CHARACTERISATION OF ESKAPE PATHOGENS WITH SPECIAL REFERENCE TO MULTIDRUG RESISTANCE AND BIOFILM PRODUCTION IN A NEPALESE HOSPITAL

Rosy Pandey¹*, Shyam Kumar Mishra ², Angela Shrestha¹

Address:
1 Tribhuvan University, St. Xavier's College, Department of Microbiology, Kathmandu, Nepal.
2 Tribhuvan University, Institute of Medicine, Department of Microbiology, Kathmandu, Nepal.

E-mail:
Rosy Pandey- roseypandey@gmail.com,
Shyam Kumar Mishra - shyammishra@iom.edu.np
Angela Shrestha- shrestha.a@sxc.edu.np

*Corresponding author: roseypandey@gmail.com

ABSTRACT

1. Background: “ESKAPE” is an acronym for group of organisms as Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter calcoaceticus baumannii complex, Pseudomonas aeruginosa and Enterobacter spp. They are associated in causing life threatening infections. Global efforts on controlling multidrug resistant (MDR) organisms have been hampered by their rapid emergence, inadequate tests for rapid detection and their ability to escape the antibacterial drugs. The objective of this study was to determine the prevalence of ESKAPE pathogens with prime focus on biofilm production and antibiotic resistance.

2. Methods: A total of 8756 clinical specimens were processed for the isolation and identification of ESKAPE pathogens following standard microbiological protocol. These isolates were subjected to antibiotic sensitivity test as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Detection of resistance phenotypes, viz., extended-spectrum-beta-lactamase (ESBL), metallo-beta-lactamase (MBL), Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant enterococci (VRE) was done by disk diffusion method and E- test method as applicable. The VRE isolates were subjected for detection of Van A and Van B genes. All the isolates were processed for biofilm detection by tube adherence method.

3. Results: The percentage distribution of Staphylococcus aureus was 33.5%, followed by Klebsiella pneumoniae 33.0%, Pseudomonas aeruginosa 18.3%, Acinetobacter calcoaceticus baumannii complex 8.7%, Enterococcus faecium 5.6% and Enterobacter aerogenes 0.9%. MRSA was 57.6% and Vancomycin resistance among Enterococcus
faecium was 20%. ESBL and MBL producing Klebsiella pneumoniae were 16.1%, and 8.1%, Acb complex 10.3% each and Pseudomonas aeruginosa 10.7% and 8.3% respectively. A total of 42.3% of isolates were biofilm producers. Linezolid was drug of choice for VRE isolates. Piperacillin- tazobactam was found to be effective against Pseudomonas aeruginosa, Klebsiella pneumoniae and Enterobacter aerogenes; Ampicillin-sulbactam was the most effective drug against Acb complex excluding polymyxins. Van A gene was detected in all the VRE isolates.

4. Conclusion: This study estimates the burden of the ESKAPE organisms and their antibiotic resistance pattern in a Nepalese hospital. The increasing percentages of drug resistance among these biofilm-producing pathogens pose great threat in medical setting. Surveillance targeting ESKAPE pathogens should be incorporated in infection control policy in Nepal.

Keywords: ESBL, ESKAPE Pathogens, MBL, MRSA, VRE

Background
Emergence of bacterial pathogens with acquired resistance to almost all available antibiotics thus limiting therapeutic choices is one of the major concerns today. These pathogens are also named as 'superbugs', particularly Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter calcoaceticus baumannii complex, Pseudomonas aeruginosa and Enterobacter spp. (ESKAPE) are capable of escaping the bactericidal effect of antibiotics \(^1\). Inefficiency of antibiotics is due to various phenotypic and genotypic mechanisms as drug inactivation, modification of drug binding sites/ targets, changes in cell permeability and mutation \(^2,3\).

Methicillin resistant Staphylococcus aureus (MRSA) emerged in 1960s and currently its infection rate in hospital setting is around 60-70% and in community setting it is up to 50% \(^4\). Staphylococcus aureus with auxiliary PBP-2a encoded by Staphylococcal cassette chromosome mec (SCC mec A, C) mediate β-lactam resistance due to low affinity \(^5,6\).

Beta-lactamase is the enzyme produced by organisms which hydrolyze β-lactam antibiotics mediating resistance and making the drug in treatment impotent. It is classified into two categories, i.e. Ambler molecular classification scheme and Bush-Jacoby-Medieros functional classification system \(^7\). Ambler classification scheme divides beta lactamase into four major classes (class A-D), where A, C and D are serine β-lactamase and class B enzyme
is MBL. Ambler class B enzyme is characterized by its ability to hydrolyze carbapenems and its resistance to all available beta-lactam but is inhibited in presence of metal chelators. ESBL is defined as an enzyme that mediates resistance to penicillin, third generation cephalosporins and aztreonam (but not cephemycins and carbapenems). They are inhibited by β-lactamase inhibitors as clavulanic acid, sulbactam and tazobactam. The most common variant of ESBL is CTX-1 type. A variety of carbapenemases have been described belonging to three classes of β-lactamase, the Ambler class A, C and D. This has brought urgent threat in utility of carbapenem in clinical laboratory, which is the major drug used for treatment of serious infections caused by ESBL-producing organisms (particularly, gram-negative members of ESKAPE pathogens). Carbapenems possess broad spectrum of activity against Gram-negative organisms and is often referred to as the last resort drug for treatment of gravely ill or suspected drug resistant infection. Aminoglycoside resistance and reduced susceptibility to glycopeptides as vancomycin in Gram positive bacteria as Enterococcus faecium and S. aureus have also been reported.

The misuse of antibiotics and the proneness of organisms to carry the resistant genes mediating multidrug resistant infection is a major issue these days. Due to the capacity of these potential pathogens to escape from antibacterial agents causing infection, there is an outcry for more potent antibiotics. Antibiotic resistance among Gram-negative organisms as ESBL, MBL (IMP, VIM, SPM, GIM, and SIM types), AmpC, production of Ambler class A enzymes (KPC and GES) and class D enzymes (OXA-48), along with MRSA, VRE, among Gram-positive organisms have widely emerged. This demand arises due to the fact that the pathogenic microorganism, in one way or another, are able to resist the effect of drugs in course of time and evolution. On the same ground, drug resistant strains of Staphylococcus aureus, mainly MRSA, Vancomycin Resistant Enterococcus faecium (VRE) and β-lactam resistant pathogens have proved to be the leading threats in clinical arena. These pathogens can survive in the hospital setting for longer duration, escape the biocidal effect easily and can be transported from one individual to other, hence spreading in community and/or hospital.

Biofilm is layer of cell clusters embedded in a matrix of extracellular polysaccharide, called polysaccharide intercellular adhesins (PIA), which consists of β-1, 6-N-acetylglucosamine synthesized by N-acetylglucosaminyl transferase. This creates a layer hindering antimicrobial
agents from penetration causing treatment failure. All member of ESKAPE pathogens are potential biofilm producers.

In this study prevalence of ESKAPE pathogens, drug ineffectiveness due to production of biofilms or enzymes like beta lactamases, methicillin resistance among *Staphylococcus aureus*, vancomycin resistance among *Enterococcus faecium* was studied. Among the nine phenotypic variants of vancomycin resistance in *Enterococci* (Van A, B, C, D, E, G, L, M and N), strains possessing Van A and Van B are found to be responsible for human infections. Therefore, in this study detection of Van A / Van B gene was done on the clinical isolates of VRE.

**Materials and Methods**

This was a cross-sectional descriptive study carried out from January to July 2018. A total of 8756 specimens (urine, swab and bodily fluids as pus, blood, sputum, tracheal aspirates, ear, wound throat swabs, pleural fluid, endotracheal secretions, fluid of ovarian cysts, cerebrospinal fluid, semen) were processed from Outpatient department (OPD) and Inpatient department (IPD) of a hospital in Kathmandu. During sample processing, all the tests were carried out appropriately in aseptic conditions. Clinical and microbiological details were recorded of each patient. A repeated specimen from the same patient within 48 hours was not included in the study to exclude selection bias.

**Sample Collection and Processing**

Samples as urine, Sputum, other bodily fluids and swabs were collected, transported and processed following standard laboratory operating protocol.

**Antibiotic Susceptibility Testing**

Antimicrobial susceptibility testing (AST) was carried out on the isolates by Kirby-Bauer disc diffusion method. Determination of isolates exhibiting MRSA, VRE, ESBL, Carbapenemase and MLSB characteristics were done following procedures recommended by Clinical and Laboratory Standards Institute, 2016. Standard international terminology has been ascribed for MDR, XDR and PDR (Pan-drug resistance) by European Centre for Disease Prevention and Control (ECDC), Centers for Disease Control and Prevention (CDC) and are well defined. MDR is defined as acquired non-susceptibility to at least one agent in three or more selective antimicrobial agents.
XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories and PDR is defined as non-susceptibility to all agents in all antimicrobial categories \(^{21}\). For detection of VRE and MBL, minimum inhibitory concentration (MIC) determination by E-strips was performed.

**Detection of ESBL**

**i. Double Disk Synergy Test (DDST)**

DDST was used as primary screening method and combined disk diffusion method was used as the confirmatory method for detection of ESBL producers. The diameter of zone of inhibition with third generation cephalosporin (Cefotaxime 30 µg) alone and with combination of β-lactam inhibitor (Amoxicillin-Clavulanate 20+10 µg) on an inoculated Mueller-Hinton agar (MHA) plate was measured after overnight incubation at 37°C. ESBL production was inferred positive if synergy was seen in the zone of inhibition between Cefotaxime disk and clavulanate containing disk \(^{23}\).

**ii. Cephalosporins/Clavulanate Combinational Disk Method**

As per CLSI guideline, when the difference in zone of inhibition of Cefotaxime (30µg) and Ceftazidime (30µg) in comparison with Cefotaxime clavulanate and Ceftazidime-clavulanate (30 µg + 10 µg) is \(\geq 5\) mm, then the isolates were considered ESBL positive \(^{20}\).

**iii. The E- Test Method (Epsilometer Test)(HiMedia, India)**

In this method, E-strip in which one end consisting of stable gradient of Cefotaxime and other end consisting of gradient of Cefotaxime with constant concentration of clavulanate was used. The isolate was confirmed as ESBL producer if the MIC ratio of Cephalosporin alone compared to Cephalosporin + Clavulanate MIC was \(\geq 8\) \(^{20}\).

**Tests for Metallo-Beta Lactamase Detection**

**A. Screening Test for MBL Detection**

Carbapenem resistant isolates were screened for production of metallo-β-lactamase (MBL) test \(^{20}\).

**B. Combined disk diffusion method**

Phenotypic MBL detection was done by combined disk method where two Imipenem disks (each 10µg), one containing 10 microliter of 0.1M (292 µg) anhydrous EDTA (Thermo Fisher Scientific India Pvt. Ltd) and another without EDTA were placed 25 mm apart.
An increase in zone diameter of >4mm around the IPM-EDTA disk compared to IPM disk alone was considered positive for MBL.

**C. E- Test Method (Epsilometer Test)**

This test uses E-strip in which one end consists of stable gradient of Imipenem and other end consists of gradient of Imipenem with constant concentration of EDTA. MBL production was inferred positive if the MIC ratio of Carbapenem alone vs. Imipenem+EDTA MIC was ≥ 8. The test was done according to manufacturer’s instructions (bioMérieux SA, France).

**Phenotypic detection of VRE**

*Enterococcus faecium* isolates showing insusceptibility to Vancomycin disk (30 µg) were screened as VRE. Then they were subjected to Vancomycin E-test according to manufacturer's instruction (E-Test technical manual, Hi-Media, India; 2018).

![Figure 1: Epsilometer test of Vancomycin Resistant Enterococcus faecium](image)

**Molecular Detection of VRE**

Phenotypically confirmed VRE isolates were processed for molecular detection as follows.

**A. DNA Extraction**

Plasmid extraction of the VRE isolates was done by alkaline hydrolysis method as described in Sambrook and Russell (2001).
B. Polymerase Chain Reaction (PCR)

Primers for amplification of Van A and Van B gene were used following Kirkan et al. 26. For Van A and Van B, amplification, reaction was performed in thermocycler under following conditions. Primers used were

Van A amplicon size 732 base pair
(Forward- GGGAAAAACGACAATTGC)
(Reverse- GTACAATGCGGCGGTAA) and

Van B amplicon size 300 base pair
(Forward- ACCTACCCGTCTTTGTA)
(Reverse- AATGTCTGGCTGGAACGTA) 26.

Initial denaturation at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 54°C for 1 minute and 72°C for 1 minute. Final elongation at 72°C for 10 minutes 27.

C. Gel Electrophoresis

After PCR, the amplicon were analyzed by agarose gel electrophoresis method and the DNA bands were analyzed in UV trans-illuminator.

Statistical Analysis

All the results were entered in statistical package for social science (SPSS) version 16.0 software packages. The P-value <0.05 was assumed significant for analysis.

Results

Culture Positivity of Specimens

Out of 8756 clinical specimens processed, 2384 (27.2%) showed significant growth indicating infection among which 452 (18.96%) showed infection caused by ESKAPE pathogens. The most common isolate was Staphylococcus aureus (n=151, 33.4%) followed by K. pneumoniae (n=149, 33%), P. aeruginosa (n=84, 18.6%) Acinetobacter calcoaceticus baumannii complex (n=39, 8.6%), Enterococcus faecium (n=25, 5.5%) and Enterobacter aerogenes (n=4, 0.9%).

Distribution of ESKAPE in various specimens

Among the different clinical specimens processed, ESKAPE pathogens were most commonly isolated from urine specimens followed by sputum, pus and other bodily fluids (Table 1).
Table 1 Distribution of ESKAPE in various specimens.

| Specimen          | E. faecium (N) | S. aureus (N) | K. pneumoniae (N) | Acb complex (N) | P. aeruginosa (N) | E. aerogenes (N) | Total (N) | %     |
|-------------------|----------------|---------------|-------------------|-----------------|-------------------|-----------------|-----------|-------|
| Urine             | 23             | 60            | 101               | 9               | 30                | 2               | 225       | 49.9% |
| Pus               | 1              | 65            | 13                | 2               | 20                | 0               | 101       | 22.3% |
| Sputum            | 1              | 8             | 29                | 25              | 32                | 2               | 97        | 21.5% |
| Blood             | 0              | 9             | 2                 | 1               | 0                 | 0               | 12        | 2.7%  |
| Semen             | 0              | 5             | 1                 | 0               | 0                 | 0               | 6         | 1.3%  |
| High vaginal swab | 0              | 2             | 1                 | 0               | 0                 | 0               | 3         | 0.7%  |
| Wound swab        | 0              | 1             | -                 | 0               | 0                 | 0               | 2         | 0.4%  |
| E.T tube          | 0              | 0             | 0                 | 1               | 1                 | 0               | 2         | 0.4%  |
| Ear swab          | 0              | 1             | 0                 | 0               | 0                 | 0               | 1         | 0.2%  |
| Broncho alveolar lavage | 0          | 0            | 1                 | 0               | 0                 | 0               | 1         | 0.2%  |
| Suction tip       | 0              | 0             | 0                 | 1               | -                 | 0               | 1         | 0.2%  |
| Oral swab         | 0              | 0             | 0                 | 0               | 1                 | 0               | 1         | 0.2%  |
| Total             | 25             | 151           | 149               | 39              | 84                | 4               | 452       | 100%  |

On the basis of gender, 249 (55.1%) ESKAPE pathogens were isolated from female and 203 (49.9%) from male. The maximum number of patients infected were of the age group 61-70 years (16.4%) followed by 21-30 years of age 14.9%. There was a significant association found in between age group and ESKAPE isolates (p=0.001).

**Antibiotic Susceptibility pattern of ESKAPE pathogens.**

**A. Antibiotic susceptibility pattern of Klebsiella pneumoniae**

Only 51% of K. pneumoniae isolates were sensitive to third generation cephalosporin (Cefixime). It is noteworthy that 17.4% of the isolates showed resistance to Meropenem. Approximately, 70% urinary isolates were susceptible to Nitrofurantoin.

**B. Antibiotic susceptibility pattern of Acb complex**

Acinetobacter calcoaceticus baumannii complex were most commonly sensitive to ampicillin-sulbactam (64.1%) followed by Piperacillin-tazobactam (53.8%), Amikacin and Doxycycline (51.3% each).
C. Antibiotic susceptibility pattern of *Pseudomonas aeruginosa*

Majority of *P. aeruginosa* were sensitive to Piperacillin-tazobactam 91.7% followed by to Meropenem 84.5%, to Amikacin 82.1% to Ceftazidime 70.2% and to Cefepime 60%.

D. Antibiotic susceptibility pattern of *Enterobacter aerogenes* (n=4)

The *Enterobacter aerogenes* isolates were sensitive to Amikacin (75%), Ciprofloxacin 75%, Trimethoprim-sulphomethoxazole 50% and all were susceptible to Piperacillin-tazobactam, Cefepime, Chloramphenicol, Meropenem and Tigecycline.

**Table 2: Antibiotic susceptibility pattern of Gram negative Isolates of ESKAPE pathogens**

| Antibiotic category | Antimicrobials | *K. pneumoniae* | *P. aeruginosa* | Acb complex |
|---------------------|----------------|-----------------|-----------------|-------------|
|                     | Sensitive n (%) | Resistant n (%) | Sensitive n (%) | Resistant n (%) | Sensitive n (%) | Resistant n (%) |
| Aminoglycosides     | Amikacin       | 112 (75.2)      | 37 (24.8)       | 69 (82.1)      | 15 (17.9)      | 20 (51.3)       | 19 (48.7)       |
| Nitrofurans*        | Nitrofurantoin | 71 (69.6)       | 31 (30.4)       | 23 (76.7)      | 7 (23.3)       | 9 (100%)        | 0 (0)           |
| Folate pathway      | Cotrimoxazole  | 99 (66.4)       | 50 (33.6)       | -              | -             | 14 (35.9)       | 25 (64.1)       |
| inhibitors          |                |                 |                 |                |               |                |                |
| Fluoroquinolones    | Ciprofloxacin  | 101 (67.8)      | 48 (32.2)       | -              | -             | 18 (46.2)       | 21 (53.8)       |
|                     | Levofloxacin   | -               | -               |                |               | 19 (48.7)       | 20 (51.3)       |
| Penicillins+β-lactamase inhibitors | Amoxicillin-clavulanic acid | 88 (59.1) | 61 (40.9) | - | - |                |                |
|                     | Ampicillin-Sublactam | - | - | - | - | 25 (64.1) | 14 (35.9) |
| Extended spectrum β-lactamase (3rd generation cephalosporins) | Cefixime | 76 (51) | 73 (49) | - | - | - | - |
| *Antipseudomonas Cephalosporin (4th generation)* | Ceftazidime | - | - | 59 (70.2) | 25 (29.8) | 14 (35.9) | 25 (64.1) |
|                     | Cefepime       | -               | -               | 60 (71.4)      | 24 (28.6)      | 16 (41)         | 23 (59)         |
| *Antipseudomonal penicillins+β-lactamase inhibitors* | Piperacillin tazobactam | 117 (78.5) | 32 (21.5) | 77 (91.7) | 7 (8.3) | 21 (53.8) | 18 (46.2) |
| Phenicol            | Chloramphenicol | 132 (88.6) | 17 (11.4) | - | - | - | - |
| Carbapenems         | Meropenem      | 123 (82.6)      | 26 (17.4)       | 71 (84.5)      | 13 (15.5)      | 19 (48.7)       | 20 (51.3)       |
| Glycylclines        | Tigecycline    | 142 (95.3)      | 7 (4.7)         | -              | -             |                |                |
| Polymyxins          | Colistin       | 149 (100)       | 0 (0)           | 84 (100)       | 0 (0)          | 39 (100)        | 0 (0)           |
|                     | Polymyxin B    | 149 (100)       | 0 (0)           | 84 (100)       | 84 (100)       | 39 (100)        | 0 (0)           |
| Tetracyclines       | Doxycycline    | 129 (86.6)      | 20 (13.4)       | -              | -             | 20 (51.3)       | 19 (48.7)       |

E. Antibiotic susceptibility pattern of *Staphylococcus aureus* (n=151)

All 151 *S. aureus* isolates were susceptible to Vancomycin, Teicoplanin, Tigecycline and Linezolid. Nearly 92% of *S. aureus* isolated from urine specimens were sensitive to Nitrofurantoin. Among 151 isolates, 0.7% was XDR and 68.2% were MDR (Table 3).
Table 3: Antibiotic susceptibility pattern of *Staphylococcus aureus*

| Antimicrobial category | Antibiotics used | Sensitive | Resistant |
|------------------------|------------------|-----------|-----------|
|                        |                  | No.       | (%)       | No.       | (%)       |
| Glycopeptides          | Vancomycin $     | 151       | 100       | 0         | 0         |
|                        | Teicoplanin      | 151       | 100       | 0         | 0         |
| Oxazolidinones         | Linezolid        | 151       | 100       | 0         | 0         |
| Glycylcyclines         | Tigecycline      | 151       | 100       | 0         | 0         |
| Phenicols              | Chloramphenicol  | 149       | 98.68     | 2         | 1.32      |
| Tetracyclines          | Tetracycline     | 149       | 98.68     | 2         | 1.32      |
|                        | Doxycycline      | 148       | 98.02     | 3         | 1.98      |
| Nitrofurans*           | Nitrofurantoin   | 55        | 91.7      | 5         | 8.3       |
| Aminoglycosides        | Amikacin         | 118       | 78.1      | 33        | 21.9      |
| Folate pathway inhibitors | Trimethoprim   | 68        | 45        | 83        | 55        |
|                        | sulphomethoxazole|           |           |           |           |
| Cephamycins            | Cefoxitin        | 63        | 42.4      | 88        | 57.6      |
| Fluoroquinolones       | Ciprofloxacin    | 63        | 41.7      | 88        | 58.3      |
| Lincosamides #         | Clindamycin      | 33        | 39.3      | 51        | 60.7      |
| Macrolides #           | Erythromycin     | 21        | 25        | 63        | 75        |

*For urinary isolates only
# For isolates other than from urine specimen.
$ E$- test for vancomycin.
F. Antibiotic susceptibility pattern of Enterococcus faecium

Out of total 25 isolates Enterococcus faecium subjected for antibiotic susceptibility test with 10 different antibiotics, 100% were sensitive to Linezolid, 88% to Teicoplanin 80% to Vancomycin, 52% each Tetracycline and Tigecycline, 48% Gentamicin (high level). A high percentage of resistance (92%) was seen in case of fluoroquinolones (Ciprofloxacin and Levofloxacin). Nitrofurantoin was found effective against 56.5% of urine isolates (Table 4).

Table 4: Antibiotic susceptibility pattern of Enterococcus faecium (n=25)

| Antimicrobial category | Antibiotics used          | Susceptibility Pattern |
|------------------------|---------------------------|------------------------|
|                        |                           | Sensitive No. (%)       | Resistant No. (%) |
| Oxazolidinones         | Linezolid                 | 25 100                 | 0 0 |
| Glycopeptides          | Teicoplanin               | 22 88                  | 3 12 |
|                        | Vancomycin                | 20 80                  | 5 20 |
| Nitrofurans*           | Nitrofurantoin            | 13 56.5                | 10 43.5 |
| Tetracyclines          | Tetracycline              | 13 52                  | 12 48 |
| Glycylcyclines         | Tigecycline               | 13 52                  | 12 48 |
| Aminoglycosides        | High-level Gentamicin     | 12 48                  | 13 52 |
| Fluoroquinolones       | Ciprofloxacin             | 2 8                    | 23 92 |
|                        | Levofloxacin              | 2 8                    | 23 92 |
| Carbapenem#            | Meropenem                 | 0 0                    | 25 100 |
| Penicillins            | Ampicillin                | 0 0                    | 25 100 |

Nitrofurans* = for urine isolates only  
Carbapenem# = intrinsic resistant (tested for species identification).

MDR AND XDR Producing Gram Positive Isolates of ESKAPE Pathogens

Among the Gram-positive isolates of ESKAPE pathogens, more than 68% were MDR and almost 5% were XDR.

Molecular Characterization of Van A and Van B Genes among VRE isolates

Molecular screening was done by PCR using gene specific primers for both Van A and Van B genes using gene specific primers specific for forward and reverse regions of Van A and Van B genes that encoded resistance in VRE isolates. All 5 VRE isolates were found to carry Van A gene. However, none of the isolates were found to harbor Van B gene.
Figure 2: Gel Electrophoresis of PCR amplification of Van A gene

Lane 1 indicates DNA Ladder (1 Kbp), Lane 2: Blank (Negative Control), Lane 3: Positive Control, Lane 4,5,6,7,8 VRE clinical isolate positive with Van A gene.

**MDR, XDR, ESBL and MBL Producing Gram-negative members of ESKAPE Pathogens**

Nearly 27% (n=74) of the Gram-negative isolates were found to be MDR and 14% (n=37) were XDR. The major drug resistant pathogens among Gram-negative member of ESKAPE was found to be Acb complex (MDR 35.3%, XDR 35.3%) followed by *K. pneumoniae* (MDR 32.2%, XDR 12.8%), *P. aeruginosa* (MDR 14.3% and XDR 7.1% each). In case of *E. aerogenes*, 2 among 4 isolates were found to be MDR (Table 5).

**Table 5 MDR and XDR Gram-negative members of ESKAPE pathogens**

| Organism            | MDR | Percentage | XDR | Percentage |
|---------------------|-----|------------|-----|------------|
| Acb complex (n=39)  | 12  | 30.76      | 12  | 35.76      |
| E. aerogenes (n=4)  | 2   | 50         | 0   | -          |
| K. pneumoniae (n=149)| 48  | 32.2       | 19  | 12.8       |
| P. aeruginosa (n=84)| 12  | 14.3       | 6   | 7.1        |
| **Total**           | 74  |            | 37  |            |

**ESBL- and MBL-producing Gram negative ESKAPE pathogens**

Eighty-three isolates of Gram-negative ESKAPE pathogens were resistant to third generation cephalosporin among which 37 isolates were ESBL positive by both DDST and CDT methods. Fifty three isolates of ESKAPE pathogens were resistant to carbapenem (Imipenem) among which 23 were phenotypically confirmed to be MBL by CDT and E-test Method. *K. pneumoniae* was major ESBL (16.1%) and MBL (8.1%) producer in number followed by
*P. aeruginosa* (10.7% ESBL, 8.3% MBL) and *Acb* complex (10.3% ESBL and MBL each). No ESBL or MBL producers were isolated among *Enterobacter aerogenes* (Table 6).

**Table 6: Gram negative members of ESKAPE Isolates**

| Organism          | ESBL  | Percentage | MBL  | Percentage |
|-------------------|-------|------------|------|------------|
| *Acb* complex (n=39) | 4     | 10.3       | 4    | 10.3       |
| *E. aerogenes* (n=4) | 0     | -          | 0    | -          |
| *K. pneumoniae* (n=149) | 24    | 16.1       | 12   | 8.1        |
| *P. aeruginosa* (n=84) | 9     | 10.7       | 7    | 8.3        |
| **Total**         | 37    |            | 23   |            |

**MDR and XDR Gram Positive Isolates of ESKAPE Pathogens**

Among the Gram positive isolates of ESKAPE pathogens, more than 68% were MDR and almost 5% were XDR. The MDR and XDR *E. faecium* was higher than that of *S. aureus*.

**Table 7: MDR and XDR producing Gram Positive isolates of ESKAPE pathogens**

| Organism           | MDR   | Percentage | XDR   | Percentage |
|--------------------|-------|------------|-------|------------|
| *E. faecium* (n=25) | 18    | 72         | 7     | 28         |
| *S. aureus* (n=151) | 102   | 67.5       | 1     | 0.6        |
| **Total**          | 120   |            | 8     |            |
Biofilm

Out of total 452 ESKAPE pathogens, 42.3% (n=191) were biofilm producers. Majority of *Acb* complex (56.4%) were biofilm producers followed by *K. pneumoniae* (52.1%) (Table 8).

Table 8: Biofilm producing ESKAPE pathogens

| Pathogen     | No. | Non-producer (%) | No. | Weak producer (%) | No. | Medium Producer (%) | No. | Strong producer (%) |
|--------------|-----|------------------|-----|-------------------|-----|---------------------|-----|---------------------|
| *E. faecium* (n=25) | 17 | 68% | 8 | 32% | 0 | 0 | 0 |
| *S. aureus* (n=151) | 110 | 72.9% | 37 | 24.5% | 2 | 1.3% | 2 | 1.3% |
| *K. pneumoniae* (n=149) | 70 | 46.97% | 50 | 33.6% | 15 | 10.1% | 14 | 9.4% |
| *Acb* complex (n=39) | 17 | 43.6% | 12 | 30.8% | 5 | 12.8% | 5 | 12.8% |
| *P. aeruginosa* (n=84) | 45 | 53.6% | 20 | 23.8% | 6 | 7.1% | 13 | 15.5% |
| *E. aerogenes* (n=4) | 2 | 50% | 2 | 50% | 0 | 0 | 0 | 0 |
| **Total(t)** | **261** | **129** | **8** | **34** |

Relationship between Biofilm and Antibiotic resistance pattern among

I. Gram negative Non- Fermentative ESKAPE isolates

There was no statistical significance among biofilm and MDR neither in *Acb* complex (p=0.102) nor in *P. aeruginosa* (p=0.732) (Table 9).

Table 9: Relationship between biofilm and antibiotic resistance of *Acb complex* and *P. aeruginosa*

| Antibiotics | *Acb* Complex | *Pseudomonas aeruginosa* |
|-------------|---------------|-------------------------|
|              | Producers     | Non-producers P value    | Producers | Non-producers P value |
| MDR          | 16            | 8                       | 9         | 9                       |
| Non- MDR     | 6             | 9                       | 30        | 36                      | 0.732    |
| **Total**    | **22**        | **17**                  | **39**    | **45**                  |
II. Gram Negative Fermentative Bacterial ESKAPE isolates

Among fermentative bacterial isolates of ESKAPE pathogens, significant association in between biofilm and MDR *K. pneumoniae* (p value= 0.050) was present. However, in case of *E. aerogenes*, there was no significant association seen (p value= 1.00) (Table 10).

Table 10: Relationship between biofilm and antibiotic resistance of *Klebsiella pneumoniae* and *Enterobacter aerogenes*

| Antibiotics | Biofilm | *K. pneumoniae* | | *E. aerogenes* | |
|-------------|---------|-----------------|---|----------------|---|
|             |         | Producers | Non-Producers | P value | Producers | Non-Producers | P value |
| MDR         | 42      | 26        | 0.050          | 1          | 1 | 1 | 1.00 |
| Non-MDR     | 37      | 44        | 1               | 1          | 1 | 1 | 1.00 |
| Total       | 79      | 70        | 2               | 2          | 2 | 2 | 1.00 |

III. Relationship between Biofilm and Antibiotic Resistant *S. aureus* and *E. faecium*

There was no significant association in between biofilm and MDR isolates of *S. aureus* (p value= 0.424) and *Enterococcus faecium* (p value= 0.484).

Table 11: Relationship between biofilm and antibiotic resistance of *S. aureus* and *E. faecium*

| Antibiotics | Biofilm | *Staphylococcus aureus* | | *Enterococcus faecium* | |
|-------------|---------|-------------------------|---|------------------------|---|
|             |         | Producers | Non-Producers | P Value | Producers | Non-Producers | P Value |
| MDR         | 30      | 73        | 0.424          | 8       | 17         | 0.484 |
| Non-MDR     | 11      | 37        | -              | -       | -          | -    |
| Total       | 41      | 110       | 8              | 17      | 0.484 |

Discussion

Antibiotic resistance is a major clinical problem in treating nosocomial and community acquired infections caused by ESKAPE pathogens, and this situation is in an alarming stage in Nepal as well. All members of ESKAPE pathogens fall under WHO's critical and high priority list of pathogens for research and development of antibiotics, which further highlights the clinical importance of these organisms.

In this study among the ESKAPE pathogens, the most common isolate was *Staphylococcus aureus*; this may be due to the fact that it is also a normal commensal of human skin and are
capable of causing wide range of infection. The major proportion (55.1%) of total patients were females and maximum number of patients infected with were of 61-70 years of age which was similar to a study conducted by Bhatta et al. This result may be because of the fact that age group 61-70 consists of debilitating and immune-compromised patients.

The Vancomycin resistant Enterococcus faecium was found to be 20% which is higher than a study carried out by Acharya et al. Among the eight phenotypic variants Vancomycin resistance in Enterococci (Van A, B, C, D, E, G, L, M and N), the 5 phenotypically confirmed VRE isolates (MIC>256 mcg/ml) in this study were subjected to molecular characterisation for Van A and Van B genes, since VRE is predominantly mediated by these two genes. Van A phenotype shows high level resistance to both Vancomycin and Teicoplanin whereas Van B and Van C strains exhibit low level or variable resistance to Vancomycin but susceptible to Teicoplanin. However in this study, all the VRE isolates weren't resistant to Teicoplanin. Among the 5 VRE isolates, 2 were susceptible to Teicoplanin and the other 3 were resistant. In this study all the 5 isolates were found to possess Van A gene and none possessed Van B gene. Van A was found predominant in the isolated VRE which is similar to other findings worldwide.

The MRSA was found to be 54.6% which is higher than a study of Parajuli et al. reported in a teaching hospital in Kathmandu (45%) and 39.6% found by study of Sanjana et al. The MDR S. aureus were 68.2% (n=108) which is higher than that of Sanjana et al. where 25.4% (n=16) were MDR even though they were MSSA. Similarly, less than one percent of isolates was found to be XDR.

Multidrug resistance among Gram-negative members of ESKAPE pathogens comprised of K. pneumoniae 32.2% (n=48), which is similar to study of Llaca-Diaz et al., and XDR were 12.8% (n=19). MDR Acb complex was 16.2% (n=12) which is less than a study conducted by Shrestha et al (2015) and XDR were 32.4% which is similar to study conducted by Llaca-Diaz et al (2013). MDR P. aeruginosa comprised of 14.3% (n=12) and XDR were 7.1% (n=6) which was lower than study of Mehta and Rossolini et al. Rates of antibiotic resistance in P. aeruginosa in this study was higher than a similar study conducted in nosocomial isolates by Shrestha et al. but lower to study of Chu et al and Mehta et al. XDR 7.1% (n=6). Enterobacter aerogenes was the least prevalent isolate (0.9%) among the ESKAPE pathogens which was similar to the finding by Pathak et al.
In case of ESBL- and MBL- producers, *K. pneumoniae* were highest ESBL producer comprising of 16.1% (n=24) which is similar to a study conducted by Raut et al. 47. MBL were 8.1% (n=12) which is lower than study of Nepal et al. 48. ESBL- and MBL- producing Acb complex were 10.3% each which is similar to a study conducted by Bhandari et al. 49 but lower than a study of Shrestha et al 45. ESBL producing *P. aeruginosa* was 10.7% (n=9) and 7% were MBL which correlated with a similar study of Pathak et al. and Chander et al. 46 50

Ampicillin- Sulbactam was drug of choice for Acb complex showing 64% sensitivity. Sulbactam containing beta-lactam drug is a good therapeutic agent against Acb complex (Chu et al (2013). Higher percentage (91.7%) of *P. aeruginosa* showed susceptibility against Piperacillin- tazobactam when compared with similar study of Mehta et al 43. In case of *K. pneumoniae*, more than 82% were susceptible to Meropenem, 78.5% Piperacillin-tazobactam and 75.2% to Amikacin among first line antibiotics. These findings were in harmony with findings of other studies 48 52. There was no resistance shown by *Enterobacter aerogenes* against Meropenem which was similar to study of Praharaj et al. 53.

Among the Gram positive members, all the isolates were sensitive to vancomycin which was similar to study by Sanjana et al. 40. Nearly 20% of *E. faecium* were resistant to vancomycin and 12% to Teicoplanin. In Nepal VRE in clinical isolate was reported from Manipal teaching hospital (one isolate) by disc diffusion method 54 and one VRE (MIC=32 mcg/ml) from case of peritonitis patient in a continuous ambulatory patient in B.P Koirala institute of health science 55. These studies are probably the only available reports on clinically isolated VRE detected by agar dilution method in Nepal till date as mentioned by Amatya et al. 13. In case of other modes of transmission as from poultry and fomite-borne transmission, almost 5 - 19% resistance has been found in minced meat supply in Chitwan 56. Similarly, study conducted in a hospital in Kathmandu found 2 VRE among 9 isolates extracted from patient's medical charts Thapa et.al 57. This indicates an intense possibility of fomite borne and food borne transmission of multiple drug resistant organisms which may lead to nosocomial infections in compromised patients. Vancomycin- a last resort drug for Gram-positive bacteria was found resistant in up to 20% among clinically isolated *E. faecium* which was similar to a study of Amatya et al. 13. Linezolid was drug of choice for VRE isolates showing 100% effectiveness in vitro; however, combinational therapy is also suggested 58.

This study showed Colistin sulphate and Polymyxin B as the drug of choice among MDR Gram-negative isolates. These drugs are regarded as reserved drugs for MDR and
extensively-drug-resistant Gram negative bacteria. All the isolate in this study were susceptible to Polymyxin; however, in Nepal resistance to Polymyxin has been reported as high as 29% among Pseudomonas spp. in tertiary care hospital. Antimicrobials are used widely around the world as veterinary medicine to promote growth of livestock/poultry in animal husbandry. Similarly resistance to Colistin is seen as high as 28% in Nepalese chicken; This coexistence of MDR infection and MDROs in food chain may exacerbate antimicrobial resistance problem leading to emergence of pan-drug-resistant organism. U.S food and drug Administration (FDA) has banned the use of medically important drugs for animal growth promotion CDC. Recently health Ministry of India has also banned use of manufacture, sale and distribution of Colistin in poultry, aqua farming and animal feed supplements. It should be taken into consideration by Nepal Ministry of Health to take necessary steps in banning or limiting use of broad spectrum and strong antibiotics in animal husbandry.

Almost 43% of ESKAPE isolates were found to be biofilm producers; however, there was no statistical significance in between MDR and biofilm producing isolates of Enterococcus faecium (p=0.484), S. aureus (p=0.424), Acb complex (p=0.102), E. aerogenes (p=1.00) and P. aeruginosa (p=0.732) which concur with similar studies of Cepas et al. However, a statistical significance in between MDR K. pneumoniae and biofilm was seen (p 0.050). This propensity of MDR resistant K. pneumoniae capable of forming biofilm was found similar to a study by Vuotto et al.

This study reveals the prevalence of Multidrug resistance as ESBL, MBL, MRSA and VRE among the clinical isolates. This high level of antimicrobial resistance among the ESKAPE isolates accounts for one of the important factors for dissemination of antibiotic resistance particularly in hospital environment. This study will be helpful for clinicians to identify the most appropriate antibiotic suitable for the treatment of infected patients by prescribing appropriate antibiotic at correct dose, time and duration.

5. Conclusion

It is quite alarming to note that the status of biofilm-producing MDR and XDR ESKAPE pathogens. This increasing antibiotic resistance is an important issue to be addressed by policy makers. Formulation of strict antibiotic stewardship policies is warranted in our country. Early detection and diagnosis of MDROs is indispensable for the choice of most appropriate antibiotic therapy.
Declarations

A. Ethics Approval

18 May 2018

Ms. Row Passey
Principal Investigator
St. Xavier's College
Matighar, Kathmandu

Ref: Approval of thesis proposal entitled Characterisation of “ESKAPE” pathogens with special reference to multi-drug resistance and biofilm production in a Nepalese Hospital

Dear Ms. Passey,

It is my pleasure to inform you that the above-mentioned proposal submitted on 13 February 2018 (Reg. no. 63/2018) has been approved by Nepal Health Research Council (NHRC), National Ethical Guidelines for Health Research in Nepal, Standard Operating Procedures Section ‘C’, point no. 6.3 through Expedited Review Procedure.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made in and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is July 2018.

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/ crude human biomaterial outside the country, only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report in between and full or summary report upon completion.

As per your thesis proposal, the total research budget is Self-Funded and accordingly the processing fee amounts to NPR 3,000. It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

[Signature]

Prof. Dr. Anand Kumar Jha
Executive Chairperson

Tel: 977-1-4254420, Fax: 977-1-4263429, Ramshat Park, P.O. Box, 7616, Kathmandu, Nepal
Website: www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np
B. Consent for Publication

Date: November/21/19

To,
The Editor,
BMC Infectious Diseases,

We give our consent for the publication of data provided in the manuscript entitled "Characterisation of 'ESKAPE' pathogens with special reference to multidrug resistance and biofilm production in a Nepalese Hospital". Ethical approval for the study has been provided by Ethical committee of Nepal Health and Research Council (NHRC). This is a self-funded research done for the partial fulfillment of post of graduate study. We declare that we don't have any financial, professional or personal competing interests that might have influenced presentation of this work in manuscript. We understand that the text and pictures in this article if published will be freely available on the internet and may be seen by the general public.

Ms. Angela Shrestha
Lecturer, Supervisor,
St. Xavier's College
Shrestha.a@xic.edu.np

Ms. Rosy Pandey
Student/ Researcher,
St. Xavier's College
roseypandey@gmail.com

Mr. Shyam Kumar Mishra
Assistant Professor, Clinical Microbiologist
T.U Teaching Hospital
shyammishra@iom.edu.np

C. Availability of data and materials

The data related to this study can be made available by the authors if requested.

D. Competing Interest

The authors declare that they have no competing interest.

E. Funding

This is a self-funded research done for the partial fulfillment of post graduate study.

F. Authors Contribution

RP and SKM designed, conceived the study and carried out the research work. RP analysed data and wrote the manuscript. SKM monitored the research work. SKM and AS supervised the study. All authors read and approved final manuscript.
G. Acknowledgement

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H. Author's Information:

1* Ms. Rosy Pandey, MSc. Public Health Microbiology
   St. Xavier's College, Tribhuvan University, Nepal
   Student, Researcher
   roseypandey@gmail.com
   Contact No +9779803005426

2 Mr. Shyam Kumar Mishra, MScMLT Microbiology, MLS(ASCP)CM
   Clinical Microbiologist,
   Assistant Professor, Department of Microbiology, Institute of Medicine, Tribhuvan University, Nepal
   shyammishra@iom.edu.np
   Contact No +9779851169698
   ORCID Identifier: orchid.org/0000-0002-3888-7319

1 Ms. Angela Shrestha
   M. Tech
   Lecturer, Department of Microbiology, St. Xavier's College, Tribhuvan University, Nepal
   Shrestha.a@sxc.edu.np
   Contact No: +9779841808024

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