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ORIGINAL RESEARCH

Characterisation of ESKAPE Pathogens with Special Reference to Multidrug Resistance and Biofilm Production in a Nepalese Hospital

Rosy Pandey[1,2]
Shyam Kumar Mishra[3,4]
Angela Shrestha[1]

[1]St. Xavier's College (Tribhuvan University), Department of Microbiology, Kathmandu, Nepal; [2]National Public Health Laboratory, Kathmandu, Nepal; [3]UNSW Sydney, School of Optometry and Vision Science, Sydney, Australia; [4]Tribhuvan University, Institute of Medicine, Maharajgunj Medical Campus, Department of Microbiology, Kathmandu, Nepal

Background: "ESKAPE" is an acronym for a group of life-threatening nosocomial pathogens, viz, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. Global efforts on controlling multidrug-resistant (MDR) organisms have been hampered by their ability to escape antibacterial drugs. This study was undertaken to determine the prevalence of ESKAPE pathogens with prime focus on biofilm production and antibiotic resistance.

Methods: A total of 8756 clinical samples were processed for the isolation and identification of ESKAPE pathogens following standard microbiological procedures. These isolates were subjected to antimicrobial sensitivity test as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Test for MDR, extended-spectrum β-lactamase (ESBL), metallo-β-lactamase (MBL), methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) was done by the disk diffusion and E-test methods. In the case of VRE molecular detection was done for *vanA* and *vanB* genes. All the isolates were processed for biofilm detection by the tube adherence method.

Results: The percentage distribution of *Enterococcus faecium* was 5.5%, *S. aureus* 33.4%, *K. pneumoniae* 33.0%, *A. baumannii* 8.6%, *P. aeruginosa* 18.6%, and *Enterobacter aerogenes* 0.9%. MRSA was 57.6%, and vancomycin resistance among *Enterococcus faecium* was 20%. ESBL- and MBL-producing *K. pneumoniae* were 16.1%, and 8.1%, *A. baumannii* 10.3% each and *P. aeruginosa* 10.7% and 8.3%, respectively. A total of 42.3% of isolates were biofilm producers. Linezolid was the drug of choice for VRE. Ampicillin-sulbactam was most useful against *A. baumannii* apart from polymyxins, whereas piperacillin-tazobactam was effective against other Gram-negative bacteria. *VanA* gene was detected in all the VRE isolates.

Conclusion: This study estimates the burden of the ESKAPE organisms and their antimicrobial resistance pattern in a hospital setting. A high percentage of drug resistance and biofilm production was noted; hence antimicrobial resistance surveillance targeting ESKAPE pathogens should be incorporated in the infection control policy in Nepal.

Keywords: extended-spectrum β-lactamase; ESBL, ESKAPE pathogens, metallo-β-lactamase; MBL, methicillin-resistant *Staphylococcus aureus*; MRSA, vancomycin-resistant *Enterococcus*; VRE

Correspondence: Rosy Pandey
St. Xavier's College, Post Box: 7437, Maitighar, Kathmandu, 44601, Nepal
Tel +977-9803005426
Email roseypandey@gmail.com

Background

The emergence of bacterial pathogens with acquired resistance to almost all available antimicrobials is one of the major concerns today.[1] These pathogens are also named "superbugs," particularly *Enterococcus faecium, Staphylococcus aureus,*

Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacter* spp (ESKAPE). The inefficiency of antimicrobials against these pathogens is due to various resistance mechanisms such as drug inactivation, modification of drug binding sites/targets, changes in cell permeability and/or mutation.[2]

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in the 1960s and was first identified in 1961 in the United Kingdom.[3] Its nosocomial infection rate in the United States and the United Kingdom was around 60–70% in 2004.[4] However, the incidence of MRSA bloodstream infections in the United States dropped off 74% and 40%, respectively, in the hospitals and communities from 2005 to 2016.[5] In Nepal, the prevalence of MRSA in the hospital setting was found as high as 68% while it was around 50% in the community; a similar study showed that 42% of *S. aureus* isolates were MRSA in a tertiary care teaching hospital.[6,7]

The production of enzymes, that irreversibly inactivate β-lactam antimicrobials, e.g., extended-spectrum β-lactamase (ESBL) and carbapenemase like metallo-β-lactamase (MBL), has contributed to the success of Gram-negative members of ESKAPE pathogens in the clinical settings.[8] Carbapenems are often referred to as last resort antibiotics that are used for the treatment of multidrug-resistant (MDR) Gram-negative bacterial infections as they possess activity against ESBL-producing organisms too.[8] However, Gram-negative members of ESKAPE pathogens producing MBLs are resistant to carbapenems, thus posing urgent threat in clinical settings.

On several occasions, ESKAPE clinical isolates are MDR, extensively drug-resistant (XDR), or pandrug-resistant (PDR). The misuse of antimicrobials and the proneness of organisms to carry the resistant genes conferring MDR infection is a major issue these days. On the same ground, drug-resistant strains of *Staphylococcus aureus*, mainly MRSA, vancomycin-resistant *Enterococcus* (VRE) and β-lactam resistant pathogens have proven to be the leading threats in the clinical arena.[9] These pathogens can survive in the hospital setting for a longer period of time, escape the biocidal effect easily and can be transported from one individual to other, hence spreading in the community and hospital.[10] *Acinetobacter baumannii* isolates harboring NDM-1 and 16S rRNA methylase ArmA have been found to have disseminated in medical settings in Nepal.[11] Therefore, there is an outcry for more clinically potent antibiotics or novel antimicrobials including antimicrobial peptides and their mimics.[12]

Biofilm is a layer of microbially-derived cell clusters embedded in a matrix of extracellular polysaccharide, called polysaccharide intercellular adhesins (PIA). It has shown that ESKAPE pathogens are potential biofilm producers which make them resistant to antimicrobials by creating a layer hindering antimicrobial penetration.[13]

In Nepal, studies focusing on the collective evaluation of prevalence and antimicrobial resistance profile of ESKAPE pathogens is lacking. Therefore, in this study, status of ESKAPE pathogens, antimicrobial ineffectiveness due to production of biofilms or enzymes like β-lactamases, methicillin resistance among *Staphylococcus aureus*, vancomycin resistance among *Enterococcus faecium* have been addressed. Among the nine phenotypic variants of vancomycin resistance in *Enterococci* (*vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN*), strains possessing *vanA* and *vanB* are found to be responsible for human infections.[14] Therefore, in this study, our focus was to detect *vanA*/*vanB* gene(s) on the clinical isolates of VRE.

Methods

This was a cross-sectional descriptive study conducted from February to July 2018 at a 100-bed hospital in the capital city of Nepal. A total of 8756 specimens (urine, swabs and bodily fluids – pus, blood, sputum, tracheal aspirate, ear swab, wound swab, throat swab, pleural fluid, endotracheal secretion, cerebrospinal fluid and semen) were processed aseptically from medical, surgical and intensive care units of inpatient department (IPD). 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

The clinical specimens were collected from February to July 2018 and they were processed following standard protocol of the American Society for Microbiology (ASM) and analyzed accordingly.[15] Antimicrobial susceptibility test (AST) was done for all the clinical isolates as per the guidelines of Clinical and Laboratory Standards Institute (CLSI).[16] Wet mount microscopy was done for urine specimens and Gram's staining was done on all the other bodily fluids, respiratory aspirates and swabs. For blood sample processing, BD BACTEC FX40 was

employed with Standard Aerobic or Peds Plus medium. Urine specimens were cultured in Cystine Lactose Electrolyte Deficient (CLED) medium by the semi-quantitative standard loop method, and for other specimens, Chocolate agar (CA), 5% Blood agar (BA) and MacConkey agar (MA) were used. The CA plates were incubated in a CO_2 enriched environment (5–10% CO_2) at 37°C for 24 hours. The BA, MA, and CLED plates were incubated at 37°C for 24 hours in an aerobic atmosphere. The identification of significant isolates was done following standard microbiological techniques.[15] Composite Quality scoring was done considering the relative ratio of polymorphs and epithelial cells on the samples to rule out bacterial colonization from infection for lower respiratory tract specimens.

Bacterial Identification

Identification of bacteria was done based on standard microbiological techniques which involved Gram's staining, morphological and cultural characteristics, biochemical properties and serotyping, if required in specific cases.

Antimicrobial Susceptibility Testing

AST was carried out on the isolates by using Mueller-Hinton agar by Kirby-Bauer disk diffusion method. Categorization of antimicrobial resistant isolates as MDR and XDR was done following the definitions of Magiorakos et al and Tamma et al.[17,18]

Determination of isolates exhibiting VRE, ESBL, Carbapenemase, and MRSA characteristics was done following standard procedures.[16] Inducible-macrolide-lincosamide-streptogramin-B ($iMLS_B$) resistance in *S. aureus* was detected by the disk approximation method test by placing 2 µg clindamycin disk and 15 µg erythromycin disk 15 mm apart edge to edge. The evidence of characteristic flattening zone of inhibition (ZOI) around clindamycin disk adjacent to erythromycin disk, referred to as "D" zone, was considered to confirm that the isolate exhibited inducible clindamycin resistance.[16] For the detection of vancomycin resistance, MBL, and ESBL production, respective E-test methods were also employed.

Initial screening of ESBL among Gram-negative isolates of ESKAPE pathogens was done by using cefotaxime (CTX), ceftazidime (CAZ) and ceftriaxone (CRO) 30 µg discs (BD, USA). If the ZOI was ≤ 27 mm for CTX, ≤22 mm for CAZ and ≤25 mm for CRO, then the isolates were screened as potential ESBL-producers. These isolates were further tested for ESBL production by double disk synergy test (DDST),[19] Combination Disk Test (CDT) and Epsilometer Test (E-Test) method.[16]

Detection of MBL

Screening test for MBL Detection

Carbapenem-resistant isolates identified by the carbapenem disk diffusion method were screened for the production of MBL.[20]

Combined Disk Diffusion Method

Phenotypic MBL detection was done by Combined disk method where two imipenem (IPM) disks (each 10 µg), one containing 10 microliter of 0.1 M (292 µg) anhydrous EDTA (Thermo Fisher Scientific India Pvt. Ltd) and another without EDTA were placed 25 mm apart (center to center). An increase in zone diameter of >4 mm around the IPM-EDTA disk compared to IPM disk alone was considered positive for MBL.[20]

E-Test Method (Epsilometer Test)

This test uses an E-test strips in which one end consists of a stable gradient of imipenem and the other end consists of a gradient of imipenem with a 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 the manufacturer's instructions (bioMérieux SA, France).

Detection of Biofilm – Tube Adherence Method

Detection of biofilm by tube adherence method was done as proposed by Christensen et al.[21] Two milliliter of Brain Heart Infusion broth supplemented with 1% glucose was used to incubate one colony of the organism for 48 hours at 37°C. The contents were then decanted and washed by phosphate buffered saline (pH 7.2) thoroughly and dried. Then 1% safranin was added to the tubes and gently rotated for uniform staining. The tubes were washed with distilled water and dried by placing the tubes upside down. Presence of stained material at the inner wall of the test (liquid-air interface) was ranked by two investigators as weak biofilm producer (+), medium (++) or strong (+++).[22]

Phenotypic Detection of VRE

Enterococcus faecium isolates showing insusceptibility to vancomycin disk (30 µg) were screened as VRE. Then they were subjected to vancomycin Epsilometer test according to the manufacturer's instruction (E-Test technical manual, bioMérieux SA, France; 2018). The isolates

showing MIC ≥32 μg/mL of vancomycin were confirmed as VRE.

Molecular Detection of VRE

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

DNA Extraction

Plasmid extraction of the VRE isolates was done by the alkaline hydrolysis method as described by Sambrook and Russell.[23]

Polymerase Chain Reaction (PCR)

Specific primers for amplification of *vanA* (Forward: GGGAAAACGACAATTGC

Reverse:GTACAATGCGGCCGTTA) and *vanB* (Forward: ACCTACCCTGTCTTTGTGAA reverse: AATGTCTGCTGGAACGATA) were used.[24,25] For *vanA* and *vanB* amplification, PCR was performed in a thermocycler as follows- initial denaturation at 95°C for 5 minutes, 30 cycles for denaturation, annealing and extension at 95°C for 30 seconds, 54°C for 1 minute, and 72°C for 1 minute, respectively. Final elongation was done at 72°C for 10 minutes.[24]

Gel Electrophoresis

After PCR, the amplicons along with loading dye (bromophenol blue) were loaded onto 1.5% agarose gel stained with ethidium bromide of concentration 0.5 μg/mL and run at 60 V for 1 hour. The DNA bands were then analyzed in the UV transilluminator.

Statistical Analysis

All the data were statistically analysed using the IBM SPSS Statistics for windows version 16.0 (IBM Corp, Armonk, NY, USA). The P-value <0.05 was assumed significant for analysis.

Results

Culture Positivity of Specimens

Clinical specimens which met the criteria as recommended by ASM were processed for culture and susceptibility test. After receiving specimens from sample collection site, they were immediately transported to microbiology laboratory for further processing. Out of 8756 specimens processed, 2384 (27.2%) showed significant growth. Among 2384 positive tested samples implicating infection, 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%), *A. baumannii* (n = 39, 8.6%), *Enterococcus faecium* (n = 25, 5.5%) and *Enterobacter aerogenes* (*K. aerogenes*) (n = 4, 0.9%). A higher number of ESKAPE pathogens were isolated from patients admitted to medical department (n = 281) followed by patients from surgical department (n = 129) and ICU (n = 42) (Table 1).

Distribution of ESKAPE Pathogens Among the Diverse Clinical Specimens

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

Considering gender, 249 (55.1%) ESKAPE pathogens were isolated from females and 203 (44.9%) from males. 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%).

Antimicrobial Susceptibility Pattern of ESKAPE Pathogens

The *Enterococcus faecium* isolates were subjected to AST with 10 different antimicrobials. High percentage of resistance (92%) was seen against ciprofloxacin followed by gentamicin (high level) (52%), tetracycline (48%) and tigecycline (48%), vancomycin (20%) and teicoplanin (12%). However, all the isolates were susceptible to linezolid (Table 2).

Seventy-five percent of non-urinary isolates of *S. aureus* were resistant to erythromycin. A large number of *S. aureus* isolates were resistant to trimethoprim-sulfamethoxazole (55%), and ciprofloxacin (58.3%). Almost 58% of the isolates were MRSA and 68.2% were MDR. None of the *S. aureus* isolates were resistant to vancomycin, teicoplanin, tigecycline and linezolid (Table 2).

Forty-nine percent of *K. pneumoniae* isolates were resistant to cefixime. It should be noted that 17.4% of the isolates showed resistance to meropenem. Approximately, 65% of urinary isolates were resistant to nitrofurantoin (Table 3).

A. baumannii showed 64% resistance to ceftazidime and cotrimoxazole each, 59% to cefepime and more than 50% to fluoroquinolones. However, they were most susceptible to ampicillin-sulbactam (64.1%) followed by

Table 1 Distribution of ESKAPE pathogens in Various Specimens

Specimens	Wards	*E. faecium* (N)	*S. aureus* (N)	*K. pneumoniae* (N)	*Acinetobacter baumannii* (N)	*P. aeruginosa* (N)	*E. aerogenes* (N)	Total (N)	Percentage
Urine	Medical Surgical ICU	16 4 3	46 11 3	78 16 7	9 – –	22 2 6	2 – –	225	49.9%
Pus	Medical Surgical ICU	1 – –	10 52 3	3 10 –	– 2 –	2 17 1	0 – –	101	22.3%
Sputum	Medical Surgical ICU	1 – –	6 – 2	23 1 5	22 – 3	27 – 5	2 – –	97	21.5%
Blood	Medical Surgical ICU	0 – –	3 6 –	– 1 1	– – 1	0 – -	0 – -	12	2.7%
Semen	Medical Surgical ICU	0 – –	- 5 –	1 – –	0 – –	0 – –	0 – –	6	1.3%
High vaginal swab	Medical Surgical ICU	0 – –	2 – –	1 – –	0 – –	0 – –	0 – –	3	0.7%
Wound swab	Medical Surgical ICU	0 – –	– 1 –	– 1 –	0 – –	0 – –	0 – –	2	0.4%
Endotracheal tube	Medical Surgical ICU	0 – –	0 – –	0 – –	– – 1	– – 1	0 – –	2	0.4%
Ear swab	Medical Surgical ICU	0 – –	1 – –	0 – –	0 – –	0 – –	0 – –	1	0.2%
Broncho alveolar lavage	Medical Surgical ICU	0 – –	0 – –	1 – –	0 – –	0 – –	0 – –	1	0.2%
Suction tip	Medical Surgical ICU	0 – –	0 – –	0 – –	1 – –	– – –	0 – –	1	0.2%
Oral swab	Medical Surgical ICU	0 – –	0 – –	0 – –	0 – –	1 – –	0 – –	1	0.2%
Total		**25**	**151**	**149**	**39**	**84**	**4**	**452**	**100%**

piperacillin-tazobactam (53.8%). (Table 3). Almost 49% of *P. aeruginosa* were resistant to ciprofloxacin, 29% to ceftazidime and 28% to cefepime. However, the majority were susceptible to piperacillin-tazobactam (91.7%) and meropenem (84.5%) (Table 3). All isolates of *Enterobacter aerogenes* (*K. aerogenes*) were resistant to cefixime and 50% were resistant to cotrimoxazole.

Among the Gram-positive ESKAPE pathogens, more than 68% (n= 122) were MDR and almost 4% (n=6) were XDR (Table 4). Nearly 27% (n = 74) of the Gram-negative isolates were found to be MDR and 14% (n = 37) XDR. The major drug-resistant pathogens among Gram-negative members of ESKAPE was *A. baumannii* (MDR and XDR 30.7% each) followed by *K. pneumoniae* (MDR 32.2%,

Table 2 Antimicrobial Resistance Pattern of Gram-Positive Isolates of ESKAPE Pathogens

Antimicrobials	*Enterococcus faecium* (N=25)		*Staphylococcus aureus* (N=151)	
	N	%	N	%
Linezolid	0	0	0	0
Teicoplanin	3	12	0	0
Vancomycin	5	20	0	0
Nitrofurantoin	10	43.5	5	8.3
Tetracycline	12	48	3	1.32
Doxycycline	-	-	3	1.98
Tigecycline	12	48	0	0
High-Level Gentamicin	13	52	-	-
Amikacin	-	-	33	21.9
Ciprofloxacin	23	92	88	58.3
Meropenem	25	100	-	-
Ampicillin	25	100	-	-
Chloramphenicol	-	-	2	1.32
Trimethoprim Sulphomethoxazole	-	-	83	55
Cefoxitin	-	-	88	57.6
Clindamycin	-	-	51	60.7
Erythromycin	-	-	63	75

Notes: Nitrofurantoin= For urine isolates only; Meropenem= Intrinsic resistance in *E. faecium* (tested for species identification); Vancomycin= E-test for vancomycin; Cefoxitin= Screening of MRSA; Clindamycin/Erythromycin= For other than urinary isolates.

XDR 12.8%) and *P. aeruginosa* (MDR 14.3% and XDR 7.1% each). In case of *E. aerogenes (K. aerogenes)*, 2 out of 4 isolates were found to be MDR (Table 4).

Molecular Characterization of *vanA* and *vanB* Genes Among VRE Isolates

Molecular screening for *vanA* and *vanB* genes from genomic DNA/plasmid was amplified by PCR using vancomycin specific primers. All 5 VRE isolates were found to carry *vanA* gene (Figure 1).

Antimicrobial Resistant Gram-Negative Members of ESKAPE Pathogens

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 double disk synergy test, combination disk and E-test methods. Fifty-three isolates were resistant to carbapenem (imipenem) among which 23 were phenotypically confirmed to

Table 3 Antimicrobial Resistance Pattern of Gram-Negative Isolates of ESKAPE Pathogens

Antimicrobials	*Klebsiella pneumoniae* (N = 149)	*Acinetobacter baumannii* (N = 39)	*Pseudomonas aeruginosa* (N = 89)	*Enterobacter aerogenes* (N = 4)
	N %	N %	N %	N %
Amikacin	37 (24.8)	19 (48.7)	15 (17.9)	-
Nitrofurantoin	31 (64.58)	0 (0)	-	2 (100%)
Trimethoprim-Sulfametoxazole	50 (33.6)	25 (64.1)	-	2 (50%)
Ciprofloxacin	48 (32.2)	21 (53.8)	43 (48.31)	-
Levofloxacin	-	20 (51.3)	-	-
Amoxicillin-Clavulanic Acid	61 (40.9)	-	-	-
Ampicillin- Sulbactam	-	14 (35.9)	-	-
Cefixime	73 (49)	-	-	4 (100%)
Ceftazidime	-	25 (64.1)	25 (29.8)	-
Cefepime	-	23 (59)	24 (28.6)	-
Piperacillin Tazobactam	32 (21.5)	18 (46.2)	7 (8.3)	-
Chloramphenicol	17 (11.4)	-	-	-
Meropenem	26 (17.4)	20 (51.3)	13 (15.5)	-
Tigecycline	7 (4.7)	-	-	-
Colistin	0 (0)	0 (0)	0 (0)	
Polymyxin B	0 (0)	0 (0)	0 (0)	-
Doxycycline	20 (13.4)	19 (48.7)	-	
				-

Note: Nitrofurantoin= For urine isolates only.

Table 4 Frequency of ESBL, MBL, MDR and XDR Among ESKAPE Pathogens

Organism	MDR		XDR		ESBL		MBL	
	N	%	N	%	N	%	N	%
E.faecium	20	80	5	20	–	–	–	–
S.aureus	102	67.5	1	0.6	–	–	–	–
K. pneumoniae	48	32.2	19	12.8	24	16.1	12	8.1
A. baumannii	12	30.76	12	30.76	4	10.3	4	10.3
P. aeruginosa	12	14.3	6	7.1	9	10.7	7	8.3
E. aerogenes	2	50	0	0	0	0	0	0

Abbreviations: ESBL, extended-spectrum β-lactamase; MBL, Metallo-β-lactamase; MDR, multi-drug resistant; XDR, extensively-drug resistant.

be MBL by the combination disk and E-test methods. *K. pneumoniae* was the major ESBL- (16.1%) and MBL- (8.1%) producer followed by *P. aeruginosa* (10.7% ESBL, 8.3% MBL) and *A. baumannii* (10.3% ESBL and MBL each). No ESBL- or MBL-producers were isolated among *Enterobacter aerogenes* (*K. aerogenes*) (Table 4).

Biofilm Production in ESKAPE Pathogens

Of total 452 ESKAPE pathogens, 42.3% (n = 191) were biofilm-producers. Among the Gram-negative isolates, *A. baumannii* (56.4%) was the major biofilm-producer, and among Gram-positive isolates, 32% of *Enterococcus faecium* were biofilm producers (Table 5).

Relationship Between Biofilm and Antibiotic Resistance

There was no statistical significance among biofilm and multidrug resistance in *A. baumannii* (p = 0.102), *P. aeruginosa* (p = 0.732) and *E. aerogenes* (*K. aerogenes)* (p value = 1.00). However, in the case of *K. pneumoniae* significant association between biofilm and MDR (p value = 0.050) was present. In case of Gram-positive isolates there was no significant association between biofilm and multidrug resistance in *S. aureus* (p value = 0.424) and *Enterococcus faecium* (p value = 0.484) (Table 5).

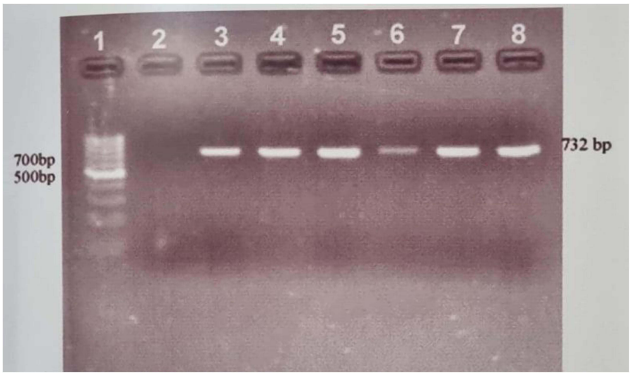

Figure 1 Gel Electrophoresis of PCR amplification of *Van A* gene. Lane 1 indicates DNA Ladder (1 Kbp), Lane 2: Blank (Negative Control), Lane 3: Positive Control, Lane 4,5,6,7,8 VRE clinical isolate positive with *van A* gene.

Table 5 Biofilm Producing ESKAPE Pathogens

Organisms	Producers		Non-Producers		P-value
	N	%	N	%	
Enterococcus faecium	8	32	17	68	0.484
Multidrug-resistant	8	-	17	-	
Non-multidrug-resistant	0	-	0		
Staphylococcus aureus	41	27.1	110	72.9	0.424
Multidrug-resistant	30	-	73	-	
Non-multidrug-resistant	11	-	37	-	
Klebsiella pneumoniae	79	53.03	70	46.97	0.050
Multidrug-resistant	42		26	-	
Non-multidrug-resistant	37		44	-	
Acinetobacter baumannii	22	56.4	17	43.6	0.102
Multidrug-resistant	16	-	8	-	
Non-multidrug-resistant	6	-	9		
Pseudomonas aeruginosa	39	46.4	45	53.6	0.732
Multidrug-resistant	9	-	9	-	
Non-multidrug-resistant	30	-	36	-	
Enterobacter aerogenes	2	50	2	50	1.00
Multidrug-resistant	1	-	1	-	
Non-multidrug-resistant	1	-	1	-	

Discussion

Antimicrobial resistance is a major clinical problem worldwide in treating nosocomial and community-acquired infections caused by ESKAPE pathogens,[26] and this situation is in alarming stage in Nepal as well.[27,28] All members of ESKAPE pathogens fall under WHO's critical and high priority list of pathogens for research and development of

antimicrobials,[29] which further highlights the clinical importance of these organisms.

Among various clinical specimens processed, the incidence of infection with ESKAPE pathogens was found highest in urine (49.9%) followed by pus (22.3%) and sputum (21.5%) specimens. In the urine specimen, major organisms isolated were *K. pneumoniae* 101 (44.8%) followed by *S. aureus* 60 (26.6%) and *P. aeruginosa* 30 (13.3%). In the pus specimen, *S. aureus* (64.4%) was the most frequent bacteria. This result is supported by a similar study of Pandeya et al, which was done in 164 bacteria isolated from pus specimens in a tertiary care hospital in Nepal, where the most common isolates were *S. aureus* (32.3%), *K. pneumoniae* (9.1%) and *P. aeruginosa* (6.1%).[30] In the sputum specimen, the major isolates were *P. aeruginosa* (32.9%), *K. pneumoniae* (29.8%) and *A. baumannii* (25.7%) which is similar to the findings of Mishra et al, which was done on 113 bacterial isolates of lower respiratory tract specimens, the major isolate being *P. aeruginosa* (37.2%), *K. pneumoniae* (21.2%) and *A. baumannii* (31.9%).[31]

Staphylococcus aureus was the most common bacteria isolated among ESKAPE pathogens in this study. This may be because it is also a normal commensal of human skin and is capable of disseminating and causing a wide range of infections.[32]

In this study, 57.6% of *S. aureus* were methicillin-resistant which is higher than studies conducted in two different teaching hospitals of Nepal which showed MRSA to be 45% and 39.6% respectively.[33,34] The MDR *S. aureus* was 68.2% (n = 108) which is higher than a similar study by Sanjana et al.[34] However, less than 1% of *S. aureus* were XDR. All the isolates of *S. aureus* were susceptible to vancomycin.

VRE have transferred vancomycin-resistant gene (v*anA*) to *S. aureus* through horizontal gene transfer containing VRE genes as transposon introducing vancomycin-resistant *S. aureus* (VRSA),[35] thus compromising treatment by the last resort antibiotic against Gram-positive bacteria. Five out of twenty-five (20%) isolates of *Enterococcus faecium* were found vancomycin resistant in this study which is similar to the finding of Amatya et al,[36] but higher than a study carried out in eastern Nepal by Acharya et al.[37] In this study, five phenotypically confirmed VRE isolates (MIC>256 μg/mL) were subjected to molecular characterisation for *vanA* and *vanB* genes because VRE is predominantly mediated by these two genes in humans. [35] The *vanA* genotype is associated with high-level resistance to both vancomycin and teicoplanin whereas *vanB* and *vanC* strains exhibit low level or variable resistance to vancomycin but they are susceptible to teicoplanin. [38] However, in this study, 12% of the VRE were resistant to teicoplanin. Among the 5 VRE isolates, 2 were susceptible to teicoplanin, while all were found to possess *vanA* gene. The predominance of *vanA* was seen similar in other studies.[35,38,39] Therefore, excessive control measures should be initiated to prevent the emergence of VRE which may lead to global crisis of antimicrobials.[35]

MDR among Gram-negative members of ESKAPE pathogens comprised of *K. pneumoniae* (32.2%), which is similar to the findings by Llaca-Diaz et al.[40] MDR *A. baumannii* were 30.7% which is higher than findings by Shrestha et al,[11] and XDR were 30.7% which is similar to the findings by Llaca-Diaz et al.[40] The prevalence of MDR and XDR *P. aeruginosa* was lower than the reports by Mehta and Rossolini et al.[41,42] *Enterobacter aerogenes* (*K. aerogenes*) was the least prevalent isolate among the ESKAPE pathogens which was similar to the finding by Pathak et al.[43]

In the case of ESBL- and MBL-producers, *K. pneumoniae* was the highest ESBL- producer comprising of 16.1% which complies with the findings by Raut et al;[44] MBL were 8.1% which is lower than a study of Nepal et al.[45] ESBL- and MBL-producing *A. baumannii* were 10.3% each which is similar to the results by Bhandari et al.[46]

Acinetobacter are largely known to produce carbapenem-inactivating OXA enzymes which also effectively hydrolyse cephalosporins.[47] This study found 10.7% ESBL-producing *P. aeruginosa* and 8.3% MBL-producers which correlated with a similar study of Pathak et al.[43] However, it should be noted that no phenotypic methods can reliably detect ESBL production in *Acinetobacter* sp. or *Pseudomonas aeruginosa*. Moreover, MBL detection in *Acinetobacter* with EDTA can result in false positive tests.[48]

Considering *A. baumannii*, ampicillin-sulbactam was the drug of choice with high susceptibility (64%) excluding polymyxins. The increased susceptibility of ampicillin-sulbactam against *A. baumannii* is because sulbactam containing β-lactam drug is a good therapeutic agent against *A. baumannii*[49] as it inhibits penicillin-binding proteins (PBPs)- PBP1 and PBP3, with a very low frequency of resistance.[50] A higher percentage (91.7%) of *P. aeruginosa* showed susceptibility against piperacillin-tazobactam

when compared with other studies.[41,42] In the case of *K. pneumoniae*, 82.6% were susceptible to meropenem, 78.5% piperacillin-tazobactam and 75.2% to amikacin among the first-line antibiotics. These findings were in agreement with the findings of other studies.[51.] There was no resistance shown by *Enterobacter aerogenes* (*K. aerogenes*) against meropenem.

Nearly 20% of *Enterococcus faecium* were resistant to vancomycin and 12% to teicoplanin. VRE of 5–19% was found in minced meat showing the probability of poultry and food-borne transmission in Nepal.[52] Similarly, a study conducted in a hospital in Kathmandu found two VRE among nine isolates from patient's medical charts. This indicates an intense possibility of fomite-borne and food-borne transmission of MDR organisms which may lead to nosocomial infections in immuno-compromised patients.[53,54] The increasing rates of VRE in clinical samples have been observed in Europe and America too.[35] In our study, linezolid was the drug of choice for VRE isolates showing 100% effectiveness in vitro.

This study showed colistin sulphate and polymyxin B as the effective drugs against MDR Gram-negative isolates. These drugs are regarded as reserved drugs for MDR and XDR Gram-negative bacteria.[41,51] All the isolates in this study were susceptible to polymyxin; however, in Nepal resistance to polymyxin has been reported as high as 29% among *Pseudomonas* spp.[55] These antimicrobials are used widely around the world as veterinary medicine to promote the growth of livestock/poultry in animal husbandry.[56] Similarly, resistance to colistin is seen as high as 28% *Escherichia coli* isolates from chicken in a Nepalese study. This co-existence of MDR infection and MDR organisms (MDROs) in the food chain may exacerbate antimicrobial resistance problem leading to the emergence of PDR organisms. The U.S Food and Drug Administration (FDA) has banned the use of medically important drugs for animal growth promotion.[57] Recently the health Ministry of India has also banned the manufacture, sale and distribution of colistin in poultry, aqua farming and animal feed supplements.[58] It is imperative that the Nepalese government take necessary steps to ban or limit the use of broad-spectrum and strong antimicrobials in animal husbandry.

Almost 42% of ESKAPE isolates were found to be biofilm producers; however, there was no statistical significance between MDR and biofilm-producing isolates of *Enterococcus faecium* ($p = 0.484$), *S. aureus* ($p = 0.424$), *A. baumannii* ($p = 0.102$), *E. aerogenes* (*K. aerogenes*) ($p = 1.00$) and *P. aeruginosa* ($p = 0.732$) which concur with similar studies of Cepas et al, and Sanchez et al.[59,60] However, statistical significance in between MDR *K. pneumoniae* and biofilm was seen (p 0.050). This propensity of MDR *K. pneumoniae* capable of forming biofilm was seen in a study by Vuotto et al too.[61]

This study provides insight into antimicrobial resistance in bacterial pathogens in Nepal. Almost 70% (122/176) of the Gram-positive isolates were MDR. In the case of Gram-negative isolates, 26.8% (74/276) and 13.4% (37/276) were MDR and XDR respectively. A high level of ciprofloxacin resistance (32–54%) in Gram-negative isolates and (58–92%) in Gram-positive isolates was found suggesting the increase in the resistance of fluoroquinolones which is commonly used as empirical treatment for many infections.

Conclusion

This study shows a high level of antimicrobial resistance and biofilm formation among the ESKAPE isolates accounting for one of the important factors for the dissemination of antimicrobial resistance. These findings may alert clinicians when dealing with infections by ESKAPE group. Therefore, increasing antibiotic resistance is an important issue to be addressed by policymakers. Formulation of strict antibiotic stewardship policies is warranted in hospitals.

Data Sharing Statement

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

Ethics and Consent to Participate

The ethical approval for this research work was obtained from the Government of Nepal, Nepal Health Research Council (NHRC) (Reference number: 2741). Written informed consent was obtained from each patient before enrollment. For the participants under 16-years-old, consent was taken from their parent or guardian. This study was conducted in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors declare that they have no competing interest.

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