ORIGINAL ARTICLE

CHARACTERIZATION OF VIRULENCE FACTORS IN MULTIDRUG-RESISTANT ESCHERICHIA COLI ISOLATED FROM INTESTINAL AND EXTRA - INTESTINAL CLINICAL SAMPLES

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ABSTRACT

Introduction: The global issue of antimicrobial resistance to human health is becoming increasingly concerning. *Escherichia coli (E. coli)* is a widespread pathogen that causes a wide range of illnesses. This is because multidrug-resistant (MDR) *E. coli* bacteria have emerged, proliferated, and remained persistent. Antimicrobial resistance is more predominant among pathogenic organisms compared to the commensal ones. The correlation between resistance and virulence factors could be a result of the successive exposure of pathogenic organisms to antibiotics. Therefore, the goal of our study was to detect some virulence factors in multidrug-resistant *E. coli* that were isolated from fecal and other clinical samples.

Methods: A laboratory-based cross-sectional study was conducted from October 2023 to March 2024, a period of six months. E. coli was isolated using standard microbiological methods from a range of clinical samples. Antibiotic susceptibility was done to identify MDR *E. coli* by Kirby-Bauer disk diffusion method. Different phenotypic assays were used to detect virulence factors.

Results: Out of 318 isolated *E. coli* from different clinical samples, 160 (50.3%) were found to be multidrug-resistant. Higher distribution of MDR *E. coli* (51.9%) was found in extra-intestinal samples than fecal specimen. MDR *E. coli* strains were highly resistant to most antimicrobials. The most common virulence factor was cell surface hydrophobicity (100%) followed by motility (83.7%), biofilm production (36.2%), serum resistance (25%),hemolysin production (15%) and gelatinase production (2.5%). There were multiple virulence factors found in 156 (97.5%) MDR *E. coli* isolates.

Conclusion: It was discovered that MDR *E. coli* strains obtained from patient intestinal and extra-intestinal samples were more virulent and more resistant to drugs.

Keywords: ***Virulence factors, motility, hemolysin production, cell surface hydrophobicity, serum resistance, biofilm production, gelatinase production***

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BACKGROUND

Escherichia coli, a member of the Enterobacteriaceae bacterial family, despite its ubiquity as a commensal, can lead to various intestinal as well as extra-intestinal infectio[1]. Antimicrobial resistance (AMR) poses a serious global threat of growing concern to human, animal, and environment health. This is due to the emergence, spread, and persistence of MDR bacteria[2]. This growing prevalence of MDR *E. coli* poses escalating challenges in infection treatment, as it limits the available choices for effective treatment options. The global incidence of MDR *E. coli* has consistently been on the rise[3].

AMR in E. coli has been reported worldwide. In 2014, the World Health Organization (WHO) stated five out of the six regions reported >50% resistance to third generation cephalosporin by E. coli[4]. A research conducted in patients of a tertiary care teaching hospital of Nepal, 59.6% of the 136 E. coli isolates were multidrug resistant[5]. Another study showed 64.9% were MDR among 739 *E. coli* isolates in pediatric patients[6].

Escherichia coli can cause infections due to its ability to the acquisition of mobile genetic factors carrying many virulence genes[7]. Adhesions, iron uptake, toxins, flagella and capsules are the most common virulence factors responsible for attachment, adherence and invasion in the host and then lead to infection[8]. Genetic virulence factors can regulate physical attributes of the bacteria such as flagella, fimbriae, adhesions, biofilm, or biochemical factors, including host cell surface modifying enzymes, toxins, and antibiotics to provide a competitive advantage[9].

A study conducted by Sharma et al., 2007 showed a majority (68.7%) of ESBL negative strains of *E. coli* produced multiple virulence factors whereas most of the ESBL producers (68.2%) did not produce multiple virulence factors[10]. Since genetically encoded antibiotic resistance promotes host pathogenesis, enabling chronic or persistent illnesses, it can be viewed as a subtype of virulence factors in many respects[9].

Since antibiotic resistance is frequently linked to infection, it is also linked to virulence, as is the case with intracellular infections or microbes that form biofilm. The direct participation of efflux pumps, porins, cell wall modifications, and two-component systems that either activate or repress the expression of different genes, including those involved in resistance and virulence, are further traits shared by virulence and resistance[11].

Notably, distinct *E. coli* isolates with extra-intestinal and enteric pathotypes have similar virulence determinants and tactics[12]. In recent years, numerous virulence factors linked to enteric *E. coli* pathotypes implicated in intestinal and extra-intestinal illnesses have been discovered[13]. In a number of animal infection models, virulence has now been directly linked to multidrug resistance[8]. Pathogenic organisms are more likely than commensal species

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to be resistant to antibiotics. The relationship between virulence factors and resistance may be the consequence of pathogenic organisms being exposed to drugs one after the other[1]. Therefore, the aim of this study was to determine the virulence factors associated with their pathogenicity in MDR *E. coli* isolated from extra-intestinal and intestinal specimen.

MATERIALS AND METHODS

A cross-sectional study was carried out in a laboratory setting with patients who came to the Manmohan Memorial Teaching Hospital in Kathmandu, Nepal. Clinical samples that satisfied the American Society for Microbiology's (ASM) established standards for pus, sputum, urine, and stool were chosen for additional processing and analysis; those that did not were disqualified.

Isolation and Identification of *E. coli*

Standard technique[14] was used to inoculate a variety of clinical samples onto different culture media. Following a 24-hour aerobic incubation period at 370C, *E. coli* is identified by analyzing colony shape, gram staining, and other biochemical assays.

Identification of MDR *E. coli*

The antibiotic sensitivity testing of identified *E. coli* was performed by a modified Kirby- Bauer disk diffusion method on Mueller Hinton Agar using standard methods recommended by CLSI guidelines[15] . The antibiotics tested were: Amoxycillin (25µg), Ceftazidime (30µg), Cefotaxime (30µg), Cefixime (5µg), Cefepime (30µg), Aztreonam (30µg), Imipenem (10µg), Meropenem (10µg), Tetracycline (30µg), Gentamycin (30µg), Amikacin (30µg), Polymyxin-B (5µg), Cefoxitin (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Cotrimoxazole (25µg), Ceftazidime + Clavulanic acid (20µg+10µg). MDR E. coli were categorized as per the definition given by A-P Magiorakos et al., 2012[16] .

Detection of Virulence Factors

Motility Testing

A loopful of fresh liquid culture suspended in peptone water was placed in the center of the coverslip, and a small amount of Vaseline was applied to the four corners of the coverslip to create the hanging of drop. In order to make touch with the Vaseline but not the culture droplet, a depression slide (also known as a deep-weel slide) was finally placed over the coverslip. The droplet of culture hung beneath the coverslip as soon as the preparation was inverted. Thereafter, examined under a microscope [17,18].

Hemolysin Production

Haemolysin generated by MDR *E. coli* was detected using the plate hemolysis assay. The bacteria were cultured at 37 °C for the overnight after being streaked onto 5% sheep blood agar. Strains that produced a zone of partial or complete lysis of the erythrocytes around the colony of the medium were regarded as positives[19].

Cell Surface Hydrophobicity

The salt aggregation test (SAT), as described by Siegfried L. et al.[20] and Raksha R. et al.[21] , was used to assess the hydrophobicity of the MDR E. coli cell surface. On a glass slide, one loopful (10 µl) of bacterial suspension prepared in phosphate buffer was mixed with an equivalent volume of ammonium sulphate solution of varying molarity, i.e., from 0.3125 M to 5.0 M, and rotated for one minute. In the SAT, the highest dilution of ammonium sulphate solution that resulted in observable bacterial clumping was scored. In 0.002 M phosphate buffer alone (pH 6.8), strains exhibiting aggregation were deemed auto-aggregative. MDR *E. coli* strains were considered hydrophobic if their SAT value was equal to or less than 1.25 M.

Biofilm Production

The test was carried out using a polystyrene tissue culture plate (96 wells) with a flat bottom, slightly altering the procedure as outlined by Mathur et al. (2006)[22] . This procedure involved inoculating two milliliters of Luria Bertani broth with a fresh culture of the MDR *E. coli*. Then, Luria Bertani broth was incubated at 37°C for 24 hours. The growth from the broth was diluted in a ratio of 1:100 with fresh medium and 200µl of the diluted culture inoculated in the sterile wells of tissue culture plate. Broth that had been uninoculated was regarded as a negative control. The plates were incubated at 37°C for 24 hours. Then, the content from well was removed and washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. After being fixed for an hour at 60°C, the bacterial biofilm was dyed with 2% crystal violet reagent.

After rinsing three times with deionized water to remove excess stain, 30% acetic acid was used to decolorize the area. The ELISA auto-reader was used to determine the optical density (OD) of the stained biofilm at 570 nm. The biofilm production was interpreted using the standards provided by Stepanovic et al. (2000)[23] . Three standard deviations above the mean OD of the negative control was then used to define OD.

Serum Resistance

Fresh cultures of the isolates were used to investigate serum resistance as described by Sharma at al. (2007)[10]. After being cultivated overnight on blood agar at 37°C, MDR *E. coli* was harvested and suspended in Hanks balanced salt solution (HBSS). Pooled serum (0.05 ml) and bacterial suspension (0.05 ml) were then incubated for 180 minutes at 37°C. After removing ten microliters of samples and spreading them out on blood agar plates, the viable count was calculated after 18 hours of incubation at 37°C. Bacterial resistance to serum bactericidal activity was measured as the proportion of bacteria that survived after 180 minutes of serum incubation compared to the initial count. Bacteria were classified as resistant if more than 90% of organisms survived after 180 minutes and serum sensitive if the viable count fell to 1% of its starting value.

Gelatinase Production

Gelatin agar was used to test the production of gelatinase enzyme. MDR *E. coli* was inoculated to the gelatin agar plate, and it was then incubated for 24 hours at 37°C. After that, a solution of mercuric chloride was poured onto the plate. The formation of opacity in the medium and the clearing zone surrounding colonies was regarded as a sign that gelatinase was being produced[24] .

Ethical Consideration

Ethical permission was taken from Nepal Health Co-operative Limited, Institutional Review Committee (IRC), Kathmandu (number: NEHCO-IRC 080/076-13/10/2023). Prior to sample collection, informed written consent was obtained from each participant after they had been told of the study's objectives.

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 and percentage.

RESULTS

Distribution of MDR *E. coli*

E. coli growth was observed in 318 (66.6%) of the 477 total specimens. Of these, 160 isolates (43.3%) were identified as MDR (*Table 1*).

Antibiogram Profile of MDR E. coli Isolates

Amoxycillin had the highest degree of resistance (97.5%), followed by third-generation cephalosporins. Likewise, imipenem had the lowest resistance rate (11.2%), followed by meropenem (13.7%), amikacin (15%), and gentamycin (17.5%) in that order (*Table 2*).

Phenotypic Characterization of Virulence Factors in MDR *E. coli*

Virulence parameters such motility, haemolysin, surface hydrophobicity, serum resistance, and gelatinase were examined. For every isolated MDR *E. coli*, the cell surface was hydrophobic. Only 15% of isolates could make hemolysin, although 83.7% were motile. More motility (87.5%) and less hemolysin production (5%) were observed in fecal MDR *E. coli* compared to extra intestinal MDR *E. coli* (25%), as shown in Table 3a. Thirty percent of the isolates were resistant to serum, and only 36.2% of the isolates were observed to produce biofilm. Gelatinase enzyme (2.5%) was determined to be the least expressed virulence factor by MDR *E. coli*. Fecal MDR *E. coli* strains had greater distributions of serum resistance (30%) and gelatinase production (5%), while extra intestinal MDR E. coli strains produced more biofilm (52.5%) (*Table 3b).*

Table 1: Distribution of MDR E. coli Isolates

Samples	Number	E. coli isolated (%)	MDR E. coli (%)
Extra- intestinal (Urine, Pus and Sputum)	238	154 (64.7)	80 (51.9)
Intestinal (Fecal)	244	164 (67.2)	80 (48.7)
Total	477	318 (66.6)	160 (50.3)

Table 2: Resistant Profile of Antibiogram against MDR E. coli Isolates

Antibiotics	MDR E. coli (n=160)		Total Resistant n(%)
	Extra-intestinal Resistant, n (%)	Intestinal Resistant, n (%)	
Amoxycillin	(100) 80	76 (97.5)	156 (97.5)
Cefixime	78 (97.5)	56 (70)	134 (83.7)
Ceftazidime	68 (85)	66 (82.5)	134 (83.7)
Cefotaxime	64 (80)	68 (85)	132 (64.4)
Cefepime	58 (72.5)	60 (75)	118 (58.3)
Aztreonam	42 (52.5)	50 (62.5)	92 (57.5)
Polymyxin B	12 (15)	48 (60)	60 (37.5)
Cotrimoxazole	52 (65)	46 (57.5)	98 (61.2)
Ciprofloxacin	52 (65)	54 (67.5)	106 (66.2)
Levofloxacin	54 (67.5)	28 (35)	82 (51.2)
Gentamicin	24 (30)	4 (5)	28 (17.5)
Tetracycline	54 (67.5)	54 (67.5)	108 (67.5)
Amikacin	8 (10)	16(20)	24(15)
Meropenem	10 (12.5)	12 (15)	22 (13.7)
Imipenem	12 (15)	6 (7.5)	18(11.2)

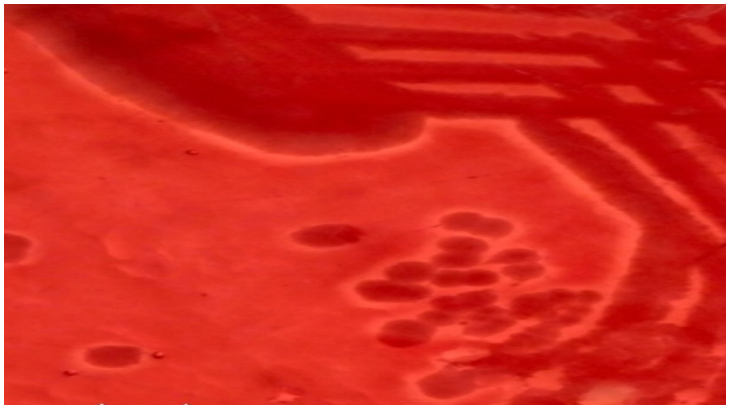


Figure 1: Hemolysin Production by MDR E. coli on Blood Agar Plate

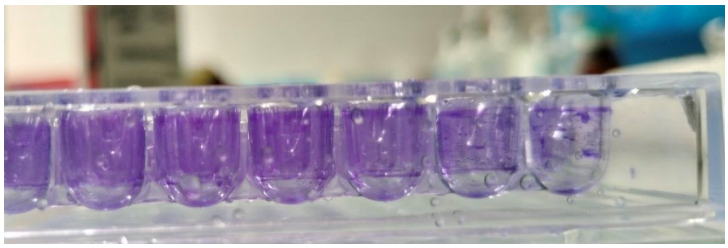
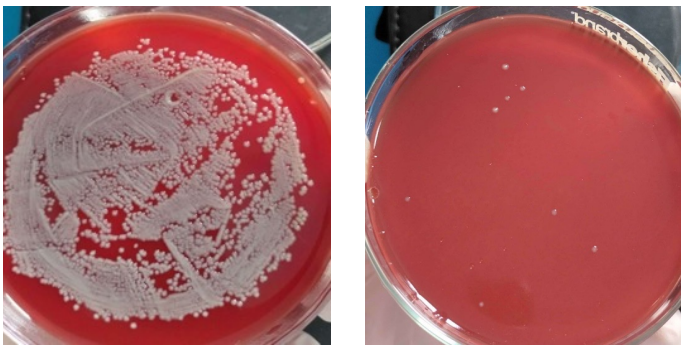


Figure 2: Biofilm Production by MDR E. coli



Initial Growth (without treating with serum) *Growth after treating with serum*

Figure 3: Serum Killing Assay, Showing Serum Sensitive MDR E. coli

Table 3a: Phenotypic Characterization of Virulence Factors of MDR E. coli

MDR E. coli (n)	Motility Test		Hemolysin Production		Cell Surface Hydrophobicity	
	Motile n (%)	Non-motile n (%)	Positive n(%)	Negative n(%)	Positive n(%)	Negative n(%)
Extra- intestinal (80)	64 (80)	16 (20)	20 (25)	60 (75)	80 (100)	0 (0)
Intestinal (80)	70 (87.5)	10 (12.5)	4 (5)	76 (95)	80 (100)	0 (0)
Total (160)	134(83.7)	26(16.3)	24 (15)	136 (85)	160 (100)	0 (0)

Table 3b: Phenotypic Characterization of Virulence Factors of MDR E. coli

MDR E. coli (n)	Motility Test		Hemolysin Production		Cell Surface Hydrophobicity	
	Motile n (%)	Non-motile n (%)	Positive n(%)	Negative n(%)	Positive n(%)	Negative n(%)
Extra- intestinal (80)	64 (80)	16 (20)	20 (25)	60 (75)	80 (100)	0 (0)
Intestinal (80)	70 (87.5)	10 (12.5)	4 (5)	76 (95)	80 (100)	0 (0)
Total (160)	134(83.7)	26(16.3)	24 (15)	136 (85)	160 (100)	0 (0)

DISCUSSION

In the past two decades, acquired MDR infections have increased due to the production of β-lactamases, leading to third generation cephalosporin and carbapenem resistance [25]. Our research revealed a 50.3% MDR prevalence among isolated *E. coli* from intestinal and extra-intestinal samples. *E. coli* has a wide range of antibiotic resistances. The MDR E. coli strains discovered in this investigation concur with other results [26,27]. The outcome is low from what was predicted by earlier research by Baral et al.[5], Parajuli et al.[28], Yadav et al.[28] and Pandit et al.[30] in this case. In a study conducted by Baral et al. showed 38.2% multidrug resistant uropathogenic E. coli. Diverse study time periods, different sample sources of isolates, and various study areas could all be contributing factors to the variations in results.

Patients who develop MDR *E. coli* infections are need to take more broad-spectrum antibiotics, stay in the hospital longer, spend more time in the critical care units, and have arterial or urinary catheterization. These resistant isolates can be challenging to identify and treat . Our study showed that MDR E. coli strains were highly resistant to most antimicrobials where amoxycillin had the highest degree of resistance (97.5%), followed by third-generation cephalosporins. Likewise, carbapenems and aminoglycosides were found to be more effective against isolated MDR *E. coli*. Almost similar effectiveness was observed by researchers in isolated *E. coli* [5, 28, 29].

The capacity of MDR *E. coli* to produce many virulence factors contributes to its pathogenicity. The pathogenicity of *E. coli* is exacerbated by the production of virulence factors, particularly since the majority of them are multidrug resistant, making therapy difficult[33]. In our study, virulence factors such motility, haemolysin, surface hydrophobicity, serum resistance, and gelatinase were included where MDR *E. coli* displayed a high diversity of virulence factors profiles. For E. coli to migrate toward host cells, motility mediated by the flagella is essential[34] . It was discovered that 83.7% of the 160 MDR *E. coli* isolates were motile. This study shows that compared to extra-intestinal samples, fecal MDR *E. coli* isolates had a higher incidence of higher-motility strains. Rana et al.1 reported only 68% motile MDR *E. coli* isolates from different clinical sources in Egypt. Peptone and other complex media have been employed extensively in the research of motility because they allow for great movement, according to Adler & Templeton. But because glucose chelates very poorly at neutral pH due to the action of a chelating agent, motility is extremely vulnerable to inhibition by tiny levels of heavy metal ions, and amino acids are good chelating agents for metal ions[35].

The ability of micro-organisms to attach to a wide range of surfaces is influenced by the hydrophobic interaction . All of the MDR E. coli isolates in our investigation were hydrophobic. This was higher than the findings of other investigations, which showed that 27.6%10, 26.4%21, 46%20, and 55% of the *E. coli* isolates were hydrophobic, respectively. The greater levels of antibiotic-resistant *E. coli* isolates in our results could be the cause of the absolute surface hydrophobicity.

The pathogenicity of *E. coli*, particularly the more severe forms of infection, is linked to the formation of hemolysin . Only 15% of the MDR *E. coli* strains used in this investigation generated haemolysin and found more common among the isolates from extra – intestinal samples. Approximately 50% of *E. coli* isolates that cause extra-intestinal infections have been reported to produce more hemolysin than our findings in earlier studies[21,39,40,41] Sharma et al.[10] found a somewhat higher hemolysin production rate (23.7%). Our result is comparable to that of earlier study carried out in Iraq .

Serum resistance is the ability of bacteria to withstand being killed by normal human serum because of the lytic activity of the complement system's alternative pathway. Thirty percent of the isolates in our study exhibited serum resistance. However, several earlier studies revealed higher serum resistance rates, such as 61.37% , 68% 20, and 86.7% [10]. Compared to our findings with clinically isolated E. coli, a study by Baral et al.[5] revealed a reduced distribution (22.8%) of serum activity. However, a similar result (32.7%) was found by Raksha et al.[21]in extra-intestinal *E. coli* isolates.

One of the key virulence factors is biofilm. When compared to bacteria without this virulence factor, it is crucial in shielding them from exposure to antibiotics[44] . Isolates that formed biofilms demonstrated a greater level of antibiotic resistance than their counterparts that did not. But, in our investigation, biofilm production was seen in only 36.2% of the MDR *E. coli* isolates. Researchers Fattahi et al.[46] and Karam et al.[45] found that biofilm development was higher in uropathogenic *E. coli*, with a higher rate of biofilm formation (92%) and (85%), respectively. However, in a prior investigation, biofilm production was reported to be 19.11% lower in E. coli isolates[5]. A study conducted in Nepal indicated that 37.8% of coagulase-negative staphylococci generated biofilm[47] , which was similar to our results.

An essential virulence factor linked to inflammation is gelatinase, which hydrolyzes collagen, gelatin, and other bioactive peptides. The virulence factor that MDR *E. coli* expressed the least in our experiment was gelatinase (2.5%). This was lower than the findings of the earlier research by Shruthi N et al.[43] (19.4%), Johnson et al.[38] (7%) and Sharma et al.[10] (6.9%). In the investigation by Shah et al.[48] and Kaira et al.[49] on uropathogenic E. coli isolates, none of the isolates generated gelatinase,. Our result was comparable to the EL-Mosallamy et al.[50] finding (2%) in uropathogenic *E. coli.*

It was discovered that each isolate found to be hydrophobic. This led to the discovery of multiple virulence factors in almost all of the MDR E. coli strains in our investigation. The majority of the isolates that produced hemolysin were also discovered to be serum resistant (80%) and hydrophobic (100%) in nature. The results[51, 10] of earlier research are almost in line with this. Our study found that 20% of the isolates had a combination of all three virulence factors, including haemolysin, surface hydrophobicity, and serum resistance. This was greater than the 11.2% of isolates reported by Sharma et al. (2007)[10]. According to Fakruddin et al. (2013)[6] the expression of several virulence factors works well together to overcome the host's natural defenses.

CONCLUSION

In conclusion, our study highlights the pervasive presence of multiple virulence factors in multidrug-resistant *Escherichia coli* strains derived from both intestinal and extra-intestinal clinical samples. These findings emphasize the critical importance of using antibiotics judiciously. Failure to do so not only contributes to the emergence of more drug-resistant strains but also enhances their virulence and pathogenic potential. To combat the growing threat of MDR *E. coli*, it is imperative to adopt responsible antibiotic stewardship practices.

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CONFLICT OF INTEREST

Authors declared, there is no conflict of interest.