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Phenotypic Characterization of Virulence Factors and Antibiotic Resistant Pattern of Uropathogenic *Escherichia coli* Isolates

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Abstract: Uropathogenic *Escherichia coli* (UPEC) is the major causative agent of urinary tract infections; responsible for > 80% of all types of Urinary tract infections (UTIs). Emergence of multi drug resistant and possession of multiple virulence factors are closely related with pathogenesis of the UPEC bacteria. The purpose of this study was to evaluate the prevalence of different virulence factors and determine antibiotic resistance pattern among Uropathogenic *E. coli* by phenotypically. UPEC isolates from patients with different clinical symptoms of UTI were collected and confirmed with standard microbiological procedure. Various virulent factors such as biofilm formation, hemolysin production, capsule production, mannose resistant, and mannose sensitive hemagglutination (MRHA and MSHA, respectively), gelatinase and slime layer production were screened. In addition, antimicrobial resistance pattern and ESBL-producing isolates were also recorded. A total of 61 non-repetitive *E. coli* isolates were collected and confirmed as Uropathogenic *E. coli*. Out of 61 UPEC isolates, serum resistance and capsular formation were seen in 55 (90.2%) and 54 (89%) isolates, respectively. Moreover hemolysin producers 54 (88%), 52(85%) and 9 (14.75%) isolates showed the presence of P fimbriae (MRHA) and Types 1 fimbriae (MSHA). The UPEC isolates showed the highest antibiotic resistance to ampicillin and 52(85%) isolates were MDR. Moreover, 43(70.49%) of isolates were ESBL producers. This study showed a remarkable rate of antibiotic resistant characteristics along with ESBLs production and phenotypic virulence traits of the UPEC isolates. Phenotypic detection of the virulence profile and antibiotic resistance may enhance the understanding of severity of UPEC pathogenesis and prescribe better medical intervention.

Keywords: Uropathogenic *Escherichia coli*, Urinary tract infections, Hemolysin, Biofilm, P fimbriae, Virulence traits, Drug resistant

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Introduction

Urinary tract infections (UTIs) are the microbial infectious diseases in genitourinary tract which cause considerable amount of morbidity and mortality (Haghighatpanah and Mojtahedi, 2019). Irrespective of age, UTIs can affect both male and female populations (Ganesh *et al.*, 2019). However, the occurrence of UTIs are higher in female, because of anatomical differences, hormonal and behaviors effects. Several epidemiological, serological and bacteriological studies revealed that *Escherichia coli* is the pathogen most frequently associated with UTI (Sorwer and Dolilur, 2018). *Escherichia coli* is the predominant agent of UTI in all patient groups, causing 80-90% UTI. *E. coli* responsible for 75-95% of uncomplicated UTI and 40-50% of complicated UTI. It accounts for about 75% - 90% of all community acquired and hospital acquired UTI infections (Fazly *et al.*, 2021).

Uropathogenic *E. coli* can grow well in urine and form colonization even in the absence of specific adherence molecules. UPEC has the ability to adapt to an environment very different from the human gut by altering its metabolism, ascend against the flow of urine and adhere to the epithelial layers and the ability to form intracellular bacterial community (Prajapati, 2018). The severity of UTI depends on the pathogenic potential of *E.coli* strains, which in turn depends on the presence and expression of the various virulent markers. For the successful colonization invasion and survival within the host, UPEC possess a vast array of virulent and fitness markers (Harwalkar *et al.*, 2015). These factors include surface structural components like lipopolysaccharide (LPS), capsules, surface virulent markers like adhesins (P fimbriae, type 1 fimbriae, S and F1C fimbriae, afimbrial adhesion, curli), flagella, outer membrane proteins as well as secreted toxins, toxins secretery systems, ToB dependent iron uptake receptors including siderophore receptors (Alieh *et al.*, 2017; El-Shaer *et al.*, 2018). They can facilitate the bacterial adherence, colonization and establishment of infection in the urinary tract by overcome the host

defense mechanism (Bunduki *et al.*, 2021). These virulence factors are usually encoded by genes that are located in special region called pathogenicity islands (PAIs), through horizontal gene transfer most of the bacteria acquire these region (Allami *et al.*, 2021; Tanabe *et al.*, 2022). A single virulent factor is not sufficient to cause an infection, instead, multiple virulent factors are required to cause and establish infection in host cell (El-Baz *et al.*, 2022).

The emergence of antibiotic resistance among pathogenic bacteria is one of the global treatment problems. The emergence of drug resistant isolates among UPEC strains increase the serious threat to global health (Shah *et al.*, 2019). Currently, many study reports show that horizontal gene transfer is one of the reason for increasing the rate of antibiotic resistance among UPEC. Determination of antibiotic resistance patterns in common pathogenic bacteria is important to guide experimental and specific therapies against specific pathogens, including UPEC strains (Al-Naqshbandi *et al.*, 2019).

Most of the *E. coli* have plasmids that carry gene encoding for antibiotic resistance enzymes specially β lactamase enzymes. Extended spectrum β lactamase (ESBLs) are extremely broad spectrum β lactamase enzymes which promote bacterial resistance to all beta-lactams except carbapenems, cephamycins and clavulanic acid (Subedi *et al.*, 2020). Antibiotic resistance associated with ESBLs are associated with the difficult of treatments and high morbidity and mortality rates, health care cost and longer antibiotic therapy in comparison with non-ESBL producing pathogens. Hence, this study was designed to characterize the antibiotic resistance, the prevalence of ESBLs and the occurrence of different phenotypic virulence markers in UPEC isolates.

Materials and Methods

Processing of collected isolates:

A total of 61 non-repetitive urine *E. coli* isolates

were collected from various hospitals in Chennai, India. All the collected isolates were inoculated by streaking on differential and selective medias such as Cysteine Lactose Electrolyte Deficient (CLED) Agar, MacConkey, UTI agar and Eosin Methylene Blue (EMB) agar. Plates were incubated overnight at 37°C. After incubation, the individual colonies were examined and characterized by colony morphology, Gram staining and biochemical identification methods.

Antibiotic susceptibility assay:

Antibiotic susceptibility test was determined by Kirby–Bauer disk diffusion methods on Mueller–Hinton agar using 0.5 McFarland standard turbidity of bacterial inoculum according to the CLSI guidelines (2018). Antimicrobial agents used in this study were ampicillin (10 µg), cefazolin (30 µg), cefotaxime (30 µg), Cefoxitin (30 µg), amikacin (30 µg), nitrofurantoin (300 µg), Nalidixic acid (10 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), co-trimoxazole (1.25/23.75 µg), fosfomycin, and meropenem (10 µg). According to CLSI guidelines (2018) the results were interpreted.

Phenotypic detection and confirmation of Extended spectrum β lactamase producing UPEC:

Phenotypic confirmation of ESBL producers were done as per CLSI guidelines (2018) by using double disk diffusion method on Mueller–Hinton agar. Mueller Hinton agar (MHA) plates were inoculated with bacterial suspension with turbidity equivalent to 0.5 McFarland standard. Four discs, cefotaxime (30 µg), cefotaxime/clavulanic acid (30 µg/ 10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (30 µg/10 µg) were used in this study. An increase in zone diameter of ≥ 5 mm with clavulanic acid than ceftazidime and cefotaxime alone were confirmed as the production of ESBL (Moges *et al.*, 2019).

Phenotypic characterization of virulent factors among Uropathogenic Escherichia coli:

Hemagglutination:

The test was carried out to determine the Type1 and P fimbriae of uroisolates, as per the direct

bacterial hemagglutination slide test method. One drop of 3% 'O' blood group red blood cell (RBC) suspension was added to a drop of broth culture and a drop of 2% D-mannose. The slide was rotated at room temperature for 5 min. Formation of clumping was considered as positive result for hemagglutination. The absence of hemagglutination in the presence of a drop of 2% D-mannose to the red cells and a drop of broth culture taken as Mannose-sensitive hemagglutination (Mittal *et al.*, 2014).

Biofilm formation assay by microtiter plate method:

This quantitative test is considered as the gold-standard method for biofilm detection since it is accurate than other methods. The isolates were inoculated in 2 ml of LB broth and 1% glucose added as a supplement to media. The inoculated tubes were incubated at 37°C for 24 h. The fresh LB medium were used to dilute the culture in 1:100 ratio. 200 µl of the diluted cultures were filled in individual wells of sterile 96 well-flat bottom polystyrene microtiter plates. Sterile broth in inoculated wells were taken as a negative control and incubated the plates at 37°C for 24–48 h. The contents of each well were removed by gentle tapping after incubation. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) at three times to remove free floating bacteria. The adhered bacteria to the wells were fixed by staining with 0.1% crystal violet. Excess stain was removed by using deionized water and plates were kept for air drying. Optical density (OD) was taken by using micro ELISA auto reader at wavelength 550 nm. Based on the O.D value, strains were classified into the following categories: moderate or strong biofilm producers, weak and non-biofilm producers (Karigoudar *et al.*, 2019).

Slime layer production assay by Congo red agar method:

The simple qualitative method to detect slime layer production by using Congo Red Agar (CRA) medium have been described by Hrv *et al.* (2016) with slight modifications. CRA medium prepared with BHI broth supplemented with 3% sucrose

and Congo red indicator and autoclaved (121°C for 15 min). The CRA plate was inoculated with the test organism from an overnight culture plate and incubated at 37°C for 24–48 h aerobically. Black colonies with a dry crystalline consistency indicated slime production.

Hemolysin assay:

The *E. coli* isolates were inoculated on 5% sheep blood agar and incubated over night at 37°C. Hemolysin production was detected by complete hemolysis or Beta hemolysis of the erythrocytes on blood agar indicated by a clear zone around the colony. Plate was also kept at 4°C overnight to clearly observe the hemolysis (Shah *et al.*, 2019).

Serum resistance:

The property of serum resistance was studied using a fresh culture of the isolates. Overnight cultures of UPEC, grown on blood agar were suspended in Hank's balanced salt solution (HBSS). The bacterial suspension (0.05 ml) was incubated with serum (0.05 ml) at 37°C for 3h. 10 µl of samples were withdrawn and spread on blood agar plates and incubated at 37°C for 18-24 hours. Isolates were termed as serum- sensitive if the viable count dropped to 1% and resistant if viable count > 90% of organisms survived after 24 hours of incubation (Harwalkar *et al.*, 2015).

Capsular staining:

Fresh and pure colonies of UPEC isolates were transferred on a clean glass slide, mixed with nigrosin stain and allowed to air dry. Again the slide was stained with few drops of methylen blue and allowed to dry again. The nigrosin stain provides a dark background to unstained capsule and metylene blue stain provides blue color to the cells under light microscope (Ahmed and Qassim, 2017).

Gelatinase test:

Gelatin medium was prepared by adding 3% of gelatin into nutrient agar. The plate was inoculated with UPEC isolates and incubated at 37°C for 24 h. After incubation, the plate was flooded with 1% tannic acid solution.

Development of opacity around colonies was considered as positive for gelatinase (Mittal *et al.*, 2014).

Results

All the collected 61 uropathogenic *E. coli* strains were identified and confirmed based on their morphology on Cystiene Lactose Electrolyte Deficient Agar (CLED), HiChrome UTI agar, MacConkey agar and Eosin methelene blue agar plates. The isolates produced yellow coloured flat as well as mucoid colonies (on CLED agar), purple coloured colonies (HiChrome UTI agar), flat or mucoid lactose fermenting pink colonies (on MacConkey agar) and green metallic sheen colonies on Eosin methelene blue agar plates (Fig. 1). The isolates were also identified as gram negative *Bacilli* by Gram's staining. The isolates were further confirmed based on their biochemical profile for IMViC (+++-) and TSI (Not produced H₂S) (Table1, Fig. 2).

Antibiotic sensitivity assay:

Antibiotic susceptibility pattern was studied for all the identified *E.coli* isolates (Table 1). Maximum isolates showed resistance to ampicillin (n=60, 98%) followed by cefazolin (n=58, 95%), Trimethoprim sulfamethoxazole (n=55, 90%), cefoxitin (n=53, 86%), cefotaxime (n=53, 86%), ceftazidime 53(86%) and nalidixic acid (n= 52, 85%). It was found that Out of 61, 52 (85%) isolates were designated as multiple drug resistance (MDR) based on their resistant to three or more than three groups of antibiotics (Table 2).

Phenotypic detection and confirmation of extended spectrum β-lactamase (ESBL) producers:

The cephalosporin resistance directly correlates with the production of extended spectrum β-lactamase (ESBL); all cephalosporin third generation (ceftazidime, cefotaxime and ceftriaxone) resistant isolates were analysed for the production of ESBL by double disk diffusion method. Out of 61 UPEC isolates that were studied, 43 (70.49%) were detected as ESBL producers (Fig. 3).

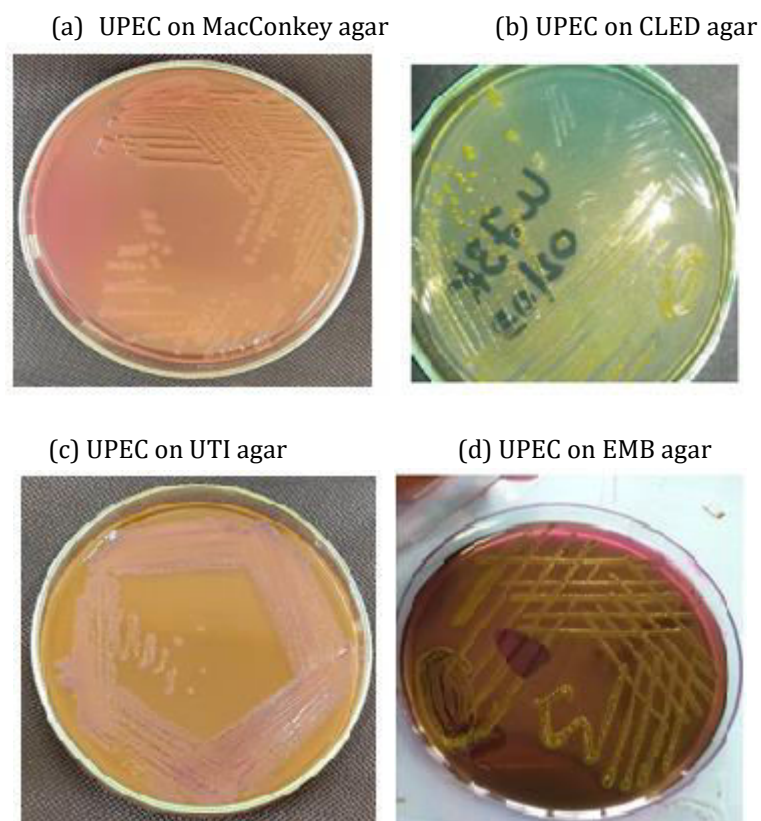


Fig. 1: Morphological identification of UPEC on CLED, MacConkey, UTI agar and EMB agar.

Table 1: 1Bilochemical characterstics of *Escherichia coli*

Biochemical test	Characteristics of <i>E. coli</i>
Indole	+
Methyl red	+
Voges – Proskauer Test	-
citrate	-
Triple Sugar Iron agar (TSI).	+/-
urease	+/-
Motility	++-

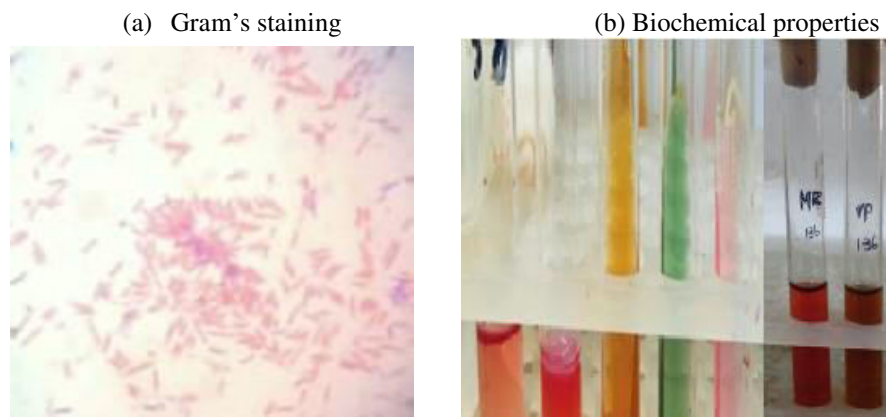


Fig. 2: Confirmation of UPEC by biochemical characterization.

Table 2: Antibiotic susceptibility assay of Uropathogenic *E. coli* isolates

S. No.	Name of antibiotic	No. and % of resistance
1	Ampicillin	60 (98%)
2	Cefazolin	58 (95%)
3	Cefoxitin	53 (86%)
4	Cefotaxime	53 (86%)
5	Ceftazidime	53 (86%)
6	Ceftriaxone	51 (83%)
7	Nalidixic acid	52 (85%)
8	Nitrofurantoin	32 (52%)
9	Fosfomycin	3 (5%)
10	Amikacin	16 (24%)
11	Meropenam	4 (6%)
12	Levofloxacin	46 (75%)
13	Ciprofloxacin	46 (75%)
14	Trimethoprim sulfomethoxazole	55 (90%)

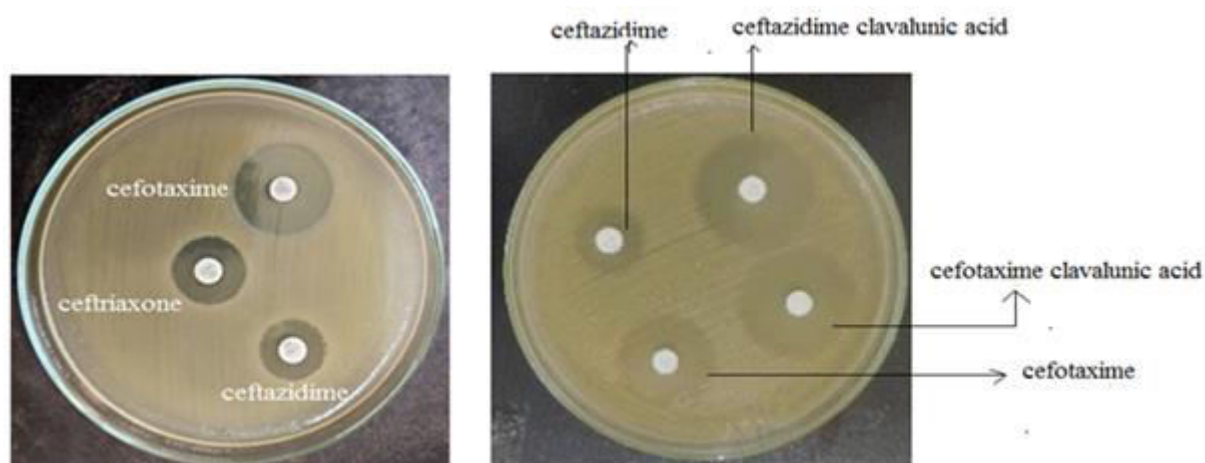


Fig. 3: Phenotypic confirmation of ESBL producers.

Phenotypic detection of virulence factors in Uropathogenic *Escherichia coli*:

In the current study, predominant virulent markers among Urinary *E.coli* isolates were detected as serum resistance (n=55, 90%), capsules (n=54, 88%) and hemolysin (n=54, 88%), MRHA (n=52, 85%), slime layer producers (n=47, 77%), gelatinase (n=40, 66%), biofilm producers (n=17, 28%) and MSHA (n=9, 14.8%) (Table 3, Fig. 4).

Discussion

Uropathogenic *Escherichia coli* (UPEC) is the predominant etiological factor of more than 80% of urinary tract infection (UTI). The study of uropathogenic *E. coli* is very important due to its

increased rate of mortality and morbidity of UTIs. Pathogenesis of urinary tract infection is influenced by host biological and behavioral factors, as well as by the expression of various virulent characteristics in the uropathogen. The prevalence of various virulence markers in UPEC strains further strengthens the concept of association of UPEC with its pathogenicity. Many research studies concluded that not only a single virulent factor is sufficient to cause an infection, but an array of virulent markers interacting with host immune system to cause infection. UPEC isolates that are able to hemeagglutinate, produce toxins like gelatinase, aspartyl proteinase and hemolysin. They are strong biofilm formers, capable of forming capsules, slime layers and also

Table 3: Phenotypic characterization of various virulent factors among Uropathogenic *E. coli*

Virulence factors	Positive (n)	Percentage %
Hemolysin assay	54	88
Hemagglutination	MRHA =52	85
	MSHA =9	14.8
Biofilm by microtiter plate method	17	28
Capsule productions	54	88
Serum resistance	55	90.2
Gelatinase test	40	66
Slime layer production	47	77

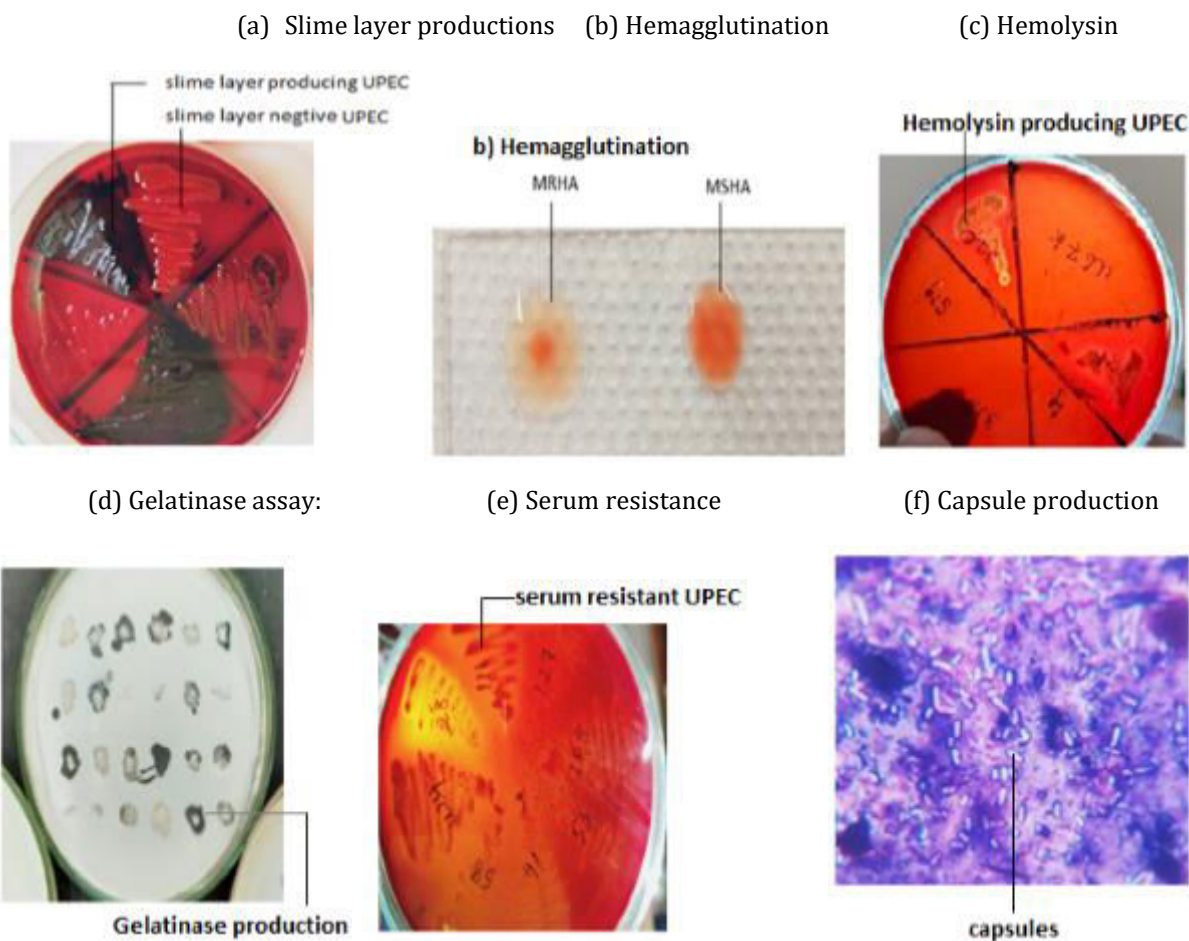


Fig. 4: Phenotypic characterization of various virulent factors among UPEC.

motile.

The present study was performed on 61 UPEC isolates, confirmed by standard microbiological procedure. Nowadays the emergence of antibiotic resistance is the major problem associated with treating infections. Antibiotic resistance of uropathogens against commonly prescribed antibiotics has increasing both in developing as well as developed countries. Our result showed that UPEC that mentioned in this study were highly resistant to ampicillin (98%), cefazolin (95%), Trimethoprim sulfamethoxazole (90%), cefoxitin (86%), cefotaxime (86%), ceftazidime (86%) and nalidixic acid (85%). Similar reports of Elsayed *et al.* (2017) stated that urinary *E. coli* isolates were highly resistant to the Ampicillin 95%, Cephalothin 93%, Nalidixic acid 70%, Norfloxacin 59%, Trimethoprim sulfamethoxazole 69% and Nitrofurantoin 16%. Sharma *et al.* (2013) reported that UPEC were resistant to Ampicillin 81.7%, Cephalexin 92.7%, Nalidixic acid 78.9%, Ciprofloxacin 49.5%, Norfloxacin 78.9%, cotrimoxazole 54.1% and Nitrofurantoin 5.5%.

In our study it is clear that ampicillin was the least effective drug against UPEC. A similar study was conducted at Kathmandu University School of Medical Science by Acharya *et al.* (2015) who have also reported that ampicillin showed least effect against Uropathogenic *E. coli*. One of the important results in this study was the high incidence of Multidrug resistance (MDR) 52 (85%) and remarkable proportion of ESBL producing 43(70.49%) UPEC. Extended spectrum β lactamases are enzymes produced by variety of Gram negative bacteria. Commonly emergence of ESBLs in Enterobacteriaceae limits treatment options because the ESBL positive isolates often co-resistance to fluoroquinolones, tetracycline, TMP-SMX, and aminoglycoside thus are often classified as multidrug resistant. Haghighatpanah and Mojtahedi (2019) reported that 51.2% *E. coli* isolates were ESBL producers. Currently, β lactamases are the leading cause of resistance to β lactam drugs. The ability of these enzymes to

cause antimicrobial resistance is primarily due to its activity, site of production, volume produced, and the permeability of the producer strain.

The ability of MRHA by UPEC is considered as an important virulence factor, because it facilitates microbial colonization and invasion of urinary tract and leads to infection. We observed a notable difference in MRHA and MSHA expression in tested isolates. Present study revealed highest rate of MRHA 52 (85.24%) and 9 (14.8%) of isolates were considered as MSHA in UPEC. Shah *et al.* (2019) demonstrated expression of 52.3% of MRHA and 5.3 % of MSHA in their study. Kaira and Pai (2018) reported 51% MRHA and 7% MSHA in their study; the results are in agreement with our findings.

Hemolysin production is associated with pathogenicity of *E. coli*, especially in more severe forms of infection. Hemolysis, though not essential for establishment of acute pyelonephritis, mainly contribute to inflammation, tissue injury, survival in renal parenchyma and entry into blood stream. Hemolysin production of UPEC was classified into alpha (Complete Hemolysis), beta (Partial Hemolysis) and gamma hemolytic (Non Hemolytic). In this study 54 (89%) out of 61 strains of *E. coli* produced hemolysin. In other studies conducted by Shah *et al.* (2019) and Mittal *et al.* (2014), hemolysin production was detected in UPEC were 45.3% and 52.3%, respectively. The toxin hemolysin is one of the major virulence factor of UPEC which targets multiple host pathways to increase infection.

In the current study gelatinase enzyme production was studied in 61 UPEC isolates. 40 (66%) isolates showed activity against gelatin in agar plate. A study conducted by Mittal *et al.* (2014) showed gelatinase activity as 53%. None of the isolates showed gelatinase activity in the study conducted by Kaira and Pai (2018) and Shah *et al.* (2019). El-Mosallamy *et al.* (2015) stated that only 1 (2%) UPEC showed gelatinase production in their study. Gelatinases is one of the important virulence factor of *E. coli* responsible for patho-

genicity in different diseases, particularly in urinary tract infection.

Bactericidal activity of human serum acts as a major first-line defense against bacterial infections. Usually normal serum possesses bactericidal activity against a wide range of gram-negative bacteria. 90.2% of *E. coli* isolates showed the bactericidal activity against human serum in our study. Similar reports were found out by Bhattacharyya *et al.* (2015), about 90% of *E. coli* isolates were serum resistant in their study. Serum resistance is the property of bacteria, it resist killing by normal human serum by lytic action of complement system.

Presence of capsule in bacteria helps survival as well as causing infection, because capsules promotes antiphagocytic and anticomplement effect in bacteria. It has been suggested that K (capsular) antigen of *E. coli* plays an important role to escape from host immune system by serum resistance and inhibiting phagocytosis. In our study a total of 54 isolates among 61 were found to be capsulated. According to the study report of Ahmed and Qassim (2017), 18 *E. coli* out of 50 were positive for capsule production. Certain capsules like k1 and K5 are showing a molecular mimicry to tissue components of infected host and prevent a proper humeral immune response. Expression of group 2 capsules is one of the reason for invasive disease caused by *E. coli*.

Biofilm formation capacity is one of the important factor increasing the pathogenicity of bacteria and their resistance to antimicrobial agents. Biofilm formation of UPEC by microtiter plate method detected 17/61 (28%) were strong biofilm formers and remaining strains had moderate to weak biofilm forming capacity. Abad *et al.* (2019) reported that among 79 *E. coli* isolates from urinary tract infections, 10 isolates (12.7%), 5 isolates (6.3%), 59 isolates (74.7%) and 5 isolates (6.3%) showed strong, moderate, weakly and non-biofilm formation ability, respectively. Afreenish *et al.* (2011) stated that microtiter plate is a quantitative and reliable

method to detect biofilm forming capacity of microorganisms. When compared to Tube method, microtiter method can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories. Biofilm is considered as one of the important virulence factor and protect UPEC from host immunity as well as from antimicrobial agents and cause persistent infections.

Slime layer production was detected by Congo Red Agar (CRA) in this study. Our results showed that 47 (77%) UPEC isolates were slime producers. Congo red agar or broth method is a very simple, sensitive, and specific method that can be used for screening the strains for the presence of slime or slime-like substances. According to the reports of Hrv *et al.* (2016), out of 103 slime producers, 39 (78%) strains of *E. coli*, 18 (94.7%) strains of *Klebsiella pneumoniae*, 21 (84.0%) strains of *S. aureus* and 25 (65.7%) strains of coagulase-negative *Staphylococci* from various specimens were positive for slime or slime-like substances by Congo red agar/broth method.

Conclusion:

This study revealed the antibiotic resistance pattern, production of Extended β lactamase enzymes (ESBL) and the expression of various virulent factors in Uropathogenic *E. coli*. Our results showed remarkable rate of drug resistance and the contribution of ESBL enzymes for the emergence of MDR *Uopathogenic E.coli* isolates. Knowledge of the prevalence of ESBLs and resistance patterns of UPEC isolates are very important in the prevention of the emergence and spread of resistance among bacteria. According to phenotypic characterization, most of the isolates expressed the virulent characteristics like serum resistance, capsules and hemolysin in this study. This study concluded that prevalence of various virulence factors determine the (pathogenic potential of UPEC isolates in Urinary Tract Infection. It is also concluded that ESBL detection along with antibiotic susceptibility testing is mandatory in all the laboratories to prevent

morbidity and mortality associated with UTI. These findings help for earlier detection and effective treatment of UPEC associated Urinary Tract Infection. The information may be helpful for future studies, to collect more Urinary *E. coli* isolates and to investigate the expression of various virulent encoding genes.

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