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Original Article

Extended spectrum, AmpC & metallo β-lactamases producing *Escherichia coli* in urinary isolates: A prospective study in north India

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***Background & objectives*: One of the most prevalent bacterial illnesses in humans is the urinary tract infection (UTI), which is frequently brought on by *Escherichia coli*. The purpose of this study was to assess the antibiotic sensitivity pattern of *E. coli* that causes UTIs and identify the presence of extended spectrum β-lactamases (ESBL), AmpC β-lactamases (AmpC), and metallo β-lactamases (MBL) using a variety of phenotypic techniques.**

***Methods*: After urine samples were inoculated on cysteine lactose-deficient agar culture media, isolated colonies were identified using standard biochemical tests. These isolates were then screened using different phenotypic confirmatory methods for β-lactamase detection and the Clinical Laboratory and Standards Institute (CLSI) guidelines.**

***Results*: Out of the total urine samples, 7.08 per cent (177/2500) were positive for the growth of *E. coli,* out of which 40.11 per cent (71/177) were multi-drug resistant. Among the 71 isolates, 31 per cent were ESBL producers, 62 per cent were AmpC producers, and 7.04 and 4.22 per cent were co-producers of ESBL and AmpC, and AmpC and MBL, respectively. The *E. coli* isolates were found to be highly resistant to ciprofloxacin (83.61%), amoxicillin/clavulanate (81.92%), and gentamicin (61%). However, these were sensitive to imipenem (98.3%), fosfomycin (97.17%), and nitrofurantoin (94.35%).**

***Interpretation & conclusions*: Early detection of various resistance patterns as well as understanding of the local susceptibility patterns among *E. coli* strains causing UTIs, are imperative for accurate treatment modalities. This knowledge would subsequently contribute to the management of antibiotic resistance and surveillance.**

Key words AmpC - β-lactamases - *E. coli* - ESBL - MBL - resistance - urinary tract infection

Urinary tract infections (UTIs) make up 40 per cent of healthcare-associated and 50 per cent of bacterial infections that extend hospitalisation and increase morbidity[1]. Beta-lactam antibiotics, which are a class of drugs that contain a -lactam ring in their chemical structure, are used for the treatment of UTIs[2]. However, in recent years, antimicrobial-resistant *Escherichia coli* (*E. coli*) strains, among other *Enterobacterales*, have

emerged and spread worldwide, even showing resistance to third-generation cephalosporins in our country[3].

To withstand the harmful effects of antibiotics, bacteria employ a variety of strategies. The main mechanism is the bacterial enzyme β-lactamase, which hydrolyses the β-lactam ring of the antibiotic and renders it inactive[3]. There is a significant clinical risk associated with the fast global spread of *E.coli* that harbours plasmid-borne extended spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases (AmpC), and metallo β-lactamases (MBL)[3]. Except for cephamycins and carbapenems, ESBLs are β-lactamases that provide bacterial resistance to penicillins, cephalosporins (1st, 2nd and 3rd generation), and aztreonam. However, these are inhibited by β-lactamase inhibitors such as clavulanic acid[3]. Although some extended-spectrum cephalosporins may show susceptibility to ESBL-producing bacteria *in vitro*, using these antibiotics to treat infections brought on by ESBL-producing strains may result in treatment failure. Similarly, organisms that manufacture AmpC β-lactamases are resistant to cephamycins and cephalosporins (narrow, broad, as well as expanded-spectrum) and are not susceptible to inhibition by clavulanate, sulbactam, and tazobactam[4]. Additionally, MBL synthesis is responsible for the gradual spread of carbapenem resistance among clinical isolates of *E. coli*[2].

The detection of β-lactamases in *E. coli* is a significant obstacle in microbiological laboratories, particularly in low-resource settings and countries. Consequently, feasible and reliable phenotypic confirmatory approaches have the potential to offer substantial contributions. Early identification of isolates that produce β-lactamase may help lower patient mortality and treatment duration, as well as prevent the spread of these infections inside hospitals. Determining the yield of several phenotypic techniques for the detection of ESBL, AmpC, and MBL in *E. coli* isolated from urine samples in an environment with limited resources was, thus, the primary goal of this work. Evaluation of the antibiotic susceptibility profile of multi-drug resistant (MDR) *E. coli* in the region was another goal of the study.

Materials & Methods

This study was undertaken by the department of Microbiology, Shaheed Hasan Khan Mewati Government Medical College (SHKM GMC), Nuh, Haryana after obtaining approval of the Institutional Ethics Committee. Informed consent was obtained, and data confidentiality was maintained.

In this observational prospective study, 177 *E. coli* isolates were collected from 2500 midstream clean catch urine samples in sterile universal containers, which were received in the microbiology laboratory of SHKM GMC, Nuh, Haryana, India. The study lasted for one year, from February 2020 to January 2021, and included all inpatient department (IPD) and outpatient department (OPD) patients of who were advised to have a urinary examination, due to suspicion of UTIs. Individuals who refused to participate and samples that were repeated were excluded.

Isolation and identification: The urine samples were subjected to a macroscopic examination and wet mount preparation before semi-quantitative culture plating on cysteine lactose-deficient agar (CLED) and overnight incubation at 37°C. Colony morphology was assessed on plating media, and isolated bacteria were identified. *E. coli* was identified using Gram staining, catalase, oxidase, hanging drop motility, and standard biochemical tests like indole, methyl red, Voges-Proskauer, citrate utilisation test (IMViC), triple sugar iron (TSI), urease, mannitol motility, and nitrate reduction[4,5]. All culture media and consumables were purchased from HiMedia Laboratories, Mumbai, India.

Antimicrobial susceptibility testing (AST): Kirby Bauer disc diffusion on Mueller Hinton Agar (MHA) was used to test the antimicrobial susceptibility of *E. coli* isolates in accordance with CLSI standards. As a control, *E. coli* ATCC 25922 strain from the American Type Culture Collection (ATCC) was used. Amoxycillin-clavulanate (20/10 μg), cefazolin (30 μg), ceftazidime (30 μg), cefoxitin (30 μg), cefotaxime (30μg), co-trimoxazole (1.25/23.75 μg), piperacillin-tazobactam (100/10 μg), gentamicin (10 μg), ciprofloxacin (5μg), aztreonam (30 μg), imipenem (10 μg), nitrofurantoin (300 μg), and fosfomycin (200 μg) were used in the antimicrobial susceptibility tests. The plates were incubated through the night at 37°C. The inhibition zone diameter was measured and categorized into sensitive, moderate, or resistant categories in accordance with CLSI 2020 guidelines[6,7].

Screening: Isolates showing reduced susceptibility to ceftazidime (zone diameter ≤14), cefoxitin (zone diameter ≤14), or imipenem (zone diameter ≤19) were further tested for confirmation of ESBL, AmpC beta lactamases, and MBL production[6].

Phenotypic confirmatory methods for detection of ESBL:

Double disc diffusion method: The study used ESBL positive (ATCC 700603) *Klebsiella pneumoniae* and *E. coli* ATCC 25922 (ESBL negative) as controls. The test isolate's standard inoculum (0.5 McFarland) was applied to MHA. After placing the ceftazidime (30 µg) and clavulanic acid (30 µg/10 µg) discs 20 mm apart, they were incubated for the entire night at 35ºC±2ºC. ESBL generation was indicated by an increase of at least 5 mm in the inhibition zone width around the ceftazidime + clavulanic acid disc when compared to the ceftazidime disc alone[6-9].

ESBL E-Test strip: The ESBL E-test was performed as per the manufacturer's instructions (Himedia Laboratories Pvt. Ltd.). Minimum inhibitory concentration (MIC) values were noted for ceftazidime plus clavulanic acid and ceftazidime alone, where the margin of the eclipse cut the strip. ESBL production was ascertained when the ratio of ceftazidime to ceftazidime plus clavulanic acid was ≥8, as illustrated in supplementary figure 1, or when no zone was obtained for ceftazidime and a zone was obtained for ceftazidime plus clavulanic acid. The results were considered inconclusive when no zone of inhibition was observed on either side[8,9].

Phenotypic confirmatory methods for detection of AmpC ß-Lactamases:

AmpC disc test: The AmpC disc test was performed as described by Chanu *et al*[10]. A 0.5 McFarland suspension of *E. coli* ATCC 25922 (lawn and negative control) was inoculated on the MHA[11]. A sterile or a Tris-EDTA disc (6 mm) was rehydrated with 20 µl of saline, and colonies from a pure culture were added. A 30 µg cefoxitin disc was placed on the MHA's inoculated surface with AmpC disc nearby. The plate was inverted and incubated at 35°C overnight. Indentation or flattening of the cefoxitin inhibition zone near the test disc indicated AmpC β-lactamase generation (Supplementary Fig. 2)[7,10,12].

Cefoxitin-Cloxacillin disc diffusion test: This test was conducted in accordance with the methodology explained by Peter-Getzlaff *et al*[11]. Cefoxitin (30 µg) and cloxacillin (200 µg) discs were placed 20 mm apart on a lawn culture of the test isolate on an MHA plate and incubated overnight at 37°C. An AmpC β-lactamase producer was identified when the zone diameter around the cefoxitin plus cloxacillin disc was ≥4 mm larger than that around the disc alone (as depicted in Supplementary Fig. 3)[12-14].

Phenotypic confirmatory methods for detection of metallo ß-lactamases:

Combined disc synergy test with 0.1 M ethylene diamine tetra acetic acid (CDST): The combined and double-disc synergy tests were performed as stated by Panchal *et al*[15] *Pseudomonas aeruginosa* ATCC 27853 was used as a negative control. A standard inoculum of the test isolate was inoculated on MHA, followed by two imipenem (10 µg) discs placed 30 mm apart. To achieve the necessary concentration of 292 µg, 10 µl of 0.1M EDTA solution was added to one imipenem disc. The inoculation plates were then incubated overnight at 37°C. The inhibition zone of imipenem alone and with EDTA was measured. MBL production was considered positive if the zone diameter of imipenem plus EDTA was ≥7 mm larger than that of imipenem alone (Supplementary Fig. 4)[15,16].

Double-disc synergy test with 0.1 M ethylene diamine tetra acetic acid (DDST): Imipenem disc (10 µg) and sterile blank disc were put 20 mm from edge to edge after inoculating MHA with 0.5 McFarland of the test isolate. 10 µml of 0.1M (292 µg) EDTA was applied to the blank disc and incubated. MBL producers would have an enhanced zone of inhibition between the imipenem disc and the EDTA disc (Supplementary Fig. 5)[15,16].

Statistical analysis: The data was compiled and subsequently tabulated into a Microsoft Excel spreadsheet. The analysis was performed using descriptive statistics and presented in the form of figures and tables.

Results

Multi-drug resistance in the bacterial isolates: Out of a total of 2500 urine samples that were received in the laboratory, 177 (7.08%) samples were confirmed to have *E. coli*. There were 71 MDR (40.11%) isolates of *E. coli*, whose resistance patterns to antibiotic classes are shown in table I. A male-to-female ratio of 23:48 indicated that the majority of the resistant isolates came from urine samples of females.

Table I. Antibiotic susceptibility pattern of the *E. coli* isolates by Kirby Bauer disc diffusion method

Antibiotics	Sensitive (n=177), n (%)	Resistant (n=177), n (%)
Amoxicillin-clavulanate	32 (18)	145 (81.92)
Cefazolin	36 (20.33)	141 (79.66)
Ceftazidime	106 (59.88)	71 (40.11)
Cefotaxime	88 (49.71)	89 (50.28)
Cefoxitin	133 (75.14)	44 (24.85)
Piperacillin-tazobactam	139 (78.53)	38 (21.46)
Aztreonem	94 (53.1)	83 (46.89)
Imipenem	174 (98.3)	3 (1.69)
Gentamicin	69 (38.98)	108 (61)
Co-trimoxazole	109 (61.58)	68 (38.41)
Ciprofloxacin	29 (16.38)	148 (83.61)
Nitrofurantoin	167 (94.35)	10 (5.64)
Fosfomycin	172 (97.17)	5 (2.82)

Beta-lactamase detection by various phenotypic methods: The MDR profile of the *E. coli* strains was slightly higher in the specimens of outdoor) patients (OPD) (50.7%) than that of the indoor patients (IPD) (49.29%). Out of the 71 MDR *E. coli* isolates, 22 (31%) were ESBL producers, as detected by the double-disc diffusion method. Of these 22 ESBL-positive isolates, 7 (32%) were from the IPD, and 15 (68%) were from the OPD. Out of the same 71 MDR isolates, 44 (62%) were positive for AmpC production, of which there were 27 (61.4%) IPD samples and 17 (38.6%) OPD samples. There was a co-occurrence of ESBL and AmpC in 7.04 per cent (5/71) of the isolates. Among the isolates showing the coexistence of ESBL and AmpC production, 20 per cent (1/5) were from the IPD, whereas 80 per cent (4/5) were from the OPD.

No isolate exhibiting just MBL production was found; rather, 4.22 per cent (3/71) of the isolates existed as co-producers with AmpC, all of which were recovered from the IPD.

The combination of ESBL, AmpC, and MBL-resistant strains was not found during our study period. Table II displays the MDR *E. coli* isolates identified for ESBL, AmpC, and MBL production using various phenotypic methods.

Discussion

In this study, 7.08 per cent UTIs were caused by *E. coli*, which is comparable to another study carried out in rural Haryana that found a prevalence of 8.1 per cent[17,18]. In contrast, studies conducted in Assam and Uttarakhand in India reported 34.21 per cent and 52 per cent presence of *E. coli* in community-acquired UTIs, respectively[19,20]. The observed discrepancies could be attributed to variations in the investigated population, patient screening protocols, and the predominant bacterial strains responsible for causing UTIs in each geographical area[20].

An additional concern is the escalating incidence of infections due to multidrug resistance, which complicates the development of effective treatments. MDR refers to the acquired resistance to at least one agent in three or more antimicrobial groups[20]. After screening for ESBL, AmpC, and MBL resistance, 40.11 per cent of *E. coli* were found to be multidrug resistant, which is comparable to the findings of a study in a tertiary care centre in Rajasthan (47.55%)[21]. The present study revealed an occurrence of 31 per cent ESBL-generating *E. coli*, which is in contrast to other studies, the findings of which ranged between 20[17], 41.6[18], and 73.3[22] per cent in North India, Central India, and New Delhi, respectively. The incidence of ESBL in clinical isolates varies widely by region and time, which may explain this range of results[20].

Our study found 50.7 per cent AmpC-producing *E. coli*, which is a higher rate than in other countries (2-10%)[21,22]. Phenotypic test methods, material quality, and location variances appear to be the primary causes of discrepancies in the results[20].

In the present study, no *E. coli* isolates were found to be individual producers of MBL but rather coexisted with AmpC-producing isolates (4.22%). This may be due to the geographical distribution of these β-lactamases. A prevalence of 2-70 per cent of MBL-producing *E. coli* strains was reported from various parts of India[21].

The present study found the simultaneous production of ESBL and AmpC (7.04%) and that of AmpC and MBL (4.22%) in the urinary isolates. This coexistence may be owed to the spread of plasmid-mediated AmpC β-lactamase among *Enterobacterales*, because of which phenotypic ESBL testing may be negative for isolates. Different classes of β-lactamases in a single bacterial isolate can complicate identification and treatment. The AmpC-producing organisms may serve as an ESBL reservoir. High-level AmpC synthesis may hide ESBLs, resulting in lethal and ineffective antimicrobial therapy[23].

Table II. Beta lactamase production by *E. coli* strains causing UTI detected by various phenotypic confirmatory methods

	ESBL		AmpC		MBL	
	Double-disc diffusion method (n=71), n (%)	E-test (n=71), n (%)	AmpC disc test (n=71), n (%)	Cefoxitin-cloxacillin disc diffusion test (n=71), n (%)	Combined disc synergy test- 0.1 M EDTA test (n=71), n (%)	Double disc synergy test- 0.1 M EDTA (n=71), n (%)
Positive	22 (31)	32 (45)	44 (62)	6 (8.5)	3 (4.2)	3 (4.2)
Negative	49 (69)	39 (55)	27 (38)	65 (91.6)	68 (95.8)	68 (95.8)

ESBLs, extended spectrum β-lactamases; AmpC, AmpC β-lactamases; MBL, metallo β-lactamases; EDTA, ethylenediaminetetraacetic acid; E-test, epsilometer test

In the present study, the prevalence of *E. coli* causing UTI was higher in females (67.6%) than in males (32.4%). The increased susceptibility of females to UTIs can be due to several factors, including the relatively short urethra, proximity to the anus, sexual intercourse, childbirth, and the presence of normal flora in the vagina[24].

Out of the 71 MDR *E. coli* isolates in this study, 50.7 per cent were those of OPD patients and 49.3 per cent were those of IPD patients, which was not significantly different. However, the occurrence of ESBL-producing *E. coli* was notably greater in OPD patients (68%) compared to IPD patients (32%). This scenario exemplifies the consequences of indiscriminate use of antibiotics, which includes both self-medication and the sale of these medications over the counter in the community. Additionally, a higher proportion of AmpC-producing isolates were obtained from IPD patients (61.4%) compared to OPD patients (38.6%). The higher proportion of β-lactamases observed in IPD patients as opposed to OPD patients could be due to the extended hospital stay, excessive utilization of third-generation cephalosporins in medical facilities, and the use of invasive medical devices[20]. All the MBL-producing isolates, which were found to co-exist with AmpC, were detected in the OPD patients (100%). The coexistence of ESBL and AmpC-producing isolates was found more among the OPD patients (80%) than the IPD patients (20%).

The co-occurrence of β-lactamases is a potential cause of concern because the co-production of enzymes indicates that there is horizontal transfer of several resistance genes that are found in plasmids[24]. This could pose a threat if left unchecked in a world that is already grappling with antimicrobial resistance.

Our results revealed that 31 per cent of the *E. coli* isolates were phenotypically detected as ESBL positive by the double disc diffusion method, and 45 per cent of the isolates were detected by the E-test method. This aligns with the findings of the study carried out in Puducherry[25], which showed that 29 and 35 per cent of the ESBL-producing *E. coli* strains were detected by the double disc diffusion method and the E-test method, respectively. In addition to being simple, the E-test appears to be a reliable alternative approach for phenotypic confirmation of ESBL formation.

The AmpC disc test detected 62 per cent of the isolates producing AmpC, while the cefoxitin-cloxacillin disc diffusion test detected 8.5 per cent. In the present study, 4.2 per of the MBL producing isolates were detected both by CDST-0.1 M EDTA and DDST-0.1 M EDTA. Only a few studies have been done in India on the prevalence and detection methods of AmpC and MBL-producing *E. coli* that cause UTIs.

There was a significant resistance to quinolone classes (83.61%) and the β-lactam/β-lactam inhibitor group (21.46-81.92%), followed by cephalosporins (24.85-79.66%), aminoglycosides (61%), monobactams (46.89%), and sulphonamides (38.41%). The resistance to nitrofurantoin and fosfomycin, which are exclusively used for testing urinary isolates, was 5.64 and 2.82 per cent, respectively. Over 60 per cent of the strains were resistant to amoxicillin-clavulanate, ciprofloxacin, and gentamicin. The increasing use of β-lactam antibiotics in healthcare has led to a major increase in antibiotic resistance over the past decade[26]. Improper usage of β-lactam/β-lactamase inhibitor combinations has led to resistance, with amoxicillin-clavulanate being the least effective[27].

According to the Infectious Diseases Society of America (IDSA) and the European Society of Microbiology and Infectious Diseases (ESCMID), fluoroquinolones, cotrimoxazole, nitrofurantoin, and amoxicillin-clavulanic acid are the first-line of treatments for uncomplicated UTIs[28]. Nevertheless, local susceptibility patterns should determine first-line treatment recommendations. The National Centre for Disease Control (NCDC) recommends nitrofurantoin,

cotrimoxazole, and ciprofloxacin for as first-line medicines for uncomplicated UTIs and piperacillin-tazobactam and amikacin as second-line antibiotics[29]. Only 18 per cent of the isolates in this study were sensitive to amoxicillin-clavulanate, making it an unsuitable first-line treatment in this southern part of Haryana. On the contrary, nitrofurantoin and fosfomycin showed a high sensitivity of 94.35 and 97.17 per cent, respectively. For uncomplicated UTIs, nitrofurantoin is preferable.

With 16.38 per cent sensitivity, ciprofloxacin was less sensitive. This is concerning since ciprofloxacin is still a first-line therapy[29]. The extensive usage of fluoroquinolones and their high permeability to many body compartments contribute to their high resistance rate. The NCDC recently named ciprofloxacin an 'alert drug,' meaning it is one of the most commonly prescribed antibiotics and should only be used when microbiologically indicated. The NCDC strongly advises switching to narrower-spectrum antibiotics after culture results for UTIs. These measures may prevent fluoroquinolone resistance[28].

Multiple classes of β-lactamases in a single bacterial sample hinder diagnosis and treatment. This is particularly challenging because hazardous drugs like polymyxin and colistin have restricted availability[30]. Without defined protocols for identifying β-lactamases, infections caused by many bacteria, especially *Enterobacterales*, can be fatal. Incorrect antibiotic treatment could exacerbate the current state of antimicrobial resistance[20]. Using phenotypic confirmatory methods, like the ones we used in this study, on a regular basis in microbiology labs may improve treatment by finding β-lactamase development early. These methods are simple and economical. Developing an antimicrobial stewardship programme based on local epidemiological data and worldwide guidelines has the potential to enhance patient outcomes, assure cost-effective therapy, and mitigate the adverse effects of antimicrobial use in hospitalized patients. The risk of drug resistance in healthcare settings can be reduced by implementing rigorous infection control practices[31].

The gold standard for carbapenemase detection is molecular methods, but our laboratory, which operates in a low-resource setting, lacks those facilities. As a result, we resorted to phenotypic methods, which constituted a limitation of our study.

In conclusion, this study found a large proportion of β-lactamase-generating *E. coli* in UTI patients in the region. Imipenem, nitrofurantoin, and fosfomycin are the most sensitive antibiotics for *E. coli* isolates in this region and can treat UTIs. Phenotypic approaches for ESBL, AmpC, and MBL detection were used as they are reliable, straightforward, cost-effective, and help in prompt detection. Detection of β-lactamase-producing organisms is critical for appropriate treatment, reducing patient mortality and morbidity, and preventing antibiotic resistance. With routine ESBL, AmpC, and MBL detection in an institute, an effective antimicrobial policy and prompt infection control measures can be developed.

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