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Multidrug resistant *Escherichia coli* isolated at National Public Health Laboratory, Nepal

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ABSTRACT

Background: Antimicrobial resistance in *Escherichia coli* is mostly associated with ß-lactamases and carbapenemases enzyme production resulting in treatment challenges. This study was conducted with the aim to detect and characterize antimicrobial resistance in *E. coli* isolates.

Methods: A cross-sectional study was conducted during 2018-2022, at National Public Health Laboratory where the clinical specimens (24636) received were processed for identification and characterization of antimicrobial resistance following conventional & advanced methods. Antibiotic susceptibility tests were performed by Modified Kirby Bauer disc diffusion and Minimum inhibitory concentrations using VITEK2 compact (Biomeriux). The isolates were tested for extended-spectrum β-lactamases and Carbapenemase production following Clinical Laboratory Standards Institute guidelines.

Results: Bacterial growth was observed in 9% (2166/24636) of the specimens, of which 44% (959) were *E. coli*. Among the 959 *E. coli* isolates, 320 were reconfirmed with VITEK-MS (Biomeriux). Phenotypic multi-drug resistance was observed in 75% (240/320) of the isolates with 62% (197/320) extended-spectrum β-lactamases, 12% (39/320) AmpC-ß-lactamase, 10% (31/320) serine carbapenemases and 7% (22/320) Metallo-ß-lactamase while 3% (9/320) produced three types of enzymes. The extended-spectrum-β-lactamase producing *E. coli* were sensitive to Tigecycline (100%), Amikacin (92%), Imipenem (87%), and Meropenem (84%). Carbapenemase producers were sensitive to Tigecycline (100%), with 61% to Amikacin. Extensive-drug resistance was observed in 2% (7/320) of the isolates, with Colistin resistance in one.

Conclusions: The findings highlight alarmingly high antimicrobial resistance in *E. coli* posing significant challenges in treatment. Early detection of multi-drug resistant isolates in healthcare settings is crucial to combat antimicrobial resistance.

Keywords: β-lactamase; carbapenemases; extensive-drug resistance; metallo-ß-lactamase; multidrug resistance.

INTRODUCTION

Increasing Antimicrobial resistance (AMR) highlights AMR as a global public health concern.[1] AMR in Gram-negative bacilli is a problem due to frequent transfer of antibiotic resistance genes, especially through plasmids.[2] Multidrug resistance (MDR) is a problem and difficult to treat in South-east Asia and low-middle income countries (LMICs) like Nepal.[3] ß-lactamase enzymes [Extended Spectrum ß-Lactamase (ESBL), AmpC ß-lactamases (ACBL), Metallo-ß-lactamases (MBLs)] producing and Carbapenem-resistant Enterobacterales (CRE) have become a serious threat.[4] Extensively drug-resistant (XDR) CRE[5] have limited alternatives for antibiotic therapy.

Early detection and characterization of AMR is crucial to influence antimicrobial treatment guideline, policies to contain AMR. This study characterized AMR in *Escherichia coli* isolated from clinical specimens received at National Public Health Laboratory (NPHL) of Nepal.

METHODS

This was a laboratory based cross-sectional study, where

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clinical specimens received at NPHL were processed following standard methods[6] for the identification and antimicrobial susceptibility testing of the *E. coli*.

All the laboratory testing were performed in Microbiology Laboratory of NPHL. The study population was *E. coli* isolates from the suspected patients visiting NPHL during 2018-2022.

The study adhered to Ethical Review Board. Nepal Health Research Council (NHRC) approved study-Regd. 428-2018, protocol.

E. coli isolates obtained from clinical samples processed at Microbiology laboratory at NPHL were included for further analysis. Duplicate, unlabelled, improperly transported, contaminated and samples lacking patients' clinical data were excluded.

Convenience sampling; one non-MDR isolate and 5-6 isolates representing ESBL/ACBL/Carbapenemase/MBL resistance were included every month through weekly selection, which came to be 320 in total.

The clinical specimens were processed for culture on primary isolation media blood agar, MacConkey agar, and chocolate agar, Cystine-lactose-electrolyte deficient agar for urine specimens, Bactec-automated culture system plus primary isolation for blood specimens, following standard microbiological techniques.[6] All identified isolates were stored as pure growths in Tryptic soya broth[6] with glycerol at -80°C. The isolates were reconfirmed using MALDI-TOF (matrix-assisted laser desorption ionization-time-of flight-based mass spectrometry) on VITEK-MS (Biomeriux) equipment.[7]

Antibiotic susceptibility testing was performed following modified Kirby Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines.[8] The antibiotics included in the disc diffusion were Beta-lactam (BL): Ampicillin (AMP 10 µg), BL+ Beta-lactam inhibitor (BLI) combination: Amoxycillin-clavulanic acid (AMC 20/10 µg), Fluoroquinolone: Ciprofloxacin (CIP 5 µg), Levofloxacin (LE 5 µg), Folate pathway inhibitor: Trimethoprim-sulfamethoxazole (TMP-SMX 1.25/23.75 µg), Aminoglycoside: Gentamicin (GEN 10 µg), Amikacin (AK 30 µg), 3rd generation cephalosporins: Cefotaxime (CTX 30 µg)/Ceftriaxone (CTR 30 µg)/Cefixime (CFM 5 µg)/Ceftazidime (CAZ 30 µg), Furan: Nitrofurantoin (NIT 300 µg) for urine isolates only, and Fourth-generation cephalosporin: Cefepime (FEP 30 µg), Antipseudomonal penicillin+ BLI: Piperacillin-tazobactam (PIT/PTZ 100/10 µg), Tetracyclines: Tetracycline (TE 30 µg), Glycylcycline: Tigecycline (15 µg), and Carbapenems: Imipenem (IMP 30 µg)/Meropenem (MEM 30 µg).

Additional antibiotics for MIC were 2nd-generation cephalosporin: Cefuroxime Axetil, Quinolone: Nalidixic acid, Carbapenem: Ertapenem, Cephalosporin plus Beta-lactam inhibitor: Cefoperazone-sulbactam and the Polymyxin: Colistin were tested.

Minimum Inhibitory Concentration (MIC) analysis: AST N280 card for VITEK 2 compact was used for MIC testing for urine isolates and AST N281 for other isolates, following manufacturer's instructions and interpretative standards of the CLSI.[8]

Screening and confirmation of ESBL producers: ESBL production was screened using third-generation cephalosporins (3GC), Ceftazidime (CAZ) (30µg) and Cefotaxime (CTX) (30µg). An isolate was considered as a potential ESBL-producer when zone of inhibition (ZOI) was ≤ 22mm for CAZ and/or ≤27mm for CTX.[8] The combined disc method was used for confirmation where lawn culture of 0.5 MacFarland inoculum of the *E. coli* isolates was made on MHA and antibiotic discs CTX (30 µg), CAZ (30µg), CTX+Clavulanic acid (CA:10µg) and CAZ+CA (CA:10µg) were placed at a distance of 25 mm from each other. A difference of ≥5 mm in the ZOI for either antibiotic tested in combination with CA versus its ZOI when tested alone was confirmed as ESBL.[8]

Screening of AmpC ß-lactamase and carbapenemase: Isolates showing <18mm ZOI to Cefoxitin were taken as potential AmpC ß-lactamase producers and confirmed by AmpC disc test method described previously.[9] Briefly, AmpC disks containing Tris-EDTA, were used to indicate enzymatic inactivation of cefoxitin (AmpC positive), or the absence of a distortion, indicating no significant inactivation of cefoxitin (AmpC negative).

All isolates that were resistant to any one of the carbapenems were chosen for further testing. Metallo-ß-lactamase (MBL) production was screened by using Ceftazidime (CAZ) and Imipenem (IPM)10µg antibiotic discs. When ZOI was ≤ 18mm for CAZ and/or ≤ 19mm for IPM, the isolate was considered as a potential MBL-producer. Modified Carbapenem-inhibition method (mCIM)[8] was used for carbapenemase testing and eCIM used to confirm MBLs.

Multi-drug resistance (MDR) was defined as an acquired non-susceptibility to at least one agent of three or more antimicrobial classes. Extensively-drug resistance (XDR)

was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories.[10] Intermediate susceptibility against tested antibiotics were grouped as "resistant" for the purposes of analysis except for Colistin.

ATCC® *E. coli* 25922 was used for standardization of media, manual biochemical identification, antibiotic susceptibility testing procedures, and as a negative control in ESBL testing. *Klebsiella pneumoniae* ATCC® 700603 was used as positive control for ESBL testing. *K. pneumoniae* ATCC® BAA 1705 and BAA-1706 respectively were used as positive and negative controls for mCIM test, while *E. coli* ATCC® 8739 strain was used for the VITEK-MS procedures.

Descriptive analysis was done as frequency and percentages, while Chi-square test was used for comparison of percentages as inferential statistics. P-value less than 0.05 was considered significant.

RESULTS

Distribution of bacterial growth in clinical specimens: Out of 24,636 clinical specimens processed, significant bacterial growth was observed in 9% (2166/24,636) only. *E. coli* was the predominant isolate 44% (959/2166) and majority 98% (935/959) of the *E. coli* isolates were obtained from urine samples which was statistically significant (p-value <0.001) (Table 1). Among the 959 *E. coli* isolates, purposefully selected 320 isolates (6-7 isolates per month) were further analysed.

Table 1. Distribution of *Escherichia coli* isolates in different specimen types.

Specimen type	No. of Specimens processed (%)	No. of significant growth (%)	No. of mixed growth (%)	No. of *Escherichia coli* isolates (%)	p-value
Urine	20,306 (82.4)	1,761 (8.7)	541 (2.7)	935/1761 (53.1)	<0.001
Blood	3,007 (12.2)	108 (3.6)	6 (0.2)	5/108(4.6)	
Respiratory specimens	1091 (4.4)	203 (18.6)	0 (0)	8/203 (3.9)	
Pus/wound swabs	167 (0.7)	86 (51.5)	1 (0.6)	10/87 (11.5)	
Body fluids	55 (0.2)	7 (12.7)	0 (0)	0 (0)	
Genital swabs	10 (0.04)	1 (10)	0 (0)	1/1 (100)	
Total No.	24636 (100)	2166 (100)	548 (100)	959	

Antibiotic Susceptibility Pattern: Low proportion of *E. coli* were susceptible to Ampicillin 24% (73), Fluoroquinolones 24% (76), 3GC 40% (128) and trimethoprim-sulfamethoxazole 48% (155). High proportion were susceptible to Tigecycline 99% (316), Carbapenems ≥90% (90-92), Aminoglycosides: ≥82% (82-94), Cefoperazone-sulbactam 86% (276) and Piperacillin-Tazobactam 79% (254) with moderate susceptibility of urinary isolates to Nitrofurantoin 80% (242) (Table 2).

Table 2. Antibiotic resistance pattern of *E. coli* with disc diffusion and MIC methods. (n=320)

Antibiotic class	Antibiotics	Disc diffusion results No. (%)		MIC results No. (%)		P-value
		Susceptible	Resistant	Susceptible	Resistant	
Aminoglycosides	Amikacin	192(80)	49(20)	302(94)	18(6)	<0.001
	Gentamicin	223 (74)	80 (26)	262 (82)	58 (18)	0.013
B Lactams (BL)	Ampicillin	50 (16)	270 (84)	73 (24)	237 (76)	0.012
BL+ B-lactam inhibitor (BLI) combination	Amoxycillin-clavulanic acid	29 (22)	101 (78)	203 (64)	116 (36)	<0.001
	Cefoperazone-sulbactam	ND	ND	276 (86)	44 (14)	NA
Antipseudomonal penicillin+ BLI	Piperacillin-Tazobactam	171 (72)	67 (28)	254 (79)	66 (21)	0.039
2nd Generation Cephalosporins	Cefuroxime Axetil	ND	ND	113 (35.5)	214(61)	NA
3rd Generation Cephalosporins	Ceftriaxone/ Cefotaxime	98 (31)	219 (69)	135 (42)	185 (58)	0.003
4th Generation Cephalosporin	Cefepime	38 (34)	74 (66)	177 (55)	143 (45)	<0.001
Folate pathway inhibitor	TMP-SMX	149 (47)	167 (53)	156 (49)	164 (51)	0.687
Furans	Nitrofurantoin	250(84)	47 (16)	242 (80)	62 (20)	0.146
Quinolone	Nalidixic acid	55(18)	257(82)	ND	ND	NA
Fluoroquinolones	Ciprofloxacin	85 (28)	224(72)	76 (24)	244 (76)	0.280
	Levofloxacin	71(34)	138(66)	8 (44)	10(56)	NA
Tetracyclines	Tetracycline	91(61.9)	53 (36.1)	2 (61.9)	1 (36.1)	NA
Glycylcyclines	Tigecycline	48 (96)	2 (4)	316 (99)	4 (1)	0.407
Carbapenems	Imipenem	87 (65)	46 (35)	293 (92)	27 (8)	<0.001
	Meropenem	117 (87)	17 (13)	288 (90)	32 (10)	0.400
	Ertapenem	ND	ND	290(91)	30 (9)	NA
Polymyxins	Colistin	ND	ND	NA	7(2.2)	NA

Note: ND: Not done; NA: Not applicable.

Antibiotic susceptibility evaluations by MIC method were more precise as compared to the disc diffusion method (p-value<0.05) for all antibiotics except Trimethoprim-sulfamethoxazole, Nitrofurantoin, Ciprofloxacin, Meropenem and Tigecycline (Table 2).

Antibiotic resistance enzymes production: Among the 320 *E coli* isolates, 218 were potential ESBL producers, 45 potential ACBL producers and 49 potential carbapenemase producers. ESBL production was re-confirmed in 62% (197), ACBL in 12% (39), serine carbapenemases in 10% (31) and MBL in 7% (22) of the isolates. More than one type of enzymes was produced in the isolates viz., 8% (25) ESBL and ACBL, 10% (30) ESBL and serine carbapenemases, and

4% (12) ESBL, ACBL and serine carbapenemases while 3% (9) of the isolates produced all three enzymes ESBL, ACBL and MBL.

Minimum Inhibitory Concentration in the ESBL *E coli*: ESBL producers had significantly high MICs >64µg/mL (91%) against 3GC while ESBL negative isolates had MIC value of <1 µg/mL (98%). The ESBL producers were most susceptible to Tigecycline (100%), Amikacin (92%), Carbapenems (84-87%), Cefoperazone-sulbactam (78%), Gentamicin (76%), Nitrofurantoin (76%), and Piperacillin-tazobactam (71%) which was statistically significant except for Amikacin (Table 3).

Table 3. Minimum Inhibitory Concentration of ESBL (n=197) and non-ESBL *E. coli* isolates (n=123)

Antibiotic	Non-ESBL *E. coli* MIC results		ESBL *E. coli* MIC results		p-value
	Susceptible No. (%)	Resistant No. (%)	Susceptible No. (%)	Resistant No. (%)	
Amikacin	120 (98)	3 (2)	182 (92)	15 (8)	0.051
Gentamicin	113 (92)	10 (8)	149 (76)	48(24)	<0.001
Ampicillin	71 (58)	52 (42)	4 (2)	193 (98)	<0.001
Amoxycillin-clavulanic acid	102 (83)	21(17)	101 (52)	95(48)	<0.001
Cefoperazone-sulbactam	122 (99)	1 (1)	154 (78)	43(22)	<0.001
Piperacillin-Tazobactam	114 (93)	9 (7)	140 (71)	57(29)	<0.001
Cefuroxime Axetil	94 (78)	27 (22)	10 (5)	187 (95)	<0.001
Ceftriaxone	122 (99)	1 (1)	13 (7)	184 (93)	<0.001
Cefepime	120 (98)	3 (2)	57 (29)	140 (71)	<0.001
TMP-SMX	86 (70)	37 (30)	70 (35)	127 (65)	<0.001
Nitrofurantoin	103 (88)	14 (12)	139 (74)	48 (26)	0.004
Nalidixic acid	39(34)	77 (66)	11 (6)	176 (94)	<0.001
Ciprofloxacin	59 (48)	64 (52)	17 (9)	180 (91)	<0.001
Tigecycline	119 (97)	4 (3)	197 (100)	0 (0)	0.042
Imipenem	122 (99)	1 (1)	171 (87)	26(13)	<0.001
Meropenem	122 (99)	1 (1)	166 (84)	31 (16)	<0.001
Ertapenem	122 (99)	1 (1)	168 (85)	29(15)	<0.001
Colistin	120 (98)	3 (2)	193* (98*)	4 (2)	NA

Note: *Colistin reported as intermediate and resistant only (Not reported as susceptible). NA: Not applicable

Among 31 carbapenemase producers, MICs varied with Ertapenem showing highest MIC >8 µg/mL in 77% (24), Imipenem >16µg/mL in 65% (20) and Meropenem >16µg/mL in 52% (16) isolates. Among the 22 MBL producing *E. coli* isolates, 100% resistance was observed to most tested antibiotics with 100% susceptibility to Tigecycline, followed by the aminoglycosides (Range: 41-50%). Low (41%) susceptibility to Nitrofurantoin was observed in urinary isolates.

Seven (2%, 7/320) of the analysed isolates were extensively-drug resistant (XDR) while seven showed phenotypic colistin resistance (2%, 7/320), which on microdilution confirmed only one true colistin-resistant isolate. However, not all XDR isolates were colistin-resistant.

Production of all enzymes (ESBL, sCPM, ACBL) except MBL showed significant association with MDR in the *E. coli* isolates (Table 4).

Table 4. Association between ESBL, ACBL, sCPM and MBL positivity and MDR.

Type of antibiotic resistance enzyme	Non-MDR (N=94)	MDR (N=226)	Total tested (N=320)	P-value
ESBL producer	16	181	197	<0.001
ACBL producer	4	35	39	0.005
Serine carbapenemases producer	1	30	31	0.010
MBL producer	0	22	22	0.690

Multi-drug resistance in *E. coli*: Among the 320 isolates, based on disc diffusion 75% (240) were MDR while 25% (80) were non-MDR. The resistance to different antibiotic classes varied from zero to eight and 226 MDR morphotypes were observed based on MICs to the 19 antibiotics. Distribution among the MDR (≥3 antibiotics) isolates was maximum 30% (68) to any four, 21% (47) to only three, 49% (111) to five or more classes of antibiotics (Figure 1). Extensive-drug resistance (XDR) was observed in 2% (7) of the isolates.

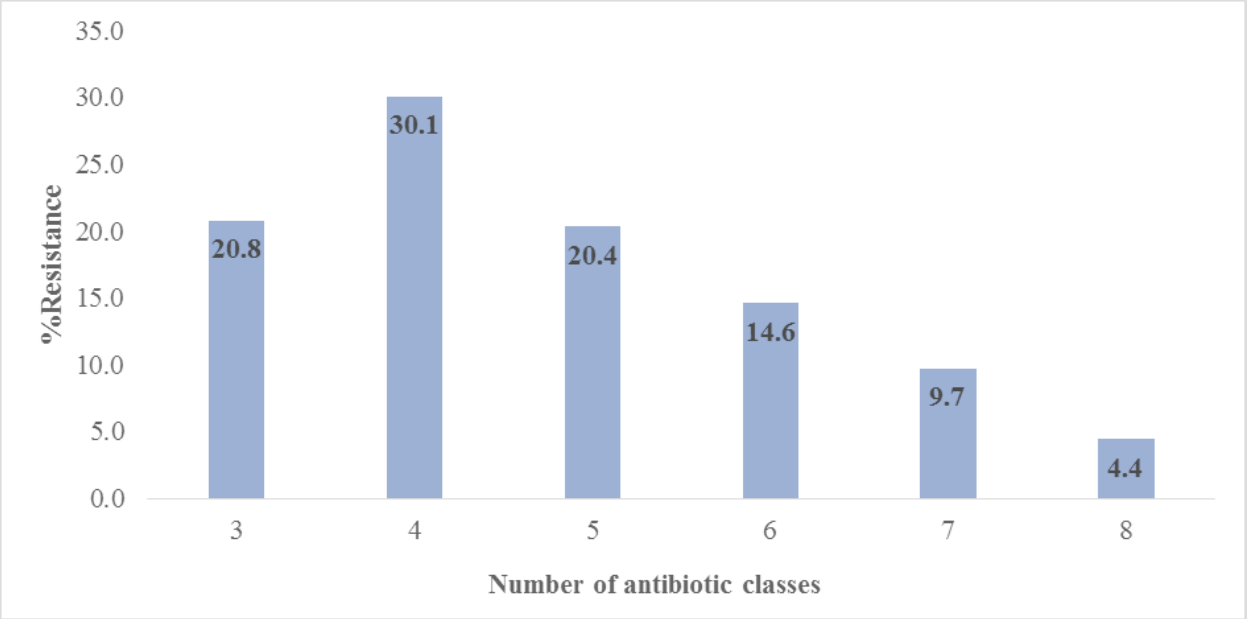

Figure 1. Multidrug resistance in E. coli isolates.

Combined resistance to ß-lactams and Fluoroquinolones 90% (213) with Ampicillin, 3rd generation cephalosporin and Fluoroquinolone morphotype (AMP, 3GC, FQ) was the most prevalent at 77% (174) among other combinations (Figure 2).

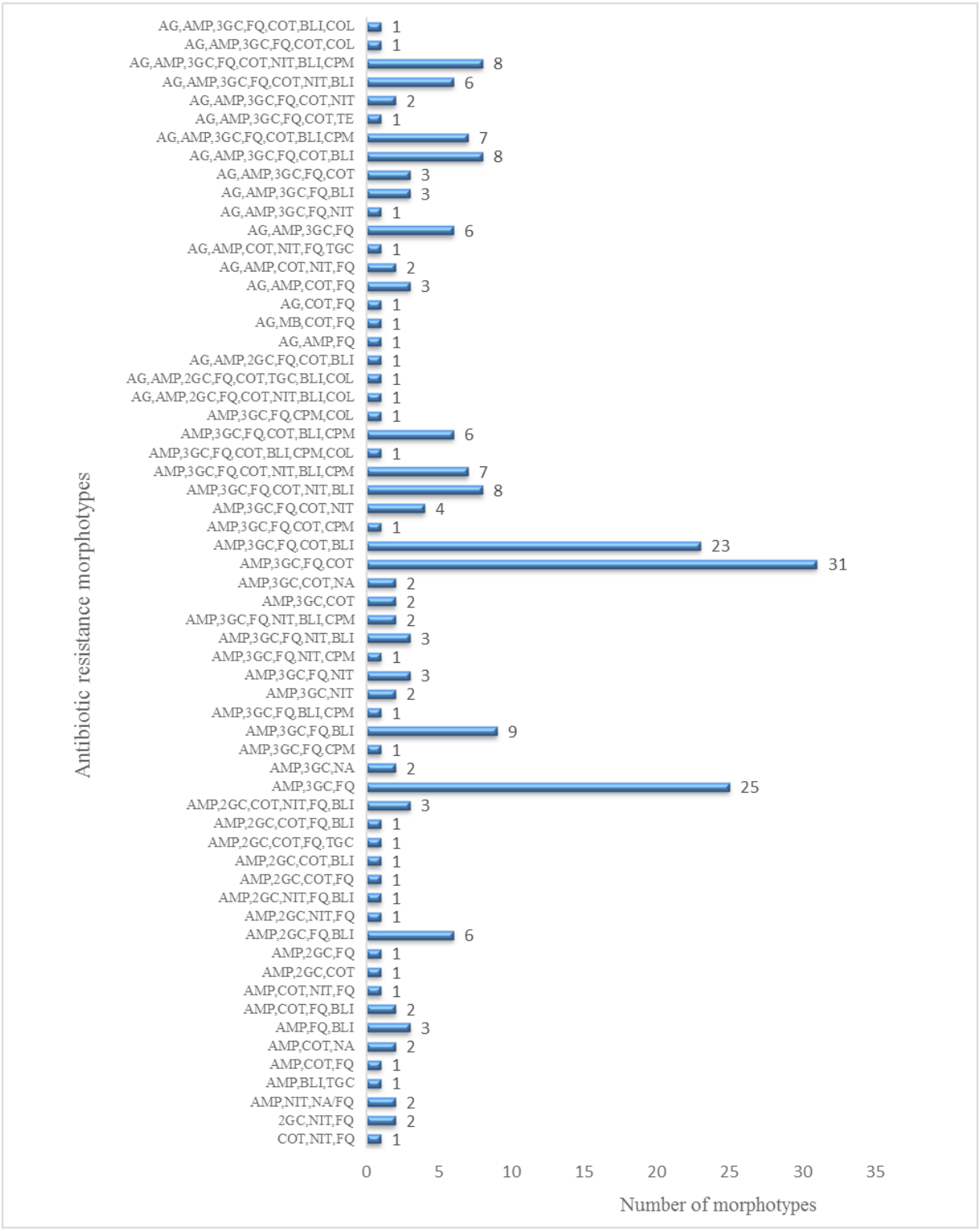

Figure 2. Antibiotic resistance morphotypes (n=226) in E. coli isolates.

Legend: AMP: Ampicillin; AG: Aminoglycoside, COT: Trimethoprim-sulfamethoxazole, COL: Colistin, CPM: Carbapenem, FQ: Fluoroquinolone, NA: Nalidixic acid, NIT: Nitrofurantoin, BL: Beta-lactam, BLI: Beta-lactam inhibitor, 2GC: Second-generation cephalosporin, 3GC: Third-generation cephalosporin, TGC: Tigecycline.

DISCUSSION

Most *E. coli* were resistant to one or more of the first-line antibiotics, with high ß-lactam and Fluroquinolone combined resistance. ESBL and MBL producers had higher multidrug-resistance but showed susceptibility to Tigecycline and Aminoglycosides. A major cause of MDR in *E. coli*, 3GC-resistant and carbapenem-resistant *E. coli* are listed as priority bacterial pathogens of concern[1], hence, the characterization and antibiotic susceptibility pattern of MDR *E. coli* in this study may provide information on circulating strains for effective evidence-based policy interventions, to counter the AMR challenge.

Identification of *E. coli* by conventional methods has been the norm in many microbiology laboratories. In this study, MALDI-TOF VITEK-MS (Biomeriux) identification results were comparable with conventional biochemical tests-based identification methods.

Urine was the most predominant specimen (82%) with *E. coli* (44%) as the predominant isolate with 53% from urine which is quite similar to other reports.[11]

MICs for most antibiotics except Trimethoprim-sulfamethoxazole, Nitrofurantoin, Ciprofloxacin, Meropenem and Tigecycline showed better precision at detecting resistance than disc diffusion which is in agreement with studies that found disc-diffusion methods to give false susceptibility results.[12,13]

In this study, 74% of *E. coli* isolates were resistant to Ampicillin and 70% to Ciprofloxacin, the most used 'Watch' group antibiotic for various infections in our country.[14] This is similar to the findings from Nepal, however higher from other countries.[15] This may be due to over-the-counter availability, and irrational use of antibiotics in Nepal.[16]

MDR was observed in 70% isolates with 90% (213) having combined resistance to ß-lactams and Fluoroquinolones which has been reported in neighbouring countries though lower in developed countries.[17,18]

MDR and XDR *E. coli* infections have increased in recent years which has resulted in limited treatment options and a challenge for clinicians. 3GCs which were rampantly used for all types of infections are now ineffective against infections caused by Enterobacterales due to the production of enzymes such as ESBL and ACBL. This scenario has worsened with an increase in rates of carbapenem resistance as high as 62% in some areas of Nepal.[19] In this study, 70% were MDR *E. coli* isolates, and 62% ESBL producers, which varies in studies from Nepal (30-80%), neighbouring countries (36-69%) and Europe (0-24%).[20-22] In this study, more than one mechanism of resistance was observed in up to 8% of *E. coli* isolates which has been reported by hospital-focused studies in (8-72%) Nepal and neighbouring countries[19,23]. The lower percentages in this study could be due to phenotypic characterization and AmpC beta-lactamases masking the ESBL.[24]

ESBL-producing *E. coli* were more prone to be MDR (82%) in this study and were resistant to Ciprofloxacin (85%) which is similar to other studies.[25,26] The ESBL producers showed higher susceptibility towards Tigecycline (100%), Imipenem (88.3%), Amikacin (73.3%) followed by Piperacillin-tazobactam (68.1%) similar to others' study findings.[19]

The MICs for third- and fourth-generation cephalosporins were very high (91%, >64 µg/mL) in ESBL producers indicating the need for testing MIC along with the breakpoint concentrations to guide the treatment.[27] Carbapenem resistance in ESBL-producing strains makes therapy even more difficult, frequently forcing clinicians to choose between drugs with lower efficacy and higher toxicity, like tigecycline or polymyxins[28].

Studies conducted in Nepal have reported a higher proportion (19.5%, 15/77)[19] of Carbapenemase/MBL production than this study findings (7%) while other countries have shown MBL production ranging from 0.3 to 65%.[29]

All Carbapenemase producers were resistant to the 'Access' and 'Watch' group of antibiotics in our study with 100% resistance towards 3GCs, Piperacillin-tazobactam, Nalidixic acid, Ciprofloxacin, Cefoperazone-sulbactam and carbapenems, while Tigecycline was the most effective. Similar reports show comparable susceptibilities[19] while others have reported resistance to Tigecycline.[30]

In Nepal, aminoglycoside resistance has been reported from various institutions but still lesser than 3GCs[11,19] and this study showed similar low resistance. Resistance to last resort drug colistin was observed in seven isolates which has been reported in *E. coli* as well as other Enterobacterales[3]. On performing MIC by broth micro-dilution, only one isolate was reconfirmed as colistin resistant with MIC>16µg/mL. This clearly indicates the need for performing MICs in re-confirming the susceptibility of the MDR isolates.

Lack of clinical data, co-morbid conditions, prior antibiotic therapy, and non-inclusion of severely-ill admitted patients were the limitations of the study, which might have resulted in lower numbers of MDR and XDR cases.

CONCLUSIONS

Most *E. coli* were resistant to one or more of the first-line antibiotics, with ß-lactam and Fluroquinolone combined resistance being the most common. ESBL and MBL producing isolates were found in significant proportions with high rate of MDR. Most of the ESBL and MBL producing *E. coli* were susceptible to Tigecycline followed by Aminoglycosides. The phenotypic detection of ß-lactamase producing *E. coli* could help detect MDR and avoid treatment failure. MICs of antibiotics (not limited to colistin) should be performed for better antibiotic susceptibility analysis. Further strict antibiotic policies and measures could be implemented to limit the indiscriminate use of antibiotics and to minimize the emergence of multiple ß-lactamases.

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CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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