VIRULENCE AND DRUG RESISTANCE PATTERN OF ESCHERICHIA COLI ISOLATES FROM VARIOUS CLINICAL SAMPLE

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ABSTRACT

Introduction

E. coli is one of the most common pathogens, and its infection has resulted in a global burden due to its increased medication resistance and virulence factors. The goal of this study was to examine the drug resistance pattern and pathogenicity properties of *Escherichia coli* isolates.

Methods

Over the course of six months (March 2019-August 2019), a laboratory-based cross-sectional study was done among patients visiting Manmohan Memorial Teaching Hospital in Kathmandu, Nepal. Standard microbiological procedures were used to identify bacterial isolates from clinical specimens. The modified Kirby Bauer disk diffusion method was used to determine antibiotic susceptibilities. The combined disk test method was used to confirm the ESBL and MBL. To determine their virulence, serum bactericidal activity and biofilm productions were determined.

Results

59.60 percent (n=81) of the 136 *Escherichia coli* isolates were multidrug resistant, 25.70 percent (n=35) were ESBL producers, and 11.80 percent (n=16) were MBL producers. Serum resistance was discovered in 22.8 percent (n=31) of the total isolates, while biofilm formation was found in 19.11 percent (n=26). Amoxycillin had the highest level of resistance (87.5%), whereas Chloramphenicol (93.4%) and Imipenem (80.9%) were the most susceptible antibiotics. Polymyxin B and Colistin sulphate were absolutely sensitive. In our investigation, 61.5 percent of biofilm producers were MDR, with non-beta lactamase types being the most common. MBL producers were discovered to be more serum resistant. Amikacin and Imipenem were found to be more sensitive to biofilm makers.

Conclusions

The expression of *Escherichia coli* virulence factors varied depending on the kind of infection. *Escherichia coli* has a high rate of multidrug resistance. To minimize the emergence and spread of antibiotic resistance in bacteria, proper identification of drug-resistant bacteria, careful use of antibiotics, and effective antibiotic policy are required.

Keywords: *Escherichia coli, Biofilm, Serum resistance, Multidrug resistance*

INTRODUCTION

Escherichia coli, being commensal is also the most important pathogen and major cause of morbidity, mortality and increased health care costs[1, 2]. *Escherichia coli* is responsible for causing broad spectrum of diseases, which include sepsis, meningitis, pneumonia, intra-abdominal infections, diverse soft tissue infections, osteomyelitis and predominantly urinary tract infection (UTI)[2, 3]. The ability of *Escherichia coli* to cause infections is increasing, while the ease of treating these infections due to multidrug resistance to first line antibiotics such as Cotrimoxazole, Ampicillin and Nitrofurantoin is becoming increasingly difficult [4] . The increment in prevalence of multidrug resistant *Escherichia coli* strains worldwide is due to the spread of mobile genetic elements, such as plasmids which is also responsible for β-lactamase production and confer resistance to broad spectrum of β-lactams[5] .

Escherichia coli possess the specific virulence factors such as diverse adhesins, polysaccharide coatings (e.g, lipopolysaccharide and capsules), toxins, siderophores, proteases, invasions necessary to cause disease[6, 7] . The key virulence factor utilized by the bacteria to overcome host defenses and cause MDR (Multidrug resistance) is serum resistance and biofilm production.Animal serum contains substances that are lethal for most bacteria and constitute important host defenses against bacterial infection but the production of protective extracellular polysaccharide capsules and expression of factors that interfere with the complement cascade are responsible for causing serum resistance in *E. coli*. Also, recent studies have highlighted structural integrity of the cell envelope as a factor that helps in serum survival of organism[8, 9]. Uropathogenic *Escherichia coli* form intracellular bacterial communities with many biofilm like properties within the bladder epithelium[10] . Biofilms causes up to 60% of human infections and biofilm producers are difficult to eradicate with antimicrobial treatment[11] . Microorganisms which develop in a biofilm are intrinsically more resistant than planktonic cells to antimicrobial agents. High concentration of antimicrobials are needed to inactivate organisms that develop in a biofilm, because antibiotic resistance can increase 1,000 fold[12] .

Above studies showed that there is constant increase in MDR and highly virulent *Escherichia coli*. In this perspective, we aimed to conduct study in order to know the status of MDR pathogens and their virulence property so that strict treatment policy can be formed for their eradication.

MATERIAL AND METHODS

A laboratory based study was conducted in Department of microbiology over the period of six months among the patients visiting Manmohan Memorial Teaching Hospital, Kathmandu, Nepal. The clinical specimens (urine, blood, pus, sputum and high vaginal swab) strictly meeting the requirements suggested by the American Society for Microbiology[13] were selected for further processing.

Inoculation, identification and antimicrobial susceptibility testing (AST)

Clinical specimens were inoculated in appropriate culture media and then incubated aerobically at 37°C for 24 hours. The colonies were identified by using standard microbiological technique which involved morphological appearance of colony, gram's staining and biochemical tests[13] . AST was performed on Mueller Hinton Agar (MHA) using Kirby-Bauer disk diffusion technique as recommended by Clinical and

Laboratory Standards Institute (CLSI)[14] . Isolate resistant to at least one antimicrobial from three different group of first line drugs tested was regarded as MDR[15] .

Detection of beta lactamases production

The initial screening test for the production of extended spectrum β-lactamase **(**ESBL) was performed by using Ceftazidime (30 ug) and Cefotaxime (30ug) discs. If the ZOI was ≤ 22 mm for Ceftazidime and ≤ 27 mm for Cefotaxime, the isolate was considered as a potential ESBL producer as recommended by CLSI guideline. Then the combined disk test methods for ESBL detection was performed from the suspected isolates for confirmation. An increase in zone diameter by ≥5 mm in the disk containing Ceftazidime-Clavulanic acid than Ceftazidime alone confirmed the presence of ESBL enzyme[14] .

Isolates found to be non-susceptible to Imipenem in Kirby Bauer disk diffusion were presumably regarded as Metallo β-lactamase **(**MBL) producers and were confirmed using a combined disk method. Isolates with an increase in zone size of more than or equal to 7mm for imipenem-EDTA disk compared to imipenem disk alone were confirmed as MBL producer[16] .

Detection of biofilm production

The biofilm production test was carried out in 96 welled flat bottom tissue culture plate made up of polystyrene as per the criteria given by Stepanovic et al., 2000. In this method, fresh culture of organism was inoculated in 2 ml of Luria Bertani broth. 200µl of the diluted culture was inoculated in the sterile wells of tissue culture plate and incubated at 37°C for 24 hours. Floating bacteria are removed with 0.2 ml of phosphate buffer saline (pH 7.2) four times. Biofilm formed by bacteria were fixed by keeping at 60°C for 1 hour and stained by crystal violet (2%). Excess stain was removed by rinsing with deionized water and subsequently decolorized with 30% acetic acid. Optical density (OD) of stained biofilm was obtained by using ELISA reader at wavelength 570 nm. OD was defined as three standard deviations above the mean OD of negative control [17,18] .

Serum killing assay

Sahly H. and colleagues method was used for testing the susceptibility of bacteria to human serum where serum was prepared from ten healthy human's blood. An inoculum of 25µl (adjusted to 10^6 colony forming units/ml) prepared from the mid-log phase was diluted by 0.9% saline, and was added to 75µl of pooled human sera contained in a tube. Viable counts were checked at 1, 2, and 3 hr of incubation at 37°C. Each strain was tested at least 3 times, and the mean results will be expressed as percent inoculums. The result were expressed as percentage of inoculation and responses in term of viable count were graded from 1 to 6, as a serum sensitive at grades of 1 to 2 , intermediately sensitive at grades of 3 to 4, and resistant at grades of 5 to 6. For grade 1, viable counts (VC) after 1 and 2 h was <10% of the inoculum; after 3hr, it was <0.1%.

For grade 2, VC after 1 hr was 10–100%, after 3 hr, <10%. For grade 3, VC after 1 hr was >100%, after 2 and 3 hr, it was <100%. For grade 4, VC after 1 and 2 hr was >100%, after 3 hr, <100%. For grade 5, VC after 1, 2 and 3 hr was >100%, but VC could fell some time during the 3 hr period. For grade 6, VC after 1, 2 and 3 hr was >100% of the inoculum and could rise throughout the 3 hr period [19] .

Ethical consideration

Ethical approval was obtained from institutional review committee of MMIHS, Kathmandu, Nepal. Informed written consent was taken from each and every participant after explaining the objective of the study.

Data analysis

Each sample was encoded with identification number. Finding was manually recorded and entered in Micro-soft Excel 2010. Analysis was done by SPSS Version 20 and interpreted according to frequency distribution, percentage and Chi-square test.

RESULTS

Antibiotics	Sensitive(%)	Resistant(%
Amoxycillin	17 (12.5)	119 (87.5)
Cephalexin	25 (18.4)	111 (81.6)
Cefoxitin	95 (69.9)	41 (30.1)
Cefixime	29 (21.3)	107 (78.7)
Cefotaxime	41 (30.1)	95 (69.9)
Ceftazidime	64 (47.1)	72 (52.9)
Gentamycin	86 (63.2)	50 (36.8)
Ciprofloxacin	66 (48.5)	70 (51.5)
Nitrofurantoin	103 (75.7)	33 (24.3)
Cotrimoxazole	70 (51.5)	66 (48.5)
Amikacin	103 (75.5)	33 (24.3)
Chloramphenicol	127 (93.4)	9 (6.6)
Levofloxacin	71 (52.2)	65 (47.8)
Tetracycline	71 (52.2)	65 (47.8)
Piperacillin/Tazobactam	98 (72.1)	38 (27.9)
Polymyxin B	136 (100)	-
Colistin sulphate	136 (100)	-
Imipenem	110 (80.9)	26 (19.1)

During the study period, a total of 136 *E. coli* was isolated. These *Escherichia coli* isolates were recovered from various clinical samples in which majority of them were isolated from urine (80.1%), followed by blood (8.1%), pus (6.6%), sputum (4.4%), and high vaginal swab (0.7%).

Antibiogram of *Escherichia coli* isolates

Highest level of resistance was seen with Amoxicillin (87.5%), followed by first and third generation Cephalosporins such as Cephalexin (81.6%), and Cefixime (78.7%), Cefotaxim (69.9%) respectively. Similarly, 51.5% of isolates were resistant to Ciprofloxacin and 48.5% of isolates were resistant to Cotrimoxazole. However, all isolates were sensitive to Polymyxin B and Colistin sulphate (Table 1). Out of total *E. coli* isolated, 81 were MDR, 35 were ESBL producer and 16 were MBL producer**Table 1: Antibiogram pattern of *Escherichia coli* isolates**

Incidence and categorization of biofilm formation

Out of 136 *E. coli* isolated, 110 (80.88%) were biofilm non producer and 26 (19.11%) were biofilm producer. Biofilm was predominantly produced by *E. coli* isolated from the urine sample.

Table 2: **Categorization and distribution of biofilm production among different samples**

	Biofilm							
Samples	**Neg.**	**Weak**	**Mod.**	**Str.**	**Biofilm**	**Positive**	**Isolates**	(N=26)
Urine					**MDR**	**ESBL**	**MBL**	**SRI**
	77.1%	22.0%	0.9%	-				
Blood					16	5	2	3
	90.9%	9.1%	-	-	(61.5%)	(19.2%)	(7.7%)	(11.5%)
Pus	100%	-	-	-				
Sputum	100%	-	-	-				
HVS	100%	-	-	-				
Total	**110**	**25**	**1**	**0**				
	(80.9%)	**(18.4%)**	**(0.70%)**	**(0.0%)**				

Neg. = Negative, Mod.= Moderate, Str.= Strong, SRI= Serum resistant isolates

Among the biofilm producers, 61.5% were MDR. Only 19.9% of biofilm producers were ESBL producer and 7.7% of biofilm producers were positive for MBL production. 11.5% of the biofilm producers were resistant to serum killing (Table 2).

Serum killing assay among *Escherichia coli* isolates

Among the 136 *E. coli* isolated, 31 (22.8%) were resistant with serum, 29 (21.3%) were intermediately sensitive with serum and 76 (55.9%) were serum sensitive. Among serum resistant *E. coli* isolates, majority were isolated from urine (Table 3).

Table 3: Sample wise distribution of serum killing assay

Samples	Serum killing assay		
	Highly sensitive	**Intermediate sensitive**	**Resistance**
	N (%)	**N (%)**	**N (%)**
Urine	68 (62.4)	23 (21.1)	18 (16.5)
Blood	2 (18.2)	3 (27.3)	6 (54.5)
Pus	4 (44.4)	2 (22.2)	3 (33.3)
Sputum	2 (33.3)	1 (16.7)	3 (50.0)
HVS	0 (0.0)	0 (0.0)	1 (100)
Total	**76 (55.9)**	**29 (21.3)**	**31 (22.8)**

Serum killing assay among MDR and beta-lactamase producing isolates

Among the total 81 MDR isolates, 40 (49.4%) were highly susceptible to serum killing assay, 17 (21.0%) were intermediately susceptible and 24 (29.6%) were serum resistant. Similarly, among 35 ESBL producers, 15 (42.9%) were highly susceptible to serum killing assay. Likewise, 37.5% of MBL producer were highly susceptible to serum, 6.2% were intermediately susceptible and 56.2% were serum resistant. Association between serum killing assay and resistance pattern is shown in Table 4 where there was significant association between MBL and serum killing ($p<0.05$).

Table 4: Serum bactericidal activity in MDR and β-lactamases isolates

Resistance pattern		Serum killing assay			
		Highly sensitive	**Intermediate sensitive**	**Resistant**	**p-value**
		N (%)	**N (%)**	**N (%)**	
MDR	**MDR**	40 (49.4)	17 (21.0)	24 (29.6)	*0.060*
	Non-MDR	36 (65.5)	12 (21.8)	7 (12.7)	
ESBL	**ESBL**	15 (42.9)	7 (20.0)	13 (37.1)	*0.057*
	Non-ESBL	61 (60.4)	22 (21.8)	18 (17.8)	
MBL	**MBL**	6 (37.5)	1 (6.2)	9 (56.2)	*0.003**
	Non-MBL	70 (58.3)	28 (23.3)	22 (18.3)	

Comparison of antibiotics resistance pattern among biofilm producer and biofilm non-producer Polymyxin B and Colistinsulphate were 100% sensitive to all of the *E. coli*isolates. After Polymyxin B and Colistinsulphate, Amikacin and Chloramphenicol were mostly sensitive drug followed by Imipenem (Table 5).

Table 5: Comparison of antibiotics resistance pattern among biofilm producer and biofilm non-producer

	Biofilm producer	**Biofilm non producer**
Antibiotic	**N (%)**	**N (%)**
Amoxycillin	21(80.8)	98(89.1)
Cephalexin	22(84.6)	89(80.9)
Cefoxitin	7(26.9)	34(30.9)
Cefixime	21(80.8)	86(78.2)
Cefotaxime	19(73.1)	76(69.1)
Ceftazidime	11(42.3)	61(55.5)
Gentamycin	7(26.9)	43(39.1)
Ciprofloxacin	11(42.3)	59(53.6)
Nitrofurantion	7(26.9)	26(23.6)
Cotrimoxazole	9(34.6)	57(51.8)
Amikacin	1(3.8)	3(29.1)2
Chloramphenicol	1(3.8)	8(7.3)
Levofloxacin	7(34.6)	56(50.9)
Tetracycline	12(46.2)	53(48.2)
Piperacillin/Tazobactam	6(23.1)	32(29.1)
Polymyxin B	-	-
Colistinsulphate	-	-
Imipenem	2 (7.7)	24(21.8)

DISCUSSION

E. coli is an emerging pathogen which causes invasive infections in both community and hospitalized setting mainly in debilitated host[20] . β – lactam drugs like Penicillin, Cephalosporin, Carbapenems, and Aztreonam are common antibiotics used to combat most bacterial infections and the haphazard use of these antibiotics and clinical practices lead to emergence of multidrug resistant pathogens [21] .

A total of 136 *E. coli* was collected from different clinical samples in our study. The majority of *E. coli* were collected from urine (80.1%), followed by blood (8.1%), pus (6.6%), sputum (4.4%) and high vaginal swab (0.7%) samples. In the study by Bhrulgubalda et al., the clinical samples distribution was as follows: urine (92%), pus (5%), sputum (1%), ascetic fluid (1%), and blood (1%) (22). Similarly, in the study conducted by Fakruddin Md et al., 65 clinically isolated *E. coli* were studied in which most of them i.e 35% were from urine, 18.46% from peritoneal, 18.46% from blood, 15.38% from pus and 9.23% from CSF (23). . The above data suggests that, *E. coli* commonly causes urinary tract infections. Among the various infections *E. coli* is responsible for more than 90% of UTI cases therefore, it assumes greater significance. *E. coli* is the normal human and animal intestinal colonizers and has different virulence determinants as a result it has easy transmissibility and can invade the urinary tract through the ascending route and cause UTI[24] .

Pattern of resistance was studied for all the isolates of *E. coli*. Highest resistance was observed in Amoxicillin (87.5%), followed by Cephalexin (81.6%), whereas the antibiotics Amikacin (75.5%), Imipenem (80.9%) and chloramphenicol (93.4%) were sensitive. All the isolates were sensitive to Polymyxin B and Colistinsulphate. In the study carried out by Shrestha et al. in 2019, Ampicillin showed highest resistance i.e. 89%, whereas Imipenem was sensitive by 85%, Nitrofurantion by 95% and Colistin showed 100% of sensitivity[25]. Likewise, in the study carried out by Parajuli et al. in 2017 Ampicillin, Cefotaxim, Cefepime were 100% resistance, whereas Imipenemwas sensitive by 80.7% and Colistin and Polymyxin B were 100% sensitive[26]. All the above mentioned study has almost similar resistance pattern with our study.

Multi drug resistance (MDR) is characterized as acquired non susceptibility to at least one antimicrobial agent in three or more categories[15]. The emergence of MDR organisms restricts the choices for therapy for hospital acquired infections[27]. In a study conducted by Baral et al., out of the 178 *E. coli* isolates, 38.2% were confirmed to be multidrug resistant[28]. However, our study showed higher percentage of MDR as compared i.e. 59.55%. In a study conducted by Parajuli et al. 64.9% were multidrug resistant, which is higher as compared to our study[29].

ESBLs are a group of plasmid mediated β-lactamase enzymes, that are capable of hydrolyzing and inactivating broad spectrum antibiotics such as Cephalosporin, Penicillin and Monobactams by

splitting their amide ring but cannot hydrolyze cephamycin and carbapenems[30]. In our study 52.9% of isolates were positive for ESBL screening however only 25.70% were confirmed for ESBL production by CDT method. Similar results were observed in the study conducted by Rezai et al. (30.5%) in Iran and Yadav et al. (26.87%) in Nepal[31, 32]. However, a low rate of ESBL producing *E. coli* were seen in developed countries such as 9.3% from USA[33] and 10.2% from Korea[34]. These variations in the rate of production of ESBL may be due to geographical difference, local antibiotic prescription policy, the extensive use of wide spectrum antibiotics particularly third generation Cephalosporins and endemicity of drug resistance pathogens in the community[29] .

Carbapenems are the preferred antibiotics for the treatment of infections caused by ESBL producers. However, carbapenem resistance in *E. coli* due to high consumption of carbapenem or due to the co-selection by other antibiotics is causing the serious problems and complicating the treatment[35]. In our study 11.80% were MBL producers. Our findings were similar to 15% documented by Ansari et.al from Nepal[36]. However, a study conducted by Bora et al. in Nepal showed comparatively higher prevalence of MBL producing *E. coli* i.e. 18.8% [37].

In our study, 19.11% were biofilm producers. In a study conducted in Nepal (Soma Kanta Baral, 2022) among coagulase negative staphylococci also found 37.8% biofilm producer[38] and in Iran by Tajbakhsh et al. among 130 *E. coli* isolates, 80 (61.53 %) were able to make biofilm which is higher than that reported in our study[39]. Among total biofilm producers most were multidrug resistant i.e. 61.5%. Unlike our study, higher multidrug resistance (87.5%) was seen among biofilm producers in the study conducted by Chaudhary et al., 2019[40]. The high resistance exhibited by biofilm producers could be due to the activity of exo-polysaccharide matrix which delays the antibiotics penetration in biofilm matrix, decreased growth rate and expression of resistance genes. Microorganisms mostly produce biofilm in order to survive in unfavorable conditions and makes treatment difficult because only selected antibiotics can inhibit the growth of biofilm producers[41]. Similarly, in our study among biofilm producing *E. coli*, higher antibiotic resistance was observed among Cephalexin (84.6%) and Amoxicillin (80.8%) while Imipenem (7.7%), Chloramphenicol (3.8%), and Amikacin (3.8%) showed least resistance. Similar findings were reported in a study conducted by Neupane et al. in Nepal, where biofilm producing *E. coli* were mostly resistant to

Amoxicillin (89.4%) followed by Cephalexin (84.1%) and mostly sensitive to Amikacin (87.5%) [42].

Like biofilm, serum resistance property exhibited by microorganisms has been critical for their survival and establishments of disease but several mutations in bacteria might result in loss of serum resistance making several bacterial pathogens avirulent[43]. In our study, 22.8% of isolates were resistant to serum, which is lower than that reported by Brugubalda et al. where 51% of the isolates were resistant to serum bactericidal activity[22].

In our study, biofilm producers were mostly non- beta lactamase type. In contrast, in a study conducted by Dumaru et al., biofilm production was high among ESBL and MBL producers [44]. Our study showed that ESBL producers were mostly serum sensitive. The result was matching with the previous study reported by Brugubalda et al where ESBL negative strains of *E. coli* produced multiple virulence factors[22]. There are also other studies in which antimicrobial resistant isolates were less virulent. The mechanism behind such trend is not well understood but they are supposed to do so because of causal relationships between resistance and virulence (For instance, acquisition of resistance leads to gain or loss of virulence factors)[45] or result from confounding by co-associated factors[46].

CONCLUSION

According to this study, the most prevalent *E. coli* infection is urinary tract infection. The expression of *E. coli* virulence factors varied depending on the kind of infection. Despite the fact that *E. coli* did not produce beta lactamase, it was still capable of expressing virulence factors. *E. coli* has a high rate of multidrug resistance. To minimize the emergence and spread of antibiotic resistance in bacteria, proper detection of drug-resistant bacteria, careful use of antibiotics, and effective antibiotic policy are still required.

CONFLICT OF INTEREST

Authors declared, there is no conflict of interest

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