ESBL and MBL mediated resistance in clinical isolates of non-fermenting gram negative bacilli (NFGNB) in Nepal

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Introduction
Nonfermenter gram negative bacilli (NFGNB) are a taxonomically diverse group of aerobic, nonsporing, bacilli that either do not utilize glucose as a source of energy or utilize it oxidatively [1]. They occur as saprophytes in the environment and some are also found as commensals in the human gut [2,3]. NFGNB including Pseudomonas spp., Acinetobacter spp., Stenotrophomonas maltophilia, Burkholderia cepacia complex (BCC), are emerging as important causes of blood stream infections (BSI) worldwide particularly in immune-compromised patients, patients with hematological malignancies and patients admitted in intensive care units (ICUs) [4,5]. Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen and the most common gram-negative bacterium found in nosocomial infections [6]. Despite improvements in antibiotic therapy, Pseudomonas aeruginosa is intrinsically resistant to a number of antimicrobial agents [7]. Members of the genus Acinetobacter are ubiquitous, free living, strictly aerobic, short, often capsulated, non-motile, gram-negative (or gram variable) bacilli or cocccobacilli (often diplococcosbacilli) with a DNA G+C content of 39 to 47 mol% that grow on simple media and prefer moist environment and can be easily obtained from soil, water, food and sewage [8]. Acinetobacter spp., usually considered to be opportunistic pathogen, is one of the most important notorious nosocomial health-care related pathogen especially in critically-ill hospitalized patients particularly in intensive care units and occasionally in other units too [9].

ABSTRACT
Objective: The present study interpreted the prevalence of NFGNB from clinical specimens and assessed antibiotic resistance pattern as well as incidence of Extended Spectrum Beta lactamase(ESBL) and Metallo-Beta Lactamase(MBL) producers.
Methods: A total of 3585 clinical specimens were included and all the samples were processed for routine bacterial culture and antimicrobial susceptibility test as per standard protocol. They were further subjected to ESBL production detection by phenotypic confirmatory double disk diffusion test using cefotaxime with and without clavulanic acid. Imipenem resistant isolates were selected for the detection of MBL production by Imipenem-EDTA combined disc test. All the specimens were identified by the classical Microbiological and Biochemical tests.
Results: The result showed that 304 non fermenting gram negative bacilli were isolated from various clinical specimens. Out of 304 NFGNB, 195 (64.1%) isolates were Pseudomonas spp. followed by Acinetobacter spp. i.e 109 (35.9%). Imipenem (85.1% sensitive) were the most effective drug against P. aeruginosa where as Acinetobacter spp. were most sensitive to colistin and Imipenem with 76.1% and 18.3% sensitivity respectively. Altogether, 69.1% of total NFGNB isolates were MDR and ESBL and MDR produces were determined to be 51.97% and 21.4% respectively. Fortyfive (14.8%) of the total isolated were found to produce both ESBL and MBL.
Conclusions: The study showed increasing trend of MDR, ESBL and MBL production in NFGNB so constant survey of antibiotic sensitivity and precautionary use of antibiotics is essential to control and manage spread of these isolates in different units of health institutions.
KEY WORDS: NFGNB, ESBL, MBL, Acinetobacter spp, Pseudomonas aeruginosa

NFGNB are known to account for about 15% of all bacterial isolates from a clinical microbiology laboratory [10, 11]. In recent years, due to the liberal and empirical use of antibiotics, NFGNB have emerged as important healthcare-associated pathogens. They have been incriminated in infections, such as, septicemia, meningitis, pneumonia, urinary tract infections (UTI), and surgical site infections.
(SSI) [12]. NFGNB are innately resistant to many antibiotics and are known to produce extended spectrum β-lactamases and metallo β-lactamases. Important ESBL varieties are TEM β-lactamases, SHV β-lactamases, CTX-M β-lactamases and OXA- β-lactamases [11,12]. Acquisition of drug resistance by these pathogenic strains has posed serious challenge for the therapeutic management of clinical cases. Infections that are caused by drug resistant bacteria are associated with up to five times higher mortality rates compared with infections that are caused by susceptible bacteria [13]. These resistant organisms need to be identified and appropriate action be taken before scenario gets worse. There are increasing reports of Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) producing isolates expressing multidrug resistance (MDR), defined as concomitant resistance to at least two different antibiotic classes [14,15]. Patients at high risk of developing colonization or infection with ESBL producing microorganisms are often seriously ill patients with prolonged hospital stays, e.g. undergoing hemodialysis and in whom invasive medical devices are present [16]. Extended-spectrum β-Lactamase and metallo β-Lactamase producing bacteria are emerging concern for health professionals as they are associated with more severe form of disease and antibiotic resistance. ESBL and MBL have been studied well in Nepalese community [17] yet the data remains lacking for those organisms among NFGNB. As Pseudomonas spp and Acinetobacter spp are increasing threat to global health. This study was undertaken to determine the prevalence of nonfermenter gram negative bacteria with special reference to Pseudomonas and Acinetobacter and to determine their antibiotic resistance, MDR, ESBL and MBL pattern.

Materials and Methods

This cross-sectional study was conducted at B&B Hospital Kathmandu, Nepal, from April 2014 to September 2014.

Collection, isolation, and identification of bacteria

A total of 3585 samples that were sent for routine laboratory investigation were processed in this study. These samples included urine, sputum, blood, pus, throat swab, body fluids and other miscellaneous specimens such as various types of tips (Catheter tip, suction tip, CVP tip, tracheostomy tips). Processing of the specimen and identification of the isolates were done by using standard microbiological techniques which comprises of studying the colonial morphology, staining reactions and various biochemical properties [18].

Determination of antimicrobial susceptibility pattern

Susceptibility tests of the different clinical isolates towards various antibiotics were performed by modified Kirby-Bauer modified disk diffusion method for the commonly isolated pathogens using Mueller Hinton Agar (MHA) according to Clinical Laboratory Standards Institute (CLSI) guidelines [19].

Screening and confirmation for ESBL producers

The MDR isolates were screened for possible ESBL production using ceftazidime (30µg), cefotaxime (30µg) (CLSI, 2005). According to the guidelines, isolates showing ceftazidime <22 mm, cefotaxime < 27 mm are the possible ESBL producing strains. The screen positive isolates i.e. suspected ESBL producers were subjected to Combined Disk (CD) test for confirmation of ESBL production using MASTDISCS™ extended spectrum β-Lactamase (ESBL) detection discs. The kit consisted of:

Set 1: Ceftazidime (30µg) and ceftazidime (30µg) plus clavulanic acid (10µg);
Set 2: Cefotaxime (30µg) and cefotaxime (30µg) plus clavulanic acid (10µg).

Screening and confirmation for MBL producers

Isolates showing resistance to Imipenem and ceftazidime were subjected for the EDTA combined disc method-by Imipenem detection of possible MBL production. Test organisms were inoculated onto plates of Mueller-Hinton agar. Two 10 µg Imipenem discs were placed on the plate, and 750µg of 0.5 M EDTA solution of pH 8 were added to one of them. The inhibition zones of the Imipenem and Imipenem -EDTA discs were compared after 16 to 18 h of incubation in aerobic condition at 35°C. All of the MBL-positive isolates were well separated from MBL-negative isolates by the criterion of a ≥7 mm increase of inhibition zone with the discs to which 750 µg of EDTA was added [20].
Ethics statement
Ethical approval was obtained from the Ethical Review Committee, B&B Hospital, Gaurko, Lalitpur, Nepal, and informed consent was obtained from the patients or their relatives. The research was fully compliant with the Helsinki Declaration.

Statistical analysis
All the results were entered in the worksheet of SPSS software version 20. Chi-square test was used to determine significant association of dependable variables like susceptibility to ciprofloxacin, sensitivity to carbapenems etc to different independent variables like ESBL and MBL production.

Results
Bacterial isolation and prevalence of pathogens
Out of 3585 clinical specimens processed, 1523 (42.24%) different bacterial isolates were obtained, of which 304 (19.97%) were NFGNB, 651 (42.74%) were other gram negative bacteria like E. coli, K. pneumoniae and Proteus mirabilis etc where as 568 (37.29%) were gram positive. Among NFGNB, 195 (64.1%) were Pseudomonas aeruginosa, 109 (35.9%) were Acinetobacter spp. The distribution pattern of NFGNB species according to the specimen is shown in Figure 1.

Figure 1. Distribution of NFGNB species according to clinical specimens.

Antibiotic sensitivity pattern of NFGNB isolates
As shown in Table 1, P. aeruginosa strains were most sensitive towards imipenem (85.1%) followed by colistin (71.3%), amikacin (64.1%) and gentamicin (56.4%). P. aeruginosa were most resistant towards ceftriaxone (70.3%), followed by chloramphenicol (65.6%), ciprofloxacin (53.3%) and ofloxacin (52.8%). Similarly Acinetobacter spp. were more sensitive towards colistin (76.1%) followed by imipenem (18.3%) and amikacin (12.8%). Whereas the drugs most resisted was ceftriaxone (99.1%). Altogether 210 (69.1%) of MDR isolates were found in different clinical specimen. Of the total P. aeruginosa isolated, 53.9 % were MDR and 96.4% of Acinetobacter spp isolated were MDR followed by ciprofloxacin (97%), ofloxacin (96.3%) and gentamicin (90.8%).

Pattern of ESBL production among the NFGNB isolates
Out of 245 (80.3%) screen positive for ESBL, 158 (51.97%) were tested positive for confirmed ESBL production. Eighty-nine (45.6%) of the total P. aeruginosa isolates and 69(63.3%) of the total Acinetobacter spp. isolated were ESBL producers. On statistical analysis association between ESBL production and NFGNB isolates was significant. (p=0.003)

Pattern of MBL production among the NFGNB isolates
Out of 87 (28.6%) screen positive for MBL, 65(21.4%) were tested positive for confirmed MBL production. Seventeen (8.7%) of the total P. aeruginosa isolates and 48(44%) of the total Acinetobacter spp. isolated were MBL
producers. On statistical analysis association between MBL production and NFGNB isolates was significant. (p=0.01)

Among the 158 ESBL producers 45 were also MBL co-producers. On statistical analysis the association between MBL production and ESBL production was found to be statically significant. (p= 0.002).

Table 1. Antibiotic susceptibility pattern of NFGNB isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>P. aeruginosa</th>
<th>Acinetobacter spp</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>89(45.6)</td>
<td>2 (1 %)</td>
<td>104 (53.3 %)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>90(46.2)</td>
<td>2 (1 %)</td>
<td>103 (52.8 %)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>47(24.1)</td>
<td>11 (5.6 %)</td>
<td>137 (70.3 %)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>110(56.4)</td>
<td>17 (8.7 %)</td>
<td>68 (34.9 %)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>125(64.1)</td>
<td>21 (10.8 %)</td>
<td>49 (25.1 %)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>56(28.7)</td>
<td>11 (5.6 %)</td>
<td>128 (65.6 %)</td>
</tr>
<tr>
<td>Meropenam</td>
<td>101(51.8)</td>
<td>4 (2.1 %)</td>
<td>90 (46.2 %)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>166(85.1)</td>
<td>6 (3.1 %)</td>
<td>23 (11.8 %)</td>
</tr>
<tr>
<td>Piperacillin-Tazo</td>
<td>79(40.5)</td>
<td>19 (10 %)</td>
<td>97 (49.7 %)</td>
</tr>
<tr>
<td>Colistin (Methane Sulphonate)</td>
<td>139(71.3)</td>
<td>26 (13.3 %)</td>
<td>30 (15.4 %)</td>
</tr>
</tbody>
</table>

Discussion

Of the total 3585 specimens received, 1523(42.4%) specimens had shown growth, of which gram negative bacteria predominated with 955 (62.7%) isolates. In this study, the prevalence of NFGNB among total positive isolates was 304(19.97%) which was higher compared to 14.1% in a study conducted in India [21] and 9.32% in another study conducted in India [22]. Among these 304 NFGNB P. aeruginosa (64.1%) and Acinetobacter spp. (35.9%) were most commonly isolated. This result is in accordance to various international researches [23]. Malini et al in 2009 [24] reported 64.6% prevalence of P. aeruginosa and 25.3% prevalence of Acinetobacter spp. Among 304 isolates of NFGNB in our study, the prevalence was found to be highest from pus specimens, (45.7%) followed by suction tips (13.2%), tracheostomy tips (10.9%), sputum (10.2%) , urine (6.9%), catheter tip (6.9%) and CVP tip (4.9%), body fluids (1%). Majority of isolates were obtained from pus specimen. Lowest number of isolates was obtained from various body fluids and this result was similar to study done in India [25]. The variable prevalence of NFGNB in different studies might be due to local situation, arrangement of specimens in the study groups, type of institution, local use of antibiotics, sanitary conditions, the number of specimens processed and the rates of bacterial isolation and the study period involved etc[23,24, 25]. The antibiotic sensitivity test of the isolates in this study, in overall showed higher sensitivity towards colistin, meropenem, imipenem, amikacin and gentamicin. Where as highest resistance was observed towards ciprofloxacin, ofloxacin, chloramphenicol. Hence antibiotics belonging to polymyxin, carbapenem and aminoglycosides are drug of choices for the treatment of the NFGNB infections. In a recent research [26] resistance to imipenem was observed in 40% of A. baumannii and 20% of P. aeruginosa isolates. Whereas 42.8 % [27] and 21 % [28] of P. aeruginosa and 14.2 % [27] and 23 % [28] of Acinetobacter spp. were resistant to imipenem as reported in different literature.

In our study 69.1% of the total isolates were found to be MDR. Among the total P. aeruginosa isolated, 53.9% were MDR and 96.4% of Acinetobacter spp. isolated was MDR. This result is consistent with various researches [26]. In our study most of the multi drug resistance isolates were isolated from pus sample. Aloush et al in 2006 [29] also found that the common site of isolation of MDR P. aeruginosa was pus (39%). The MDR phenotype in P. aeruginosa could be mediated by several mechanisms including multidrug efflux systems, enzyme production, outer membrane protein loss and target mutations [30]. The number of multidrug resistant P. aeruginosa ranges from 20% to 85%
in various studies throughout the world [27-29]. Over 70% of strains of *Acinetobacter* spp. Isolated showed resistance to various antibiotics similar to other studies [31].

In our study among the total isolates 51.97% of isolates were confirmed ESBL producers. 45.6% of the total *P. aeruginosa* and 63.3% of total *Acinetobacter* spp. were ESBL producers. This result is consistent with Thipperudraswamy et al in 2014 [21] in which total ESBL producers were 51.5% and 41.6% of *P. aeruginosa* and 80% of *Acinetobacter* spp. were ESBL producers. Various studies from India have reported prevalence of ESBL producers among *P. aeruginosa* ranging from 3.3% to 77.3% [32,33] and among *Acinetobacter* species ranging from 14.2% to 54.6% [32,33]. ESBL production in *Acinetobacter* has been found to be 46% in Turkey [34] and 54.6% in Korea [20].

In our study 21.4% of the total isolates were identified as MBL positive. Of the total *P. aeruginosa* isolates only 8.7% were MBL producers. Chaudhari et al in 2011 [25] found 5% of the total *P. aeruginosa* to be MBL producer and 44% of the total *Acinetobacter* spp. were MBL producer. In our study 58% of *Acinetobacter* spp. and 11% of *P. aeruginosa* were resistant to imipenem. In a recent research [26] resistance to Imipenem was observed in 40% of *A. baumannii* and 20% of *P. aeruginosa* isolates. Whereas 42.8% [27] and 21% [28] of *P. aeruginosa* and 14.2% [27] and 23% [28] of *Acinetobacter* spp. were resistant to imipenem as reported in literature. Varying rates of resistance to carbapenems 36.4% [20], 12.2% [27], 12% [35] was reported in literature. However 94% of *P. aeruginosa* and 100% of *Acinetobacter* spp. isolates were sensitive to imipenem in a study by Malini et al in 2009 [24]. In our study among imipenem resistant isolates, 73.9% of *P. aeruginosa* & 75.0% *Acinetobacter* spp. of showed MBL production whereas higher MBL production in *Acinetobacter* spp. (96.6%) and *P. aeruginosa* (100%) and lower MBL production in *Acinetobacter* spp. (56%) and *P. aeruginosa* (50%) as compared to our study was reported in literature [28,36]. A high percentage of MBL production (74.7%) was observed in our study consistent with reports where MBL production varied from 85.7% - 100% [36]. Majority of MBL positive isolates showed resistance to third generation cephalosporins, aminoglycosides and quinolones which is consistent with various studies [37]. Production of MBL has tremendous therapeutic consequences since these organisms also carry multidrug resistance genes and the only viable option remains the potentially toxic polymyxin B and colistin [26].

This study showed that the *Pseudomonas* is the most predominant NFGNB followed by *Acinetobacter* spp. However, *Acinetobacter* showed more resistant to antibiotic in compare to *Pseudomonas*. The study also revealed that the increasing trend of MDR, ESBL and MBL production in NFGNB so constant survey of antibiotic susceptibility test and precautionary use of antibiotics is essential to control and manage spread of these isolates in different units of hospitals as well as distribution in different health institutions.

**Conflict of Interest**

We declare that we have no conflict of interest.

**Acknowledgments**

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**References**

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