a common practice. ESBLs were predominantly present among *E. coli* (23.52%) followed by *Citrobacter* spp. similar to the study done by Pokhrel et al. in which 16.0% isolates were found to be ESBL producing with *E. coli* being the predominant one (11.60%) (Pokharel et al. 2006) and also study done by (Ahmed et al. 2014) in which 18.95% were

ESBL producers. 3rd generation Cephalosporins were combined with β-lactamases inhibitor Clavulanic acid (i.e. CTX30 + Clav10 and CAZ 30+ Clav10), structural analog of β-lactamase and antibiotics inhibits the action of β-lactamases and antibiotic can act on the cell wall of the bacteria, result confirmed by at least or more than 5mm increase in zone of inhibition than Cephalosporins alone (Rawat and Nair 2010). *K. pneumoniae* responsible for ESBL and MBL production resulting a potential threat to hospitalized patients by limiting therapeutic option (Bora et al. 2014). ESBLs are mostly produced by *E. coli* and *Klebsiella* spp. but may also occur in other Gram-negative bacteria including *Citrobacter* spp., *Morganella* spp., *Proteus* spp., *Pseudomonas* spp., *Salmonella* spp. *Serratia* spp. and *Shigella* spp. (Akujobi and Ewuru 2010). A (Dalela 2012) study showed that imipenem is the most active drug for the treatment of infections which are caused by ESBL producers, followed by Amikacin which is similar to our finding. 7.48% isolates were found to be MBL producers which is higher than Mishra et al. 2012 (1.3%) and lesser than Haider et al. 2014 (17.93%) highest MBL producer being *Pseudomonas aeruginosa* (Mishra et al. 2012) and (Haider et al. 2014). And similar to previous study conducted at SGNHC which showed 8.4% were MBL producers (Chaudhary et al. 2016). Our findings of *P. aeruginosa* (25%) as the most common MBLs producer followed by *Acinetobacter* spp., *K. pneumoniae* and *E. coli* being least MBL producer which is exactly similar with Kamble 2015 (Kamble 2015) in which *P. aeruginosa* (23.62%), higher than study done in India by Agrawal 2008 which showed only 8.05% (Agrawal et al. 2008). It is a nosocomial pathogen of particular clinical concern not only because of its extraordinary resistance mechanisms but also for its formidable ability to adept very well to the hospital environment.

CONCLUSION

The prevalence of Gram negative bacteria based upon observed in 977 clinical samples was found to be 25.99% and an antibiogram, revealed the presence of ESBL and MBL enzyme in multidrug resistant gram-negative isolates which reflects the extensive use of antibiotics for the treatment in hospitals. Therefore, proper identification of isolates using microbiological tools should be undertaken. These types of study could help to estimate and to employ effective antimicrobial strategy so that the emergence of resistant strains could be reduced.

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Bacteriological Profile and Antibiogram of Bacterial Isolates from Pus Samples in Tertiary Care Hospital of Kathmandu

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ABSTRACT

Objectives: The main aim of the study was to isolate and identify the bacterial agent and to determine the susceptibility pattern of isolates to different antibiotics.

Methods: Thisretrospective study was conducted from February to October 2015 in microbiology laboratoryof All Nepal Hospital Kathmandu, Nepal. The clinical specimens were processed for isolation and identification of bacteriafollowing standard microbiological procedures. Antibiotic susceptibility pattern of isolates were determined according to CLSI guidelines (CLSI 2014)

Results: A total of 271 clinical specimens were processed where 164 (60.5%) showed growth positivity. A total 164 bacterial isolates were detected among which 84 (51.22%) were Gram positive 80 (48.78%) were Gram negative bacteria. Thirteen different species of bacteria were isolated. The most prevalent isolate was *Staphylococcus aureus* 53 (32.30%) followed by *E. coli* 34 (20.80%), (CoNS) 15 (9.10%), *Klebsiella pneumoniae* 15 (9.10%), *Enterococcus fecalis* 12(7.30%), *Pseudomonas aeruginosa* 10 (6.10%), *Acinetobacter* spp. 7 (4.30%) *Citrobacter* spp., *Proteus* spp., *Klebsiella oxytoca* were less common. *S. aureus* was most susceptible to Amikacin.Vancomycin was the most effective drugs for *Enterococcus fecalis*. Among Gram negative bacteria *E. coli* was found most sensitive to Polymyxin B (100%) and Imipenem (76.5%) where *Pseudomonas aeruginosa* was sensitive to, Amikacin, Imipenem (80%). Polymyxin B was the most effective drugs for *Klebsiella pneumoniae*. *Acinetobacter* spp. was found highly resistant to different antibiotics.

Conclusion: Antibiotic susceptibility evaluation showed Aminoglycosides, Phenicols Polymyxin, and Imipenem was the most effective drugs overall.

Key words: Wound infection, *Staphylococcus aureus,* Antibiogram, Aminoglycosides

INTRODUCTION

Skin, the largest organ in the human body, plays a crucial role in the sustenance of life through regulation of water and electrolyte balance, thermoregulation, and by acting as a barrier to external noxious agents including microorganisms (Zafar et al. 2008). Agents that causes wound infection can be classified on the basis of depth of wound and likelihood that they serve as the carrier for organisms that cause infection (Shrestha 2009).

There are three major sources of wound contaminants-exogenous sources (i.e. water-borne from water related injury or microorganisms from soil in a soil-contaminated injury or air-borne), endogenous source (i.e. microorganisms colonizing sweat glands, hair follicles or mucosa of gastro intestine, oropharynx, genitourinary tract) and the surrounding skin (File and Tan 1995; Acharya et al. 2008). Presence of pathogenic bacteria in wound doesn't imply infection. Infection occurs when one or more of the contaminants evades the clearing effect of the host's defenses, replicates in large numbers, attacks and harms the host's tissues (Colle et al. 1996). Wound infection is a major problem in Nepal. A complication of wound infection is very common because of poor hospital management and poor aseptic techniques used in hospitals during surgical procedure or other hospital procedure.

MATERIALS AND METHODS

A retrospective study was conducted February to October 2015 in the laboratory of All Nepal Hospital in order to find out the causative agent of wound infection and their antibiotic susceptibility pattern. The population for this study was both inpatient (admitted to different wards) and outpatient who had been requested for culture and antibiotic susceptibility from suspected wound infections by the medical practitioners or by physicians Macroscopic examination was carried out to note the colour, consistency and the presence of granules. All wound swab specimens were inoculated on Blood Agar (BA) plate, MacConkey Agar (MA) and nutrient agar (NA) and incubated at 37^{o} for 18-24 hours. (Benson 2001; Cheesbrough 2006) Preliminary identification of bacterial isolates were done bypigmentation, haemolysis on BA and also by Gram staining Conventional biochemical tests were performed from primary cultures for identification of the isolates. Gram negative rods were identified by performing a series of biochemical tests namely: catalase test, oxidase test methyl-red (MR) test, Voges-Prouskaure (VP) test indole test, motility ,hydrogen sulphide (H_2S) production test, triple sugar iron (TSI), Citrate utilization and urease test. Gram positive cocci were identified by catalase test, oxidase test, OF test and coagulase test (Benson 2001; Cheesbrough 2006).

Antibiotic Susceptibility Testing (AST): The antibiotic susceptibility of isolates were determined by Kirby- Bauer disc diffusion method using Clinical and Laboratory Standard Institute (CLSI) guidelines (2014). Antibiotics used were Amoxicillin, Amikacin, Azithromycin, Cefixime,,Cefotaxime, Cefoxitin, Ceftazidime, Chloramphenicol, Colistin, Co-Trimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nalidxilic acid, Nitrofurontoin, Norfloxacin, Ofloxacin, Piperacillin/ Tazobactam, Polymyxin B and Tigecycline. Those isolates which were non susceptible (either a resistant or intermediate) to three or more antibiotic classes were regarded as MDR (Magiorakos et al. 2011).

RESULTS

Out of 271 samples studied 153 (56.5%) were pus swab and 118 (43.5%) were aspirated pus in which 164 (60.5%) samples showed bacterial growth while 107 (39.5%) samples showed no growth. Among 164 positive samples, 89 (54.3%) were aspirated pus and 75 (45.7%) were pus swab that shown growth positive.

Distribution of Bacterial agents

Among 164 growth positive sample most prevalent bacteria was *S. aureus* (32.3%) which was followed by *E. coli* (20.7%). The least isolated bacteria was *Proteus mirabils* (0.6%).

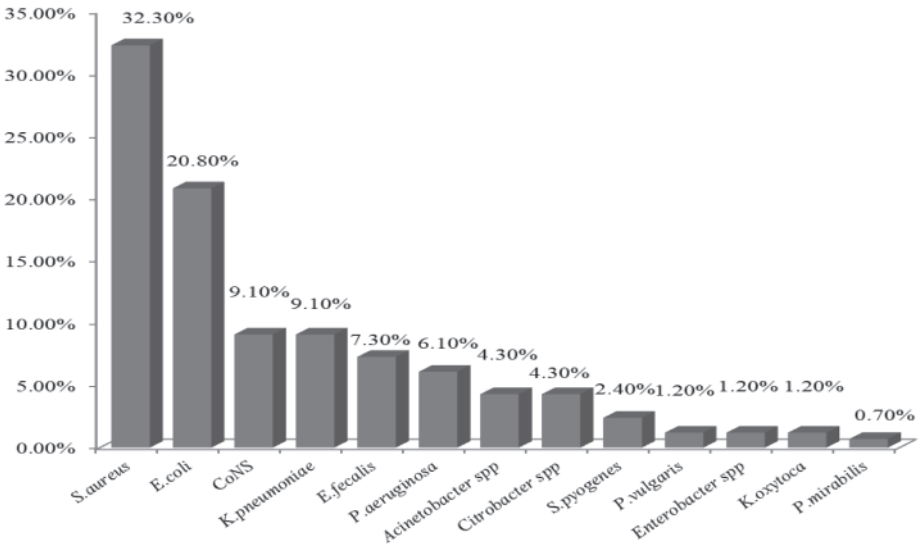

Figure 1: Percentage distribution of total bacterial isolates from wound

Antibiotic Susceptibility Pattern of the Bacterial Isolates

Antibiotic susceptibility pattern of *E. coli:* Among different antibiotics used the most effective antibiotic was Polymyxin B which was 100% sensitive followed by Imipenem (76.5%) and Amikacin (70.6%). The least effective antibiotic was Ampicillin (100%) resistant followed by Cefepime and Ceftazidime were (88.2%) resistant.

Table 1: Antibiotic susceptibility pattern of *E. coli*

Antibiotics	Sensitive		Intermediate		Resistant		Total
	No	%	No	%	No	%	
Ampicillin	-	-	-	-	34	100	34
Cotrimoxazole	9	26.5	-	-	25	73.5	34
Ciprofloxacin	6	17.6	3	8.8	25	73.5	34
Chloramphenicol	19	55.9	2	5.9	13	38.2	34
Imipenem	26	76.5	2	5.9	6	17.6	34
Amikacin	24	70.6	-	-	10	29.4	34
Gentamicin	23	67.6	-	-	11	32.4	34
Cefepime	4	11.8	-	-	30	88.2	34
Ceftazidime	4	11.8	-	-	30	88.2	34
Cefotaxime	10	29.4	-	-	24	70.6	34
Tetracycline	5	14.7	2	5.9	27	79.4	34
Polymyxin B	34	100	-	-	-	-	34
Piperacillin/Tazobactam	17	50	9	26.5	8	23.5	34

Antibiotic susceptibility pattern of *Pseudomonas aeruginosa:* The most effective antibiotic for the *Pseudomonas aeruginosa* was Amikacin, Chloramphenicol, and Imipenem having the sensitivity of 80% followed by Gentamycin and Piperacillin/Tazobactam of 70% sensitivity. The least sensitive antibiotic was Tetracycline (20%).

Table 2: Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotics	Sensitive		Intermediate		Resistant		Total
	No	%	No	%	No	%	
Ampicillin	-	-	-	-	10	100	10
Ceftazidime	3	30	-	-	7	70	10
Gentamicin	7	70	-	-	3	30	10
Amikacin	8	80	-	-	2	20	10
Cefepime	3	30	-	-	7	70	10
Ciprofloxacin	5	50	2	20	3	30	10
Chloramphenicol	8	80	-	-	2	20	10
Imipenem	8	80	-	-	2	20	10
Tetracycline	2	20	-	-	8	80	10
Piperacillin/Tazobactam	7	70	2	20	1	10	10

Among different antibiotics used the most effective antibiotic was Polymyxin B (100%) and followed by Piperacillin/Tazobactam (73.2%). The least effective antibiotic was Ampicillin (100%) and Ceftazidime (86.7%).

Table 3: Antibiotic susceptibility pattern of *Klebsiella pneumoniae*

Antibiotics used	Susceptibility pattern					
	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ampicillin	-	-	-	-	15	100
Gentamicin	6	40	-	-	9	60
Amikacin	8	53.3	1	6.7	6	40
Cefepime	5	33.3	-	-	10	66.7
Cefotaxime	4	26.7	-	-	11	73.3
Ciprofloxacin	4	26.7	-	-	11	73.3
Imepenem	8	53.3	-	-	7	46.7
Ceftazidime	2	13.3	-	-	13	86.7
Chloramphenicol	6	40	-	-	9	60
Cotrimoxazole	5	33.3	-	-	10	66.7
Piperacillin/Tazobactam	11	73.3	2	13.3	2	13.3
Polymyxin B	15	100	-	-	-	-
Tetracycline	6	40	-	-	9	60

The most effective drug against *S. aureus* was Amikacin (94%) followed by Gentamycin (92.5%) and Chloramphenicol (77.4%). Among different antibiotics used least effective was Penicillin (96.2%). CoNS were highly sensitive towards Amikacin and Chloramphenicol (80% both) and Gentamicin (66.7%).

Table 4: Comparative antibiotic susceptibility pattern of *S. aureus* and CoNS

Antibiotic	*S.aureus* (%) (N=53)			CONS (%) (N=15)		
	S	I	R	S	I	R
Penicillin	3.8	-	96.2	13.3	-	86.7
Erythromycin	37.3	9.4	52.8	20	13.3	66.7
Cotrimoxazole	32.1	7.5	60.4	33.3	13.3	53.3
Tetracycline	52.8	-	47.2	53.3	-	46.7
Chloramphenicol	77.4	-	22.6	80	-	20
Ciprofloxacin	39.6	9.4	50.9	46.7	-	53.3
Gentamicin	92.5	1.9	5.7	66.7	-	33.3
Amikacin	94.3	1.9	3.8	80	-	20

The most effective antibiotic for *E. fecalis* was Tetracycline, Chloramphenicol and Vancomycin (100%) whereas least effective antibiotic was Ciprofloxacin (58.3%).

Table 5: Antibiotic susceptibility pattern of *Enterococcus fecalis*

Antibiotics	Sensitive		Intermediate		Resistant		Total
	No	%	No	%	No	%	
Ampicillin	8	66.7	-	-	4	33.3	12
Erythromycin	6	50	2	16.7	4	33.3	12
Tetracycline	12	100		-	-	-	12
Chloramphenicol	12	100	-	-	-	-	12
Ciprofloxacin	5	41.7	-	-	7	58.3	12
Gentamicin	8	66.7	-	-	4	33.3	12
Vancomycin	12	100	-	-	-	-	12

DISCUSSION

Wound infection has been major concern among health care practitioners not only in terms of increased trauma but also in view of its burden on financial resources and the increasing requirements for cost effective management within health care system. Infection of wound delays in healing and may cause herniation of the wound and complete wound dehiscence (Alexender 1994). Wound infections are also significant in that they are the most common nosocomial infection (Dongi et al. 20011)

The study was designed with an aim to assess the prevalence of wound infection in All Nepal Hospital; the effect of age, gender and other co- morbid conditions in the prevalence as well as identifying the etiological agents and their susceptibility to antimicrobial agents. In this study a total 271 pus samples were collected and processed. The etiological agents were identified by culture and different biochemical tests and their susceptibility pattern with commonly used antibiotics were determined.

In our study, out of total samples from patients with wound infection, 60.5% showed bacterial growth whereas 39.5% didn't show any growth. Culture negative results might be difficulty in growing of fastidious organisms. Another possibility could be manual error in collection, transport of culture media and diagnosis of the infection itself and most probable reason was the sample from patient taking antibiotic. Similar studies conducted by KC et al. 2013 (60.2%), Acharya et al. 2008 (50.7%) and Bhatt et al. 2007 (80.6%) findings in Nepal. Neelima et al. (2013) 58% of the sample cultured aerobically showed positive growth. A study conducted by Giacometti et al. (2000), Manyahi (2012) in tertiary hospital >90% shows growth. Both of these studies were contrary to this study. This difference in prevalence may be due to variation in common nosocomial pathogens inhabitant, difference in policy of infection control and prevention between countries and hospitals and study designed used in the researches.

Out of total cases 46.9% were male patients where 53.1% female patients. The growth was found higher in male patients 53% and female 47%.In this study, on total sample analyzed 139 (51%) samples from outpatients and 132 (49%) samples from inpatients while on growth positive 164 cases 88 (53.7%) from inpatients and 76 (46.3%) from outpatients shown microbial growth.

On total growth positive pus samples, 84(51.22%) were Gram positive bacteria. Among Gram positive bacteria, *S. aureus* (63.1%) was the most common isolates similar study conducted by Pokhrel et al. (2004) 57.66%, Bhatt and Lakhey (2007) 50%, Acharya et al. (2008) 51.2%, showed *S. aureus* was the predominant in wound infection study conducted in Nepal, Mishra et al. (2000) reported 60.1%. But Banjara et al. 2003 (24.9%) from TUTH and De et al. 2003 (11.2%) from India that showed lower frequency of *S. aureus*. CoNS (17.9%) constituted second most prevalent bacteria among Gram positive bacteria in our study. Similar study carried by Neelima et al. (2013) CoNS (18.6%) was the second predominant organisms. According to Shah et al. (1997) CoNS was 11.4% and Manyahi et al. (2012) it was the second most predominant but Altoparlak et al. 2004 reorted 63% of prevalence of CoNS which is contradictory with our findings. Similarly *Enterococcus fecalis* (14.3%) and *Streptococcus pyogenes* (4.8%) was found in our study. Yah et al. (2007) found that *S. pyogenes* (3.3%) on kerosene burn wound.

In this study, antibiotic susceptibility test was performed for all bacterial isolates. The antibiotic discs used were Ciprofloxacin, Cotrimoxazole, Chloramphenicol, Amikacin, Gentamicin and Tetracycline was common antibiotics while Ampicillin was used for all Gram negative and *Enterococcus fecalis*. Penicillin and Erythromycin were only used for Gram positive isolates. Vancomycin was used only for *Enterococcus fecalis.* Antibiotics Ceftazidime Cefepime, Cefotaxime, Imipenem, Piperacillin/Tazobactam and Polymyxin B were used only for Gram negative. Cefoxitin disc was only used for *S. aureus*.

In our study, the most effective antibiotic for *E. coli* was Polymyxin B (100%), Imipenem (76.5%), Amikacin (70.6%), Gentamycin (67.6%) and Chloramphenicol (55.9%). Other antibiotics like Ampicillin (100%), Ceftazidime (88.2%), Cefepime (88.2%) and Tetracycline (79.4%) resistance to *E. coli*. Yakha et al. (2014) showed Imipenem (96.4%), Amikacin (86.6%) and Piperacillin/ Tazo (70.7%), Rao et al. (2014) reported Imipenem, Amikacin and Pipera/Tazo were (80%) sensitive while Ampicillin (53.34%), Ceftriaxone (73.34%), Ciprofloxacin (73.34%) show higher resistance to *Ecoli*. *E. coli* showed (90.9%) resistant to Ampicillin and Ciprofloxacin, (81.8%) to Cefotaxim and Ceftriaxone,

(72.7%) resistant to Cotrimoxazole. However, it was highly sensitive to Amikacin (100%) followed by Gentamicin (54.5%).

Regarding the sensitivity pattern of *Pseudomonas* spp., it was found that Amikacin, Imipenem, Chloramphenicol was the most effective drug (80%) sensitivity and Gentamycin and Piperacillin/ Tazobactam showed 70% sensitivity while Ampicillin, Cefepime and Tetracycline was the least effective drugs. Similarly a study carried by Amatya et al. (2015) reported Imipenem (87.9%) and Amikacin (64.6%) sensitive similarly Acharya et al. (2008) reported Amikacin was the most effective drug against *P. aeruginosa*. *Pseudomonas aeruginosa* was sensitive to Gentamicin (87.5%) and Ceftazidime (85.7%) but showed resistance to Ciprofloxacin (57.2%) (Anguzu and Ohila 2007). Mengesha et al. (2014) found that *P. aeruginosa* were 100% resistant to Ceftriaxone, Amoxicillin, Tetracycline and Ampicillin. Similar resistant pattern was also shown by Guta et al. (2014).

The most effective antibiotic against *Klebsiella pneumoniae* was Polymyxin B (100%). Antibiotics like Piperacillin/Tazobactam (73.2%), Amikacin (53.3%), Imipenem (53.3%) sensitive to *K. pneumoniae*. The least effective antibiotics are Ampicillin (100%), Ceftazidime (86.7%), Cefotaxime and Chloramphenicol (73.3%). Cotrimoxazole, Gentamicin Cefepime Tetracycline shows resistant above (60%). *Klebsiella oxytoca* is highly susceptible to Polymyxin B and Amikacin (100% both) other antibiotics like Imipenem, Cefotaxime, Chloramphenicol Tetracycline Shown (50%) sensitive by *K. oxytoca.* A study conducted by Chowdhury et al. (2013) reported *Klebsiella* are highly sensitive to Imipenem (100%) and Gentamicin Ceftazidime and Ceftriaxone are highly resistant to *Klebsiella* spp. Similarly Rao et al. (2014) have similar result with the present study, which shows maximum sensivity to Imipenem, Amikacin and Piperacillin/Tazobactam (76.92%) of each, but higher resistant to Ciprofloxacin, Ampicillin and Cefotaxime.

In the present study, isolates of *Acinetobacter* spp. was found highly resistant to commonly used antibiotics Ampicillin, Ceftazidime, Cefotaxime (100%) and Tetracycline (71.4%), Amikacin (57.1%), Gentamycin (71.4%). Whereas Imeipenem (57.1%) sensitive. Manyahi (2012) reported that *Acinetobacter* spp. were highly resistant to Ceftazidime, Ciprofloxcin and Gentamicin and 40% of them being resistant to Carbapenams. Idomir et al. (2009) also reported all tested antibiotics are resistant by *Acinetobacter* spp. except Carbapenam.

For *Citrobacter* spp. Amikacin and Polymyxin B (85.7%) was the most effective antibiotic and Imipenem and Pipera/Tazo (71.4%) and highly resistant to Ampicillin (85.7%), Cefepime (100%), Cotrimoxazole (71.4%). *Proteus* spp are highly susceptible to Imipenem, Chlormphenicol, Ceftazidime, Amikacin, Cefepime and Gentamicin (100%) but resistant to Polymyxin B, Ampicillin and Cotrimoxazole (100%).

Resistance to Penicillins and Cephalosporins (Levy and Marshall 2004) by Gram negative bacteria is most commonly due to the production of β- lactamase, either chromosomally encoded or, more often, plasmid mediated. Other important mechanisms of resistance include alteration in penicillin binding protein (PBPs), decreased penetration of the antibiotics to the bacterial cell or combinations of these resistance strategies (Deloney and Schiller 2000). Active efflux pumps in Gram negative bacteria which execrete drugs including multidrug efflux pumps, can also confer to resistance to β-lactams

The most effective antibiotic against *S. aureus* was Amikacin (94.3%) followed by Gentamicin (92.5%) and Chloramphenicol (77.4%), only Cefoxitin (56.6%). Antibiotics such as Erythromycin, Cotrimoxazle and Ciprofloxacin have sensitivity less than 40% and least effective antibiotic was Penicillin (96.2%). The result was similar in case of CoNS being most sensitive with Chioramphenicol (80%), Amikacin (80%), followed by Gentamicin (66.7%), Tertacycline (53.3%) and Cefoxitine (53.3%) sensitive.

The least effective antibiotic was Penicillin (86.7%), Erythromycin and Cotrimoxazole. A study carried out by Amatya et al. (2015) in B & B Hospital Nepal, Chloramphenicol (89.1%) and Gentamicin (52.2%) which is similar to our findings. Poudel (2013) reported that Chloramphenicol (98.9%) and Gentamicin (86.8%) was the most effective antibiotics against *S. aureus* which agreed with our findings. Andhoga et al. (2002) in Kenya have reports *S. aureus* being highly resistance to Chloramphenicol (84.8%). *S. aureus* causes clinically relevant infections mostly because of its virulence factors such as coagulase, catalase clumping factor A and leucocidines (Dissemond 2009).

CONCLUSION

In this study Gram positive bacteria was found predominant over Gram negative bacteria. The most common isolates were *S. aureus* (32.3%), *E. coli* (20.7%), *K. pneumoniae* (9.1%) and *P. aeruginosa* (6.1%). Pattern of bacterial isolates were similar in both inpatient and outpatients. The least effective antibiotic was Ampicillin (100% resistant) in case of Gram negative and Penicillin (100% resistant) in case of Gram positive. *Acinetobacter* spp. was highly resistant to different antibiotics. Polymyxin B was the most effective antibiotic against Gram negative bacteria like *E. coli, Klebsiella* spp., *Proteus* spp. and *Citrobacter* spp. Antibiotics like Amikacin, Gentamicin and Chloramphenicol were highly effective to Gram positive bacteria.

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Vancomycin Resistant *Staphylococcus aureus* Reported from Tertiary Care Hospital in Nepal

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ABSTRACT

Objectives: The study was conducted to assess the rate of Methicillin-resistant *Staphylococcus aureus* (MRSA) among patients and healthcare personnel at Manmohan Memorial College and Teaching Hospital, Kathmandu, Nepal and to evaluate the minimum inhibitory concentration of Vancomycin to MRSA isolates.

Methods: A total of 1433 different clinical specimens from patients and 33 nasal swabs from healthcare personnel were subjected to bacteriological investigation following standard protocol. *S. aureus* were isolated and identified by using standard Microbiological tools. Those isolates were subjected to Antimicrobial susceptibility testingusing modified Kirby-Bauer's disc diffusion method following CLSI guidelines.

Results: The rate of *S. aureus* carriage was found to be 65 (18.9%) in the samples from clinical patients and 24 (72.7%) in the samples from healthcare personnel. The rate of MRSA was found to be 57(85.1%) in patients and 24 (100%) in healthcare personnel. The high distribution of MRSA was found in female of age group 21-30 years (patients: 10.4%; healthcare personnel: 70.8%). Amikacin was found to be most effective antimicrobial. All *S. aureus* isolates were found to be multidrug resistant (100%).On performing D-test, 10 (17.5%) and 22 (38.6%) of MRSA from clinical specimens showed inducible and constitutive Clindamycin resistance respectively. Whereas, 11 (45.8%) and 4 (16.7%) of MRSA from nasal swabs were found to be inducible and constitutive Clindamycin resistance respectively. Upon performing minimum inhibitory concentration (MIC) test for clinical isolates, 3.5% (2) of MRSA were found to be Vancomycin resistant (VRSA), 54.4% (31) were Vancomycin intermediate (VISA) and 42.1% (24) were found to be Vancomycin sensitive (VSSA). All of the nasal swab MRSA isolates were found sensitive to Vancomycin. Congo red agar method was done for biofilm production. For clinical isolates, 32 (47.8%) were found to be strong, 6 (8.9%) moderate and 29 (43.3%) were non biofilm producer. For nasal swab isolates, 66.7% (16) and 33.3% (8) were found as strong and non-biofilm producer respectively.

Conclusion: This study reported the case of VRSA which hasn't been reported in Nepal. Though present study showed that Vancomycin remains the main choice of treatment of MRSA infection. Therefore, to preserve its value, use of vancomycin should be limited only to those cases where there are clearly needed.

Key words: *S. aureus*, MRSA, D-test, Inducible Clindamycin resistance,VRSA

INTRODUCTION

Staphylococcus aureus is one of the common human pathogens capable of causing a wide range of infections. A great deal of virulence from the organism occurs through cross infection by patient to patient in hospitals and other institutional settings. In contrast, healthy individuals have a small risk of invasive infection caused by *S. aureus*, but they can

be carriers of the organism (Foster 2004). Infection due to Methicillin-Resistant *S. aureus* (MRSA) are an increasing problem worldwide in community as well as hospital environment (Boyce et al. 2005; Skoy et al. 2006). The incidence of community-acquired and hospital-acquired *S. aureus* infections has been rising with increasing emergence of drug-resistant strains called Methicillin resistant *S. aureus* (MRSA) (Steinberg et al. 1996).

The resistance of *S. aureus* to Methicillin is caused by the *mecA* gene which codes the low affinity 78-Kda penicillin-binding protein (PBP2a). Beta-lactam antibiotic normally binds to PBPs in the cell wall, resulting in the disruption of synthesis of the peptidoglycan layer and death of bacterium. Since the beta-lactam antibiotics cannot bind to low affinity PBP2a, synthesis of peptidoglycan layer and cell wall are able to continue (Duerenberg 2007). MRSA infections often require systematic antibiotic therapy. The spread of MRSA can also be potentially minimized by prevention of the risk factors such as previous antibiotic use, contact with the healthcare workers or nursing home resident, daycare attendance, hospitalization, admission to an intensive care unit, intravenous drug use, invasive indwelling devices, haemodialysis or peritoneal dialysis, immunosuppression, chronic illness, and previous isolation of MRSA (Cohen 2007).

Following the spread of MRSA, glycopeptides (usually Vancomycin and more recently Teicoplanin) have become the mainstay of treatment for MRSA infections (CDC 2013). Vancomycin is the choice of drug for MRSA isolates. Patients unable to tolerate vancomycin have been treated with fluoroquinolones, Trimethoprim-Sulfomethoxazole, Clindamycin or Minocycline (Shah 2008). As Vancomycin is commonly used for the treatment of MRSA infections, which has resulted into development of Vancomycin-Intermediate *S. aureus* (VISA) and Vancomycin-Resistant *S. aureus* (VRSA).

Clindamycin, a lincosamide drug, has been used to treat serious infections caused by susceptible *S. aureus* in children for more than 30 years (Woods 2009). It is also an alternative choice in case of Penicillin allergic patients. Clindamycin is recommended in some European countries for suppression of panton-valentine leukocidin (PVL) toxin, along with Linezolid and Rifampin (Adaleti et al. 2010). In vitro, *S. aureus* isolates with constitutive resistant are resistant to Erythromycin and Clindamycin while isolates with inducible resistant are resistant to Erythromycin but appear susceptible to Clindamycin (Steward et al. 2005). Inducible MLSB (Macrolide, Lincosamide and Streptogramin B) resistant can be detected by discapproximation test (D-test) by placing Erythromycin and Clindamycin discs in adjacent positions (Fiebelkorn et al. 2003).

Biofilms are communities of microorganisms embedded in extracellular polymeric substances (EPS) matrix. Bacteria in biofilms demonstrate distinct features from their free-living planktonic counterparts, such as different physiology and high resistance to immune system and antibiotics that render biofilm a source of chronic and persistent infections. Extracellular polymeric matrix plays various roles in structure and function of different biofilm communities. Adhesion to the surface provides considerable advantages such as protection against antimicrobial agent, acquisition of new genetic traits and the nutrients availability and metabolic co-operability. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defense system (Donlan and Costerton 2002).

The study was thus done to determine the rate of Methicillin resistant, emergence of Vancomycin resistant and inducible Clindamycin resistant *S. aureus* among biofilm producing and non-producing isolates of *S. aureus*.

MATERIALS AND METHODS

Study design: A hospital based cross sectional descriptive study was conducted.

Study period, site and population: The study was conducted at Manmohan Memorial Medical College and Teaching Hospital, Swoyambhu, Kathmandu, Nepal in collaboration with Kantipur College of Medical Science, Sitapaila, Kathmandu, Nepal from April 2014 to October 2014. All the clinical specimens obtained from individuals of all ages and sexes visiting hospital during the study period were included in the study.

Sample size: A total of 1344 clinical specimens including blood, urine, sputum, vaginal swab, eye swab, ear swab, throat swab, wound swab, clavical swab, body fluids like pus, synovial fluid, pleural fluid, asiatic fluid, peritoneal fluid and catheter swabs and urethral discharge; were processed in the study. For the study of hospital acquired MRSA, a total of 33 nasal swabs were collected from the hospital personnel.

Laboratory diagnosis

Sample collection: Sterilized sample collection container was used for the collection of all clinical specimens. Blood was collected with sterile syringe and then poured in leak proof, dry and sterilized container.

Sample processing and bacterial identification: All the clinical samples were inoculated onto blood agar, chocolate agar and mac-conkey agar plates. Blood samples were inoculated into brain heart infusion broth and incubated at 37°C for 7 days and then further inoculated into agar media. All the culture plates were then incubated at 37°C for overnight. The plates showing growth of bacterial were processed for identification of *S. aureus* using standard microbiological procedures by inoculating the organism on mannitol salt agar and performing specific biochemical tests catalase test, coagulase test and oxidative-fermentative test.

Antimicrobial susceptibility testing and screening of multi drug resistant (MDR) *S. aureus*: All the identified isolates of S. aureus were undertaken in-vitro antibiotic susceptibility test by using modified Kirby-Bauer's disc diffusion method (CLSI 2013). The antibiotics used were Cefoxitin (5mcg), Ciprofloxacin (5mcg), Cefixime (5mcg), Tetracycline (30mcg), Amikacin (30mcg), Azithromycin (30mcg), Vancomycin (30mcg), Cloxacillin (5mcg), Cefotaxime (30mcg), Clindamycin (10mcg), Ceftriaxone (30mcg), Erythromycin (15mcg), Gentamicin (10mcg), Penicillin (10mcg), Co-trimoxazole (25mcg), Mupirocin (5mcg), and Chloramphenicol (50mcg). The organism resistant to three or more antibiotics of different classes were classified as MDR isolates (Magiorakos et al. 2012). Intrinsic resistance to any of the employed antibiotics was not counted.

Screening of methicillin resistant S. aureus: Screening for Methicillin resistant*S. aureus* was carried out by Cefoxitin disc diffusion method and interpreted according to CLSI (2013) guidelines. The growth of *S. aureus* with zone of inhibition around Cefoxin disc (ZOI) ≥ 22mm were identified as Methicillin sensitive *S. aureus* and that of ZOI ≤ 21 were identified as Methicillin resistant *S. aureus*.

Detection of Inducible clindamycin resistance (ICR): In this assay, two discs namely Erythromycin and Clindamycin were placed 18mm away edge-to-edge on Muller Hinton agar plates that were previously inoculated with 0.5 McFarland bacterial suspensions. Plates were observed after 18 hours of incubation at 35±2°C. Flattening of the zone of inhibition adjacent to the Erythromycin disc (referred to as D-zone) or hazy growth within the zone of inhibition around Clindamycin (even if no D- zone is apparent) is regarded as positive test, i.e. Inducible Clindamycin resistance (CLSI 2013).

Determination of minimum inhibitory concentration of vancomycin: Minimum inhibitory concentration (MIC) technique was performed to determine the Vancomycin intermediate and resistant strains of *S. aureus* isolates MIC to Vancomycin in isolated MRSA was done by agar dilution method following CLSI guidelines (CLSI 2013). Different concentrations ranging from 0.06-32µg/ml of Vancomycin incorporated plates was prepared. Positive growth controls were kept for each isolates and *S. aureus* (ATCC 25923) of known MIC was also included in each test as control for antibiotic potency.

Biofilm production: Biofilm detection was carried out by Congo Red Agar method (CRA**):** CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar 10 g/L and Congo Red indicator 8 g/L. Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose. CRA plates was inoculated with test organisms and incubate at 37°C for overnight aerobically. Black colonies were considered as biofilm producing isolates (Freeman et al. 1989).

RESULTS

Out of total 1433 clinical specimens, *S. aureus* was isolatesd from 67 specimens (4.7%), among them 57 (85.1%) were found to be MRSA. Whereas from 33 nasal swab specimens, 24 (72.7%) *S. aureus* were isolated and all of them were found to be MRSA (100%). All of the *S. aureus* isolates from clinical as well as nasal swab specimens were multi-drug resistant (MDR) (Figure 1).

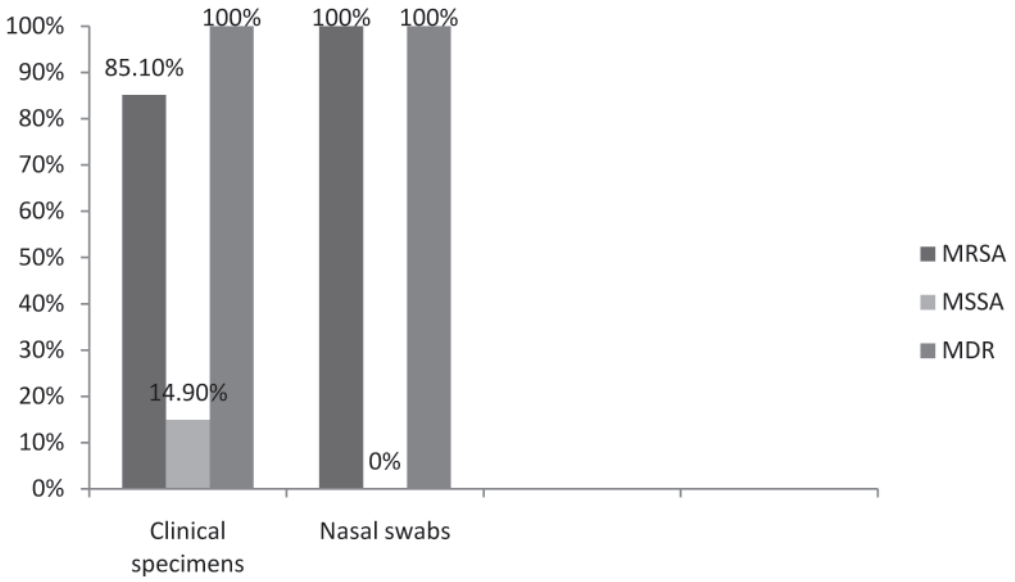

Figure 1: Distribution of MRSA, MSSA and MDR S. aureus

Among total of 67 *S. aureus* isolates, 27 (40.3%) from male and 30 (44.8%) from female. High rate of MRSA 12 (17.9%) was obtained from age group 21-30 year (Table 1).

Table 1: Age and sex wise distribution of MRSA from clinical specimens

Age group (year)	Total No. of samples	Male Number (%)			Female Number (%)			Total no. of MRSA (%)
		Samples	*S. aureus*	MRSA	Samples	*S. aureus*	MRSA	
<10	109	56	3(4.5)	2(2.9)	53	1(1.5)	1(1.5)	3(4.5)
11-20	204	99	7(10.4)	4(5.9)	105	2(2.9)	2(2.9)	6(9)
21-30	382	121	6(9)	5(7.5)	261	7(10.4)	7(10.4)	12(17.9)
31-40	202	86	3(4.5)	2(2.9)	116	4(5.9)	4(5.9)	6(9)
41-50	124	65	6(9)	6(9)	59	4(5.9)	4(5.9)	10(14.9)
51-60	128	40	1(1.5)	1(1.5)	88	6(9)	5(7.5)	6(9)
61-70	108	55	3(4.5)	3(4.5)	53	5(7.5)	3(4.5)	6(9)
71-80	87	37	2(2.9)	2(2.9)	50	3(4.5)	2(2.9)	4(5.9)
>80	89	51	2(2.9)	2(2.9)	38	2(2.9)	2(2.9)	4(5.9)
Total	**1433**	**610**	**33 (49.2)**	**27 (40.3)**	**823**	**34 (50.7)**	**30 (44.8)**	**57 (85.1)**

Among 24 nasal swab MRSA isolates, 2 (8.3%) were from male and 22 (91.7%) were from female and High rate of MRSA was 19 (79.2%) obtained from age group 21-30 year (Table 2).

Table 2: Age and sex wise distribution of MRSA from nasal swabs

Age group (year)	Total no. of samples	Male Number (%)			Female Number (%)			Total no. of MRSA (%)
		Samples	*S. aureus*	MRSA	Samples	*S. aureus*	MRSA	
<10	-	-	-	-	-	-	-	-
11-20	4	1	-	-	3	2(8.3)	2(8.3)	2(8.3)
21-30	22	3	2(8.3)	2(8.3)	21	17 (70.8)	17 (70.8)	19 (79.2)
31-40	7	-	-	-	4	3(12.5)	3(12.5)	3(12.5)
Total	**33**	**4**	**2 (8.3)**	**2 (8.3)**	**29**	**22 (91.7)**	**22 (91.7)**	**24 (100)**

MRSA from clinical specimens were 100% resistant to Penicillin G, Co-trimoxazole and Cefixime, followed by Cloxacillin (94.7%), Ceftriaxone (93%), Vancomycin (92.2%), and Cefotaxime (84.2%). Whereas MMSA isolates were 100% resistant to Cefixime, followed by Cefotaxime (90%), Ceftriaxone (90%), Penicillin G (80%) and Co-trimoxazole (80%). For nasal swab MRSA isolates, 100% showed resistance towards Cefixime, Cefotaxime, Penicillin G and Co-trimoxazole, followed by Cloxacillin (75%), Clindamycin (54.2%), and Tetracycline (50%). Whereas no MMSA isolates were obtained (Table 3).

Table 3: Antimicrobial resistance profile of MRSA and MSSA

Antibiotics	Clinical specimens		Nasal swab specimens	
	Antibiotic profile of MRSA (n=57)	Antibiotic profile of MSSA (n=10)	Antibiotic profile of MRSA (n=24)	profile of MSSA Antibiotic (n=0)
Cefoxitin	57(100%)	-	24(100%)	-
Ciprofloxacin	15(26.3%)	3(30%)	8(33.3%)	-
Cefixime	57(100%)	10(100%)	24(100%)	-
Tetracyclin	32(56.1%)	-	12(50%)	-
Amikacin	8(14%)	-	4(16.7%)	-
Azithromycin	25(43.9%)	1(10%)	6(25%)	-
Vancomycin	53(92.9%)	4(40%)	8(33.3%)	-
Cloxacillin	54(94.7%)	1(10%)	18(75%)	-
Cefotaxime	48(84.2%)	9(90%)	24(100%)	-
Clindamycin	42(73.7%)	4(40%)	13(54.2%)	-
Ceftriaxone	53(93%)	9(90%)	24(100%)	-
Erythromycin	32(56.1%)	-	8(33.3%)	-
Gentamicin	13(22.8%)	-	4(16.7%)	-
Penicillin G	57(100%)	8(80%)	24(100%)	-
Co-trimoxazole	57(100%)	8(80%)	24(100%)	-
Mupirocin	43(75.4%)	3(30%)	18(75%)	-
Cloramphenicol	11(19.3%)	-	3(12.5%)	-

Upon performing D-test, 10 (17.5%) and 22 (38.6%) of MRSA from clinical specimens showed inducible and constitutive Clindamycin resistance respectively. Whereas, 11 (45.8%) and 4 (16.7%) of MRSA from nasal swabs were found to be inducible and constitutive Clindamycin resistance respectively (Table 4).

Table 4: D-test of MRSA isolates

Phenotype	Erythromycin	Clindamycin	D-test	MRSA (%)	
				Clinical isolates	Nasal swab isolates
iMLSB	Resistance	Sensitive	Positive	10 (17.5)	11 (45.8)
cMLSB	Resistance	Resistance	Negative	22 (38.6)	4 (16.7%)

From clinical specimens, 47.8% (32), 8.9% (6) and 43.3% (29) of *S. aureus* isolates were found to be strong, moderate and non-biofilm producer respectively. Among nasal swab specimens, 66.7% (16) and 33.3% (8) of *S. aureus* isolates were found to be strong and non-biofilm producer respectively (Table 5).

Table 5: Biofilm production by *S. aureus*

Serial no.	Strong producer	Moderate producer	Non producer
1.clinical specimen (n=67)	32(47.8%)	6(8.9%)	29(43.3%)
2.Nasal swab (n=24)	16(66.7)	-	8(33.3%)

On performing MIC of MRSA from clinical specimens, 42.1% (24) of isolates were reported as VSSA (showed MIC value of 2µg/ml), 54.4% (31) as VISA (showed MIC value of 4-8µg/ml) and 3.5% (2) of MRSA isolates were reported as VRSA (showed MIC value of ≥16µg/ml) (Figure 2). Whereas all MRSA isolates from nasal swabs were found to be Vancomycin sensitive (VSSA).

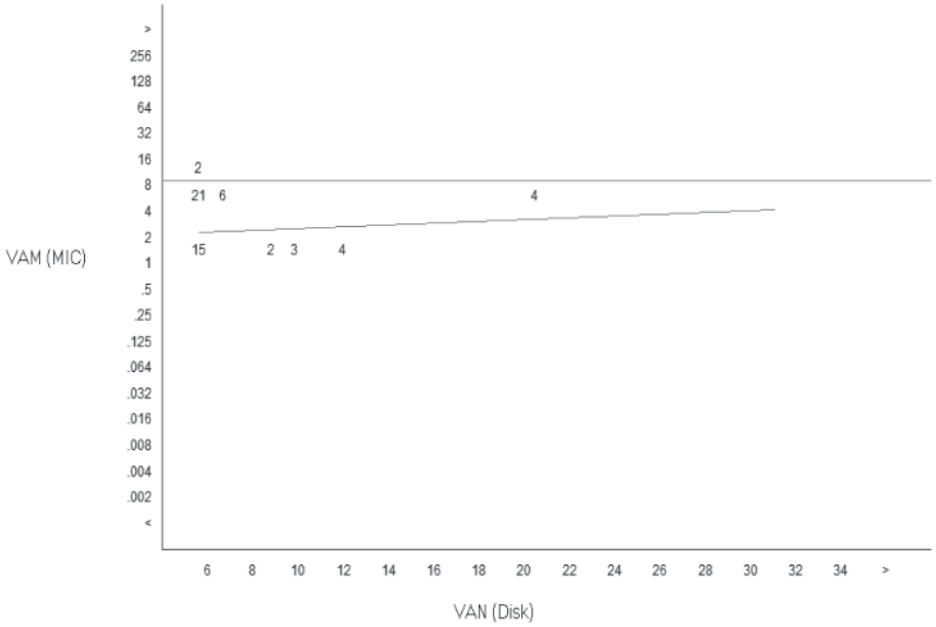

Figure 2: Scatter plot analysis of MIC of vancomycin for MRSA isolates from clinical specimens (WHONET 5.6)

DISCUSSION

S. aureus has remained a versatile and potent pathogen in humans, since it is one of the most common causes of nosocomial and community acquired infections (Rajbhandari et al. 2003). *S. aureus* is a major cause of infectious morbidity and mortality around the world, causing a wide variety of clinical manifestations ranging from localized infection to toxin mediated diseases and invasive blood stream infections(Vandecasteele et al. 2008).

In this study, the rate of MRSA isolation was found to be 85.1% from clinical specimens. This result is higher than the many other studies conducted by Kumari et al. (26.14%), Shakya et al. (12.5%) and Tiwari et al. (69.1%) (Tiwari et al. 2006; Kumari et al. 2008; Shakya et al. 2010). All of the *S. aureus* isolates from nasal swab specimens were found to be MRSA i.e. 100%. Nasal carriage rate of MRSA among health care workers in hospital setting ranges from 6-17.8% (Cesur and Cokca 2004; Pant and Rai 2007). Nasal carriage rate of 43.8% has been reported among the healthcare personnel of a Medical College Teaching Hospital in Kathmandu (Pant and Rai 2007). The nasal carriage rate of *S. aureus* in this study i.e. 72.7% was found to be greater than the study conducted by Shakya et al. i.e. 12.5% (Shakya et al. 2010). The result is also in agreement with the study by Gonsu et al. (Gonsu et al. 2013).

Regarding the sex wise distribution of MRSA clinical specimens, the study showed high incidence of MRSA from female patients (44.8%) than males (40.3%). The present study showed the opposite variation with the study conducted by Boucher and Corey (Boucher and Corey 2008) showing males (64.4%) were more predisposed than females (35.6%). The highest distribution of MRSA was found within the age group of 21-30 years (17.9%) and the lowest in the age group below 10 years (4.5%). However, the study conducted by Arch et al. (Arch et al. 2006) and Lucet et al. (Lucet et al. 2003) showed high rate of MRSA colonization among the population with age group 60 years and above.

The nasal carriage rate of MRSA reported in present study was found to be higher (72.7%) than the previous studies conducted in Nepal by Shakya et al. and Rijal et al. (Rijal et al. 2008; Shakya et al. 2010).

Penicillin was found resistant to all of MRSA isolates

i.e. 100%. This result is higher than that of Shrestha et al. who reported 91.94% (Shrestha et al. 2009). In present study, clinical MRSA isolates showed rate of resistance to antibiotics Co-trimoxazole (100%), followed by Cloxacillin (94.7%), Ceftriazone (93%), Vancomycin (92.9%), Cefotaxime (84.2%), Mupirocin (75.4%), Clindamycin (73.7%), Tetrcyclin (56.1%) and Erythromycin (56.1%). Rijal et al. reported the rate of resistance to Cloxacillin (68.8%), followed by Tetracycline (15.6%) and Erythromycin (9.4%) (Rijal et al. 2008). Resistance to Erythromycin is seen to be greater than the finding disseminated by study conducted by Mishra i.e. 14.29% (Mishra 2008)and lower than the finding disseminated by Tiwari et al. i.e. 68.7%(Tiwari et al. 2006).

All isolates were found to be multi drug resistant (MDR) in this study. The rate of MDR-MRSA (100%) is higher than that of the result reported in the studies conducted by Tiwari et al. i.e. 40.1% and Pandey et al. i.e. 75.86%. Though this study is in accordance with the previous studies from Nepal and other countries showing high percentage of MDR among MRSA; >65% by Kumari et al., 93% by Rahimi et al. and 63% by Salah et al. (Kumari et al. 2008; Salah et al. 2012; Rahimi et al. 2013).

In this study, 17.5% and 38.6% isolates were found to be inducible and constitutive Clindamycin resistance respectively. Among nasal swab specimens taken from hospital staffs, 45.5% and 16.7% were found to be inducible and constitutive Clindamycin resistance respectively. In the study conducted by Ujwol et al. (Bhomi et al. 2016), D-test positive isolates were found to be 18.03% and study also reported constitutive resistance in 36.06% of isolates.

Upon performing MIC, 3.5% (Skoy et al. 2006)) of clinical MRSA isolates were reported as VRSA, 54.4% (31) as VISA and 24.1% (Vandecasteele et al. 2008) as VSSA. Assadullah et al. (Assadullah et al. 2003); Sharma and Vishwanath (Sharma and Vishwanath 2012) reported 18.3% and 11.54% VISA among MRSA respectively. In the study carried out by Venubabu et al. (Venubabu et al. 2011), who reported 1.9% VRSA from India. Likewise Tiwari and Sen (Tiwari and Sen 2006) reported two strains of VRSA and six strains of VISA in the Northen part of India.

Biofilm production by *S. aureus* was found to be 47.8% strong, 8.955% moderate and 43.3% biofilm non producers. Whereas 66.7% and 33.3% of isolates were found to be strong and biofilm non-producer from nasal swab specimens respectively. A study, conducted by Mirani et al. (Mirami et al. 2013) reported 57% of MRSA isolates as biofilm producer. Rewatkar and Wadher (Rewatkar and Wadher, 2013) reported 90% of strong biofilm producer and remaining 10% of weak/ none producer.

CONCLUSION

Higher rate of MRSA was found in female in age group 21-30 years. Amikacin was found to be most effective drug, whereas penicillin G was found as the least effective drug. Upon performing MIC test of MRSA isolates from clinical specimens, 3.5% (2) of MRSA isolates were found as VRSA and 54.4% (31) and 42.1% (24) were as VISA and VSSA respectively. Whereas all nasal swab isolates were sensitive to Vancomycin. From this study, it could be concluded that all of the MRSA isolates i.e. 100% were multi drug resistant (MDR), which is the significant public health problem in context of Nepal, indicating the high risk of staphylococcal infections in our context. This high load of MDR organisms provokes the necessity of strictly performing susceptibility testing before starting antibiotic therapy, or there may be chance of clinical failure. Thus determination of MIC of Vancomycin is crucial. Inducible Clindamycin resistance test cannot be observed in routinely done antibiotic susceptibility testing by Kirby Bauer method hence specific D-test should be performed before treatment with Clindamycin. There are various methods for detection of biofilm production and both tube test and agar plate methods can be carried out for comparative study.

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Population Survey of Glucose-6-Phosphate Dehydrogenase(G6PD) Deficiency among People Living in Terai Districts of Nepal

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ABSTRACT

Objective: This study was carried out to determine the prevalence of Glucose-6-phosphate dehydrogenase (G6PD) deficiency among population of selected malaria endemic districts in central and eastern terai of Nepal.

Methods: Six hundred seventy whole blood samples were collected from the indigenous peoples community, identified based on district public health office records for G6PDd in the past from Jhapa, Morang and Dhanusha districts endemic to malaria, during April to June 2013. Collected blood samples were tested on the sites by using BinaxNow G6PD test kit and CareStart™ G6PD test kits.

Results: The G6PD deficiency was found to be in 6.1% and 6.3% in BinaxNow and CareStart™ respectively. In 42 G6PD deficient cases number of male to female ratio was almost equal. Higher proportions of deficient cases were from Rajbanshi and Santhal communities than others. Highest number of deficient cases was in Jhapa followed by Morang and Dhanusha districts respectively.

Conclusion: G6PD deficiency in indigenous population group in estern and central terai are heterogenous. So the testing of G6PD before initiation of radical treatment fo *Plasmodium vivax* infection would be important for reduing the risk of hemolysis following Primaquine (PQ) administration. Rational evidence based PQ administration may be helpful in contributing towards the elimination of malaria from the country.

Key words: G6PD, BinaxNow G6PD and CareStart™ G6PD

INTRODUCTION

Glucose-6-phosphaste dehydrogenase (G6PD) is an important enzyme in cellular metabolism in the first and rate-limiting step of pentose-phosphate pathway. Among the functions of this pathway is the protection of cells from oxidative stress, through its role in conversion of NADP to NADPH, thereby replenishing the levels of reduced glutathione. As erythrocytes lack other detoxifying enzymes, people with G6PD deficiency are susceptible to oxidative stress in their red blood cells (Cappellini and Fiorelli 2008). The G6PD gene is located on the X chromosome, and as a result deficiency shows X-linked inheritance, whereby a higher proportion of males suffer from the deficiency.

Malaria in humans is predominantly caused by two *Plasmodium* species, *Plasmodium falciparum* and *Plasmodium vivax* and is estimated to be spread over more than 90 countries, putting nearly 3.3 billion people at risk of disease (Guerra et al. 2010, WHO 2012). The prevalence of G6PD across countries was found to have a good correlation with those where, historically, malaria transmission has occurred (Howes et al. 2012). The explanation for this association has been that G6PD is associated with protection against *P. falciparum* (Mockenhaupt et al. 2003; Clark et al. 2009) and *P. vivax* infections (Leslie et al. 2010; Santana et al. 2013). The mechanism conferring resistance in G6PD subjects may be related to an impaired antioxidant defence in ring-stage parasitized red cells, which could lead to membrane damage, triggering increased removal of infected cells by phagocytosis before parasite maturation to the trophozoite and schizont stages (Ruwende et al. 1995). G6PD is also thought to be a protection factor against severe manifestations of malaria, although studies regarding which individuals, hemizygous males or heterozygous females, may be protected present

discrepancies (Ruwende et al. 1995; Guindo et al. 2007). G6PD deficiency is common in malaria endemic regions and is estimated to affect more than 400 million people worldwide. It is a genetic defect, which is one of the most prevalent polymorphisms and enzymopathies in humans, particularly in males.

This genetic defect was discovered in 1956 when some patients developed haemolyticanaemia after the dose of the anti-malarial drug primaquine. G6PD-deficient erythrocytes are more susceptible to destruction by oxidative stress than normal erythrocytes due to the lower NADPH levels. Individuals with this genetic defect may exhibit non-immune haemolyticanaemia in response to a number of stimuli, most commonly infections or exposure to certain medications or chemicals. Fortunately, the large majority of G6PD deficient subjects have no clinical manifestations and the condition remains asymptomatic until they are exposed to a hemolytic trigger.

The geographical distribution of malaria is remarkably similar to the world distribution of deficient G6PD variants. It is postulated that the high frequency of G6PD deficiency has arisen because G6PD deficient variants confer some protection or resistance against malaria caused by *Plasmodium falciparum* and *Plasmodium vivax*.

In Southeast Asia, a large number of G6PD deficient variants have been reported from various populations. In Nepal, previous studies have shown that G6PD deficiency in Tharu ethnic inhabitants of Chitwan districts (WHO 2013). With the development after 1960's and movement of population the scenario is likely to be changed. In a study done by Suzuki et al. 2007 it was observed that the group that has lived for many decades in malaria endemic lowland area, the Danuwar, was found to have a high prevalence of alpha-thalassaemia (79.4%) and low prevalence of haemoglobin E and G6PD deficiency. Much lower prevalence of alpha-thalassaemia were observed in the Newar (20.5%), Parbate (16.5%) and Tamang (8.8%), who, until the 1950s, all spent their hot-season nights in malaria-free areas at higher altitude.

Glucose-6-phosphate dehydrogenase deficiency poses a significant impediment to primaquine use for the elimination of liver stage infection with *Plasmodium vivax* and for gametocyte clearance, because of the risk of life-threatening haemolytic anaemia that can occur in G6PD deficient patients. Although a range of methods for screening G6PD deficiency have been described, almost all require skilled personnel, expensive laboratory equipment, freshly collected blood, and is time consuming; factors that render them unsuitable for mass-screening purposes. So commercially available RDT/ICT card tests: BinaxNow- G6PD and CareStart G6PD were used for determination of G6PD deficiency for mass screening purposes (Tinley et al. 2010, Kim et al. 2011, vonFricken et al. 2014). This cross sectional study was designed to detect the prevalence of G6PD deficiency in indigenous peoples communities of Jhapa, Morang and Dhanusha districts. Evidence from such a study would be important for taking policy decision on the use of G6PD RDTs, and use/abstinence of Primaquine in specific population/age groups, as a radical treatment of malaria.

MATERIALS AND METHODS

Study design, study site and study population: The study was designed as a descriptive cross- sectional study. The study was conducted during April to June 2013. The study sites were malaria endemic village development communities (VDCs) of Jhapa, Morang and Dhanusha districts which are in moderate malaria risk identified by the last 5 years malaria data and the recent micro-stratification study report (EDCD/DoHS/MOH Govt. of Nepal. Micro-stratification of malaria risk in Nepal 2013). Information as per the questionnaire were obtained from 670 volunteers recorded in the case record format and analyzed following statistical tools. A total of 670 blood samples were collected from volunteers representing different ethinic groups in the age groups of 5-64 years and agreeing to partiucipate in the study from Jhapa, Morang and Dhanusha districts. (NHRC ERB Reference no. 1134, 23 March 2013) and tested qualitatively for detecting G6PD deficiency using both BinaxNow G6PD and CareStart™ G6PD rapid diagnostic test kits by WHO Nepal for the survey.

Inclusion and exclusion criteria: Any person in the age group 5-64 years living in the malaria endemica area of above 3 districts were included. Any person temporarily staying in the area, however not from the indigenous ethnic groups and pregnant women or children below 5 years, and older persons above 64 years were excluded from the study.

Sample collection and testing: The blood samples from the voluteers were taken by squeezing fingertip

and piercing with lancet. The qualitative G6PD testing were performed using BinaxNow G6PD and CareStart™ G6PD test kits. All the tests were done at the collection site, maintaining aseptic condtion and universal precations.

Testing of G6PD deficiency by BinaxNow kit: BinaxNow G6PD test kits were used for follwoing manufacturer's instructions. Briefly, the device from the pouch was removed prior to use, brought to room temperature, and was recorded on the front of the device. The test was performed at the temperature between 18°C to 25°C. 70µl of reagent- A and 10µl of blood from the volunteer was mixed in the sample preparation vial with the help of sterile micropipette mixed 3 times with push and pull by micropipette. Fifty microliter of mixed blood sample was added slowly to the middle of white pad of the test. The adhesive stripe was removed and the device was closed. The result was noted after 7 minutes. A distinct color change to black/brown on the top half of the reaction pad in visible reading window was considered normal while if there was no color change in the top half of the reaction pad in 7 minutes the samples were considered as G6PD deficient (Tinley et al. 2010).

Testing of G6PD deficiency by CareStart™kit method: CareStart™ G6PD testing was performed following manufacturers' instruction. Briefly, the area of fingertip to be pierced was cleaned with alcohol pad and fingertip was squeezed and pierced using sterile lancet. Blood sample (2µl) was collected using sample pipette provided by the kit. Whole blood (2µl) was applied to the sample well in the test kit. Immediately after application of blood 2 drop (100 µl) of assay buffer was added to buffer well. The result was noted 10 minutes. Samples with normal G6PD produced a distinct purple color in the result window, ehile no color change was observed with G6PD deficient samples (Kim et al. 2011).

RESULTS

During the study period, a total of 670 whole blood samples were tested by rapid screening test for G6PD (Glucose-6–phosphate dehydrogenase) deficiency in human blood by using Carestart™ G6PD kit and BinaxNow G6PD kit.

Comparison of G6PD diagnostic test kits

Out of 670 blood samples tested for G6PD deficiency, 6.1% (41) were found to be G6PD deficient in BinaxNow while 6.3% (42) cases were found G6PD deficient in CareStart™. G6PD deficiency detected by test kit only in 30 samples. Of the total 41 G6PDd detected samples by BinaxNow kit, 11 were found by CareStart™ kit. Similarly, of the total 42 G6PDd samples detected by CareStart™ kit, 12 were found by BinaxNow kit. As both of the test kits are competing RDTs and we didnot have any gold standard to compare either of both. We only could compare the easiness of testing kits, visibility of the test results in the kits and not the sensitivity and specificity of the tests for comparison. The detail of comparison is as below (Table 1).

Table 1: Comparison between BinaxNow and CareStart™ kit

Test kits		BinaxNow G6PD kit		Total
		DEficient	Normal	
CareStart™ Kit	Deficient	30	12	42 (6.3)
	Normal	11	617	628 (93.7)
Total		**41 (6.1)**	**629 (93.9)**	**670**

Sex wise distribution of G6PD deficient cases

Among 42 G6PD deficient cases 21(7.3%) were male and 21(5.4%) were female. There is no significant association of G6PD deficiency with sex (p=0.529) (Table 2).

Table 2: Sex wise distribution of G6PD deficient cases

Sex	No. of G6PDd	Percent of G6PDd	p-value
Male (n=285)	21	7.3	0.529
Female (n=385)	21	5.4	

Distribution of G6PD deficient cases in different ethnic groups

G6PD deficiency was evaluated in the ethnic group population of the above 3 districts. The highest percentage of deficient cases were from Rajbanshi followed by Santhal, Rishidev and Tharu community among the three districts (Table 3).

Table 3: Distribution of G6PD deficient cases in different ethnic groups

Ethnic group	Total test cases	Deficient cases (%)
Tharu	151	8 (5.3)
Rajbanshi	154	18 (11.7)
Mushahar	204	7 (3.4)
Rishidev	61	4 (6.6)
Santhal	49	5 (10.2)
Others (non-ethnic)	51	0 (0)

District wise distribution of G6PD deficient cases

Highest percentage of deficient cases was noted from Jhapa district followed by Morang and Dhanusha respectively.There was significant association between G6PD deficiency and region (p=0.010).

Table 4: Region wise distribution of G6PD deficient cases

District	Total number of test cases	Confirmed deficient cases (%)
Jhapa	224	22 (9.8)
Morang	225	13 (5.8)
Dhanusha	221	7 (3.1)

DISCUSSION

Over one third of world's population lives at the risk of *Plasmodium vivax* infection. Limited evidence underpins estimation of clinical cases, however globally about 400 million clincal cases are reported anually including potentially severe illness and death. In the context of malaria elimination, therapy must target all infections including asymptomatic and sub-microscopic blood stage infections, dormant liver stage hypnozoites as well as clinical cases. One of the many consequences of neglect for last half century of *P. vivax* has been the failure to address the Primaquine toxicity problem with G6PD. No non-toxic therapeutic alternatives exist, and existing G6PDd diagnostics are largely impractical in point of care settings (Ghimire et al. 2017).

Glucose-6-phosphate dehydrogenase (G6PD) is a ubiquitously expressed enzyme that has a housekeeping role in all cells, and is particularly critical to the integrity and functioning of red blood cells (RBCs). G6PD is an important enzyme in cellular metabolism and rate-limiting step of pentose-phosphate pathway. Among the functions of this pathway is the protection of cells from oxidative stress, through its role in conversion of NADP to NADPH, thereby replenishing the levels of reduced glutathione. As erythrocytes lack other detoxifying enzymes, people with G6PD deficiency are susceptible to oxidative stress in their red blood cells (Cappellini and Fiorelli 2008).

Primaquine is the only -antimalarial drug recommended by WHO for the radical cure of malaria. However, a major barrier to widescale adoption of both of these drugs is toxicity in people with G6PD deficiency. While all people exposed to primaquine experience some drop in haemoglobin concentrations (Kellermeyer et al. 1962), people with G6PD deficiency are more likely to experience severe haemolysis, leading to severe haemolyticanaemia and, potentially, death.

The blood samples from the volunteer participants inhabiting the malaria endemic districts and consenting to participate in the study through the written consent were tested on the spot by using G6PD kits BinaxNow G6PD and CareStart™ G6PD kits. Out of 670 volunteer tested for G6PDd, 6.1% were detected as G6PD deficient were detected by BinaxNow G6PD RDT and 6.3% by CareStart™ G6PD RDT. Although the detection of G6PDd by BinaxNow is slighly more than that by CareStart™ RDT the difference is not statistically significant. Low prevalence of G6PD deficiency was reported from Latin America (Monteiro et al. 2014) whereas average prevalence of G6PD deficiency (2.1%) with apparent differences between ethnic groups and geographical regions was reported from Tanjania (Rebholtz et al. 2006). The variation is due to the geographical variation in sampling area, and ethnic groups of the people.

Compared to BinaxNow G6PD kit method sensitivity and specificity of the Carestart™ is 73.17% and 98.09%. Positive predictive value of test is 71.42% and negative predictive value is 98.24%. However, different sensitivity (86%) and nearly same specificity (98%) value were reported from a study done in UK (Tinley et al. 2010). The result was also inconsistence to the similar study in cape coast Ghana (Daniel et al. 2010). The factors responsible for the poor performance of the

kits might be the storage temperature, the temperature at which the test is performed, the ethnic community of Ghana and Nepal and also the geographical distribution of Nepal.

Among the G6PD deficient cases, male and female ratio was 1:1 and there was no significant relationship between gender and occurrence of G6PD deficiency (p=0.529). This finding is different than other studies in which number of G6PD deficient cases were more in male (Nimol et al. 2010; Julien et al. 2011). The reason for this is still unknown and requires further molecualr level investigation with more countrywide sample representation.

Looking at G6PDd distribution in different indigenous groups staying in the study area highest number of confirmed deficient cases were from Rajbanshi (11.7%) followed by Santhal (10.2%), Rishidev (6.6%) and Tharu (5.3%) in the three study districts. Higher prevalence of G6PDd was found in Jhapa (9.8%) and Morang districts (5; 8%) this result is concordance with previous studies of Hongkong, Combodia, UK and in Ghana (Nimol et al. 2010; Daniel et al. 2012). The parasite causing diseases like malaria cannot survive well in the G6PD deficient cells. One of the schools of thought is "The ethnic community or inhabitants of malaria endemic areas are being exposed to such parasites for centuries and hence deficiency is developed and inherited for generations for providing protection against deadly *P. falciparum* malaria. In context of Nepal, Tharu, Chaudhary, Rajbanshi, Santhal and Rishidev communities are majority inhabitants of malaria endemic districts since centuries, are still thought to be immune for deadly falciparum malaria. The G6PD deficiency on such community might have developed in the long run to maintain their immunity against *P. falciparum* malaria, The scientific basis of which requires further studies at molecualr level. This study simply tried to find out the qualitative information on G6PDd in different indigenous population taking in to consideration for introducing mandatory G6PDd testing in confirmed *P. vivax* cases, for effective implementation of radical treatment with Primaquine following Chloroquine as per national malaria treatment protocol.

Malaria elimination will be possible only with serious national, regional and international efforts addressing asymptomatic infection and persistent *P. vivax* infection. Currently available drugs that can radically cure *P. vivax* malaria and are able to reduce transmission of malaria parasites are those in the 8-aminoquinoline family, such as PQ. Tafenoquine, which is also an 8-aminoquinoline, is currently being assessed as a single-dose radical cure therapy, once it is approved for marketting following successful complition of clinical trials and market authorization. Unfortunately, individuals carrying the G6PDd are at risk to develop severe haemolysis if exposed to these drugs, implying that radical cure regimens will require broader testing for G6PD deficiency.

CONCLUSION

The higher prevalence of G6PDd in certain ethnic group; Rajbanshi, Santhal, Rishidev and Tharu in comparison to Brahmin and Chhetri in the eastern region clearly demonstrates the importance of G6PD deficiency screening prior to Primaquine prescription for the treatment of vivax malaria in the malaria endemic areas. Knowing the G6PDd status gives leverage to use 14 days primaquine in G6PD normal patients, while weekly primaquine under close clinical monitoring/medical supervision with ready access to blood transfusion services in G6PDd cases. In absence of appropriate G6PDd testing facility and lack of knowledge on prevalent genotype and severity, it will be prudent to err on the safe side. Rational use of such radical treatment facilitates the country in malaria elimination as planned by NMSP-2016.

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Review Article

The wastewater resistome: Lurking antibiotic resistancein the environment

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ABSTRACT

Antibiotic resistant bacteria and their resistance determinants have been frequently reported in all types of environments especially in water systems. The collection of antibiotic resistant genes and precursors in host bacteria occupying wastewater environment represents wastewater resistome. This resistome is highly potential to resist almost all types of antibiotics exposed to the environment. Since antimicrobial resistance could be transferred from environmental bacteria to clinical or veterinary pathogens and vice versa, wastewaters systems are spawning grounds for global antibiotic resistance, with potentially serious infection consequences for human and animals. Although the sub-lethal dose of antibiotics is key stimulating factor, however, physiological and environmental stress on bacteria due to various factors including non-antibiotic antimicrobials, organic pollutants like PAHs, chlorinated phenols and heavy metals may also drivethe enrichment of antibiotic resistance genes in the environment. Although actual key mechanisms for survival of antibiotic resistance bacteria, proliferation and dissemination of such genes in wastewater are still elusive, this mini review briefly describes environmental resistome, possible drivers and dissemination paths of resistance genes in wastewater systems. Considering the hazards, efficient wastewater treatment technologies are needed to be developed for mitigation of antibiotic resistance bacteria and resistance genes.

Key words: environmental resistome, wastewater, antibiotic resistant genes, mobile genetic elements

INTRODUCTION

Emergence of microbial resistance to almost all types of antibiotics has posed a serious threat to human and animal health increasing perilous risk towards microbial infections worldwide (Berendonk et al. 2015). More recently, the environment is increasingly being recognized for itsimportant role in the global spread of clinically relevant antibiotic resistance (Singer et al. 2016) because environmental microorganisms are highly antibiotic resistant and harbor responsible genetic content. Although antibiotic resistant bacteria (ARB) and genes (ARGs) are found in different environments (Allen et al. 2010; Wright et al. 2010), wastewater treatment plants (WWTPs) for example those treating sewage and municipal (Rizzo et al. 2013; Mao et al. 2015; Rodriguez-Mozaz et al. 2015), hospital (Rodriguez-Mozaz et al. 2015), pharmaceutical (Tao et al. 2016; Wang et al. 2015), swine farm (Zhu et al. 2013), livestock farm (Chen et al. 2015), tannery wastewater (Wang et al. 2013) have been reported as important reservoirs of ARGs. Since these wastewaters are normally contaminated with high or sub-lethal concentrations of antibiotics (Watkinson et al. 2007) which create a selective pressure for acquiring the resistance via horizontal spread of ARGs.Additionally, water environments are favorable for transfer of resistance genes (Aydin et al. 2016). Therefore, such WWTPs are often considered as hot spot for resistant bacteria and genes (Rizzo et al. 2013) and could be a point source to disseminate into natural environment like fresh water sediments (Czekalski et al. 2014) and other environments. These reports clearly indicate that antibiotic resistance is lurking in the wastewaters and wastewater treatment plants. It is, therefore interesting to understand increasing extent of resistome, potential drivers and their dissemination through wastewater treatment systems.

Environmental resistome

The term 'resistome'was proposed by Wright (2007) to describe the totality of all the antibiotic resistance genes and their precursors in both pathogenic and non-pathogenic bacteria. WWTPs accumulatecommunities of enteric- and environmental bacteria coupled with

residual concentrations of antibiotics, disinfectants, and heavy metals, which potentially select for ARB (Marano and Cytryn 2017) and spread antibiotic resistance.The wastewater antibiotic resistome is dynamic and ever expanding with varieties of antibiotic resistant genes and mobile genetic elements (Wang et al. 2013; Chen et al. 2015; Li et al. 2015; Mao et al. 2015; Munck et al. 2015; Rodriguez-Mozaz et al. 2015; Tao et al. 2016; Yang et al. 2016; Guo et al. 2017; Su et al. 2017) that reveal lurking nature of antibiotic resistance.

Szczepanowski et al. (2009) found 140 and 123 different resistance-gene-specific amplicons including aminoglycoside, beta-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim and sulfonamide resistance genes as well as multidrug efflux and small multidrug resistance genes on total plasmid DNA preparations obtained from bacteria of activated sludge or the WWTP's final effluents. Recently, the rapid progress in high throughput sequencing and functional gene array has allowed comprehensive profiling of ARGs in environmental samples with the metagenomics approach (Tao et al. 2016; Guo et al. 2017). A metagenomic profiling of ARGs and MGEs in tannery WWTP revealed that aerobic and anaerobic sludge contained *sul1* and *tet* genes in high abundance accompanied with over 70 types of insertion sequences and class 1 integrase (Wang et al. 2013). Su et al. (2017) detected twenty resistance gene types consisting of 381 subtypes with the three most dominant resistance gene types, conferring aminoglycoside, tetracycline, and beta-lactam resistance in sewage.Another recent metagenomic analysis reported the occurrence of 42 and 51 different subtypes of ARGsin the activated sludge and digested sludge, respectively along with abundant MGEs including plasmids, transposons, integrons (*intI*1) and insertion sequences (ISSsp4, ISMsa21, ISMba16) (Guo et al. 2017). High priority carbapenem-resistant gene (bla_{KPC-2}) and its hosts including *Klebsiella* spp., *Enterococcus* spp., *Acinetobacter* spp., *Escherichia* spp., *Shigella* spp. were also detected in all stages of WWTP (Yang et al. 2016).

Environmental drivers of antibiotic resistance

Horizontal gene transfer is a key mechanism for evolution of bacterial genomes and can disseminate the genes among microbial communities in environmental reservoirs and potential pathogens (Figure 1). It is well understood that selective pressure created by low concentration of antibiotics can promote horizontal gene transfer in environment. However, the mechanisms of antibiotic resistance may not necessarily evolve only in response to antibiotics (Piddock et al. 2006) and surprisingly, the presence of antibiotics did not significantly increase the efficiency of conjugation in many bacteria (Lopatkin et al. 2016). Instead, physiological status of microbial cell and the environmental stress over the cell could be important determinant of antimicrobial resistance (Figure 1). Bacterial stress responses due to exposure to nutrient starvation/limitation, reactive oxygen and nitrogen species, membrane damage, elevated temperature, and ribosome disruptionin wastewater treatment systems may initiate microbial response that could positively recruit the resistance determinants in microbial community enhancing their antimicrobial resistance (Poole 2012). These findings indicate that horizontal transfer of ARGs in environmental bacteria might also be enhanced due to several factors besides antibiotics. As mentioned, consistent stress on microbial cells may play important role to encourage acquiring resistance. Several toxic substances including heavy metals (Li et al. 2015;), chlorinated phenols (Baker-Austin et al. 2006; Muller et al. 2015), triclosan biocides (Karatzas et al. 2007), disinfectants (Zhang et al. 2017), herbicides (Kurenbach et al. 2015), different polyaromatic hydrocarbons (Chen et al. 2017), including pyrene (Sun et al. 2015) have been reported to co-select or enrich the ARGs mostly encoding multidrug resistant efflux pumps in the water or soil environment.Since biocide/metal resistance genes (BMRGs) for example, mercury resistance genes and the *qacE*Δ1 gene (resistance to quaternary ammonium compounds) generally co-occur with ARGs on plasmids, and cadmium/zinc and macrolide/aminoglycoside resistance genes are linked (Pal et al. 2015). Besides, efflux pumps can often provide cross-resistance to multiple chemicals.

In addition, mobile genetic elements (MGEs) including plasmids, insertion sequences and transposons play important role in resistance gene transfer (Frost et al. 2005; Li et al. 2015). So higher abundance of the MGEs enhances the risk of disseminating the antibiotic resistance in environment.

Antibiotic resistance dissemination paths

Application of antibiotics for medicine and veterinary use provides selective pressure to acquire resistance among selected bacteria in gut microbiome communities in humans and animals (Figure 2). The

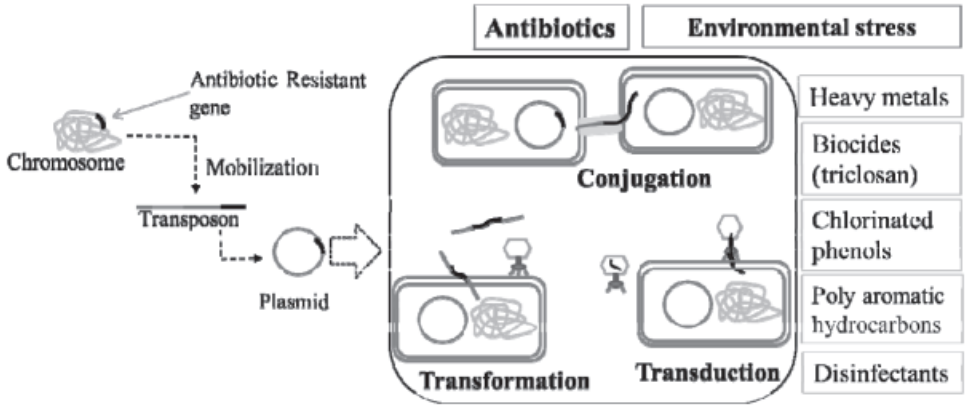

Figure 1: Antibiotic resistant gene mobilization, horizontal gene transfer and potential drivers for proliferation of antibiotic resistance in wastewater.

resistant bacteria are transferred to environment for example wastewater by various means. Once entered to the wastewater systems, ARB can disseminate their resistance carriage among other members of the endogenous microbial community. Additionally huge amount of the persistent antibiotics trigger spread of antibiotic resistanceas the hot spot in WWTPs (Singer et al. 2016). Unfortunately, WWTPs do not efficiently remove all ARGs that are subsequently released in treated effluents (Yang et al. 2014). The treated water if reclaimed for human use, the resistance genes and their host would be exposed to humans; and at same time if discharged, the effluent contaminates receiving water bodies, soil and groundwater aquifer that ultimately may transport antibiotic resistance determinants again to humans (Figure 2).

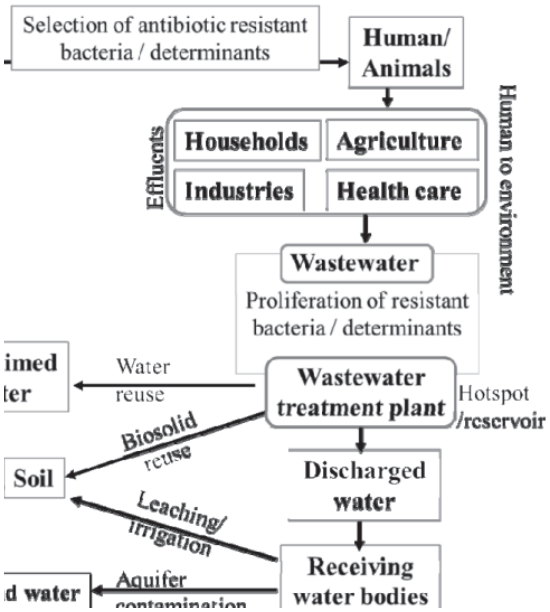

Figure 2: Potential dissemination of antibiotic resistance bacteria and their genes (modified from Guo et al. 2017)

The use of biosolids or sludge as a manureis also a potential route of human exposure to antibiotic resistance genes through consumption of contaminated crops (Rahube et al. 2016). Thus WWTPs lead to multi-lateral dissemination of antibiotic resistance in environment channeling back to humans.

In fact, this environmental resistome constituting large amount of resistant genes have the high potential to be transferred to pathogens and the evidences that at least

some clinically relevant resistance genes have originated in environmental microbes have been reported (Wright 2010). However, core resistome is unique to the WWTP environment, with 10% of the resistance genes found outside the WWTP environment (Munck et al. 2015) indicating low frequency of genetic exchange between wastewater and human resistome. Even in the case, the potential risk of acquiring antibiotic resistance to pathogens remains high enough to challenge the efficiency of commonly used antibiotics.

CONCLUSION

Water and wastewater systems are major part of environmental resistome as a great reservoir of antibiotic determinants and their host bacteria. It is now seriously realized that emergence and dissemination of the antibiotic resistant bacteria and their respective genes and mobile elements in the environment could pose much higher risk to human health than previously expected. Therefore the global antibiotic resistance control strategies should consider to recommend stringent criteria for discharge of antibiotics in wastewater. Although some factors for induction and spread of resistant determinants have been reported but the core mechanism and driving forces acting on environmental resistome are still elusive. Research efforts should focus to unravel the key factors, mechanisms and transmission paths of environmental resistome. Engineered biological systems to treat water and wastewater are reliable solutions to mitigate pollutants. Innovative treatment technologies should be developed and adopted to minimize the contents of the environmental resistome which would be great contribution in tackling the global burden of antibiotic resistance.

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