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Isolation, Identification and Serotyping Uropathogenic Escherchia Coli Siderophore Manufacturer of Hospital Patients and Health Care Centers in Bandare Anzali[1]

Mitra Doost Prast,
Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Aza University, Lahijan, Iran
Khosro Issazadeh,
Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Aza University,Lahijan, Iran
Email: atena.pan@yahoo.com
Mohamad Reza Khosh Kholgh
Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Aza University, Lahijan, Iran

ABSTRACT — Acute urinary tract infections consist of upper urinary tract infections, including pyelonephritis, prostatitis, intra-kidney abscesses, and around-kidney abscesses, and lower urinary tract infections, including urethra infection and bladder infection. These infections can independently or in combination result in irritation in various parts. They may appear as asymptomatic or as one of the clinical syndromes. Bladder and urethra infections are as superficial (mucosal) infections but prostatitis and pyelonephritis and the accumulation of pus in the kidney shows the tissue invasion. In microbiological terms, there is urinary infection when pathogenic microorganisms are found in urine, urethral, bladder, kidney, or prostate. In most cases, the growth of more than 105 microorganisms per milliliter of the samples in the middle of urine indicates the infection, but in symptomatic cases, 102-104 bacteria per milliliter of urine also suggest the infection. The symptoms of dysuria, urgent and frequent urination not associated with significant bacteriuria is called urethral syndrome. This study aimed to determine the local incidence of urinary tract infection, to identify UPEC strains, serotyping, and antimicrobial resistance, and to investigate siderophore and hemolysin and their correlation with the ability to cause infection in Escherichia coli strains isolated from these patients. In order to separate and identify UPEC bacterial factors of urinary tract infection and to determine age and sex distribution of the ones infected in a prospective study, 200 samples taken from the middle of urine were analyzed with simple random sampling. The test of sensitivity to various antibiotics was done with Kirby-Bauer method, that is, disc diffusion in agar on 21 UPEC isolates separated from these patients in the 1-86 age range, and serotyping with agglutination method on a slide and hemolysin production was evaluated on blood agar with 5% sheep blood. Also, siderophore production was studied in isolates. The whole results were analyzed by using the SPSS software. The majority of urinary pathogen UPEC isolates belonged to the serotypes O114, O26, O64, O55, O15, O44, O25, O20, O1, O126, and O157. The majority of isolates had the highest resistance to the antibiotics ampicillin, cotrimoxazole, cefixime, and cephalexin and the highest sensitivity to nitrofurantoin, amikacin, gentamicin, ciprofloxacin, and tetracycline. The present study showed that O25 serotype includes the largest number of isolates and other serotypes, in order of frequency, were O20, O44, O1, O55, O114, O126, O15, O64, O26, and O157.

KEYWORDS: *Urinary Infection, Uropathogenic Escherichia coli, Serotype, Antimicrobial Sensitivity, Siderophore*

Introduction

Urinary tract infection is one of the most prevalent infections diagnosed in outpatients and inpatients and can result in substantial fatality. Bacteriuria is the presence of bacterium in the urine. Urinary infection can be proven by the presence of bacteria in non-centrifuged urine. A minimum of 10^5 bacteria per ml of urine is referred to as urinary infection. In some cases, these infections appear as without urinary symptoms or as a clinical syndrome. Acute infection of the bladder is said to be a collection of symptoms such as dysuria, frequent urination, and emergent urination which is accompanied by lower abdominal pain in some cases(3,22). Sometimes, these very symptoms occur without finding bacteria in the urine, in which case it is called urethral syndrome. Acute pyelonephritis exhibits itself with pain in flanks, fever, often accompanied by as dysuria, frequent urination, and emergent urination while being examined. However, these symptoms may also occur in cases like kidney stones without infection. So, acute pyelonephritis is in fact defined by the presence of significant bacteriuria and acute kidney infection. The

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most prevalent microorganisms producing urinary tract infection are gram-negative bacilli. *E. coli* causes approximately 80% of acute infections in patients without catheter and without urologic disorders (8, 5,11). Among other things, gram-negative bacilli, especially *Proteus*, *Klebsiella*, and sometimes *Enterobacter*, are responsible for a lower percentage of uncomplicated infections. These microorganisms, together with *Serratia* and *Pseudomonas*, are highly important in recurrent infections, infections associated with urologic manipulations, stone, or block, and also play the major role in nosocomial infections and infections related to catheter (7,10). Among other microorganisms, gram-positive cocci have a less important role in urinary infections. *Staphylococcus saprophyticus* are responsible for 10% to 15% of urinary tract infection cases in young women. *Staphylococcus aureus* and *Enterobacter* are prevalent in patients with kidney stone or cases already catheterized. Separating *saprophyticus* form the urine should raise suspicion of kidney infections resulting from bacteremia (11). Iron makes up one of the nutritional requirements of microorganisms but its concentration is reduced in aerobic environments and neutral pH and is not enough for their maximum growth. In this situation, iron is formed as hydroxide, carbonate, and insoluble ferric phosphate. Siderophores are compounds with low molecular weights which are secreted into the environment in the case of iron deficiency, are specific absorptive factors of iron, and have been separated from many of aerobic and facultative anaerobic microbes (23). Some serotypes of *E. coli* constantly have a part in urinary pathogenesis and are determined as UPEC. These isolated are expressed as chromosomally encoded virulence markers (16,17,18).

Methods

Sampling

200 urine samples from outpatients suspicious of urinary tract infection (UTI) symptoms referred to Shahid Beheshti Hospital and Medical Health Centers of the city of Bandar Anzali during one month, who had not used antibiotics within at least the last 48 hours, were collected in sterile containers special for urine cultures by Clean Catch method. At the same time with urine culture test, special form of patient history, patient information including personal characteristics, the presence of other symptoms or diseases, clinical symptoms, and paraclinical findings were gathered in order to complete the form as much as possible. Complete urine test and routine experiments such as CBC and so on were used to complete the test results as much as possible.

Urine culture

After mixing the urine sample using the sampler, 20 µl of the sample was put at the center of the 15-cm plate containing nutrient agar (NA) medium cultures, blood agar (BA) containing 5% sheep blood, MacConkey agar, and sheep blood agar. After opening, it was cultured in a diameter of 1 cm with the help of a sterile loop in a zigzag form. Blood agar samples were stabbed in several places in order to investigate the hemolysis. Then, the cultured plates were put at 35-37 ℃ for 24-48 hours and morphology of isolated colonies was assessed in terms of purity, pigment formation, and hemolysis after incubation. After culturing and the incubation period, catalase and oxidase tests were first performed, the presence of bacilli was then confirmed with gram staining, and it was preferred to linearly culture from the above middle part of the colony in a new blood agar medium with a sterile loop in order for colony purification. Immediately after purification, conventional biochemical tests including oxidase test, culture in TSI medium, indole production, MR test, VP citrate test, urease test, and culture in SIM medium were used in accordance with standard procedures. Then, after ensuring the purity of the colony, antibiogram, serotyping, and siderophore production tests were performed for the respective isolates.

Drug susceptibility testing (antibiogram):

To do antibiogram with Disk Diffusion in Agar method, a standard concentration of microbe was first prepared. To prepare this concentration, a few (4-5) colonies were inoculated from 18-24-hour culture of the pure bacteria on blood agar with a sterile loop in 4-5 ml sterile physiological serum and incubated for at least 1-5 hours to create turbidity equivalent to 0.5 McFarland tube. Using a sterile swab, an amount of the colony was drawn on the surface of the plate containing Mueller-Hinton agar medium and all parts, even around the plate, were covered by turning the plate to a degree of 60 for three times. Selected and common antibiotic discs were put on the surface of the plate with the sterile forceps with a distance of 30 mm form one another and a distance of 10-15 mm form the edge of the plate. Next, plates were placed at laboratory temperature in a flat position for about 10-15 minutes. Then, they were incubated at 37℃ for 18 hours. Using a ruler, the diameter of growth inhibition zone was measured around each disc and compared with the standard table, and results were reported as sensitive (S), resistant (R), and Intermediate (I) (Table 2-1).

Table 1- Discs containing the antibiotic used (product of Padtan Teb Co.)

Symbol	Disc Name	Row
CIP	Ciprofloxacine	1
FM	Nitrofurantion	2
CN	Cefalexin	3
AM	Ampicillin	4
NA	Nalidixic Acid	5
TE	Tetracycline	6
SXT	Co-trimoxazole	7
GM	Gentamicine	8
AN	Amikacin	9
CTX	Cefotaxime	10
CFM	Cefixime	11

Serotyping was done by using the kit of Mast Assure Company and the respective antiserums with agglutination method on a slide as follows (Table 2-2). To study hemolysin, the bacteria isolated were cultured in blood agar culture medium containing 5-7% sheep blood and hemolysin production in the medium was studied after 24-48 hours of incubation at 37℃. We used the commercial medium Chrome Azurol Sulfonate Agar (CAS) to do the experiment so that isolates were inoculated into the blue medium CAS Agar in points and incubated at 37℃ for 24-48 hours. If the color changed, from blue to orange, it would show that ferric ion was transferred from the complex medium Blue Agar to siderophore. The size of the colored (yellow-orange) halo around the colony indicated the overall activity of siderophore production.

Results and Discussion

21 UPEC isolates were separated from the urine culture of 200 patients suspicious of urinary tract infection referred to Shahid Beheshti Hospital and laboratories of Medical Health Centers in the city. Furthermore, 20 strains of *E. coli* isolated from feces of some other people referred for a reason other than urinary infection were used as the control. Results obtained from serotyping, antimicrobial sensitivity, siderophore production, and hemolysin by the isolated strains were analyzed by using SPSS software (Version 21), T-test and Chi-square test and expressed as follows.

Among 21 uropathogenic *Escherichia coli* isolated from the urine culture of patients infected with urinary infection, the frequency of females with 17 cases (81%) was more that of males with 4 cases (19%) (Table 4-1 and Figure 4-1).

Table 2- Frequency of UPEC strains isolated from the urine of inpatients based on sex

%	Number	Frequency Sex
19	4	Male
81	17	Female
100	21	Total

The result obtained from statistical analysis in Table 3-2 according to T-test was significant, that is to say, there is a significant relationship between sex and frequency of uropathogenic *E. coli*.Age range of patients with UPEC was 1-90. Most cases of infection were in the age group 70-79 with 19%, maximum frequency, followed by the age groups 30-39, 20-29, 80-89, and 60-69 with 14.3% and then the age groups 1-9 and 50-59 with 9.5%. The minimum frequency was in the age range 40-49 with 4.8% (Table 4-3 and Figure 4-2).

Table 3- Frequency of UPEC strains isolated from the urine of patients based on age

%	Frequency	Frequency Age group (in years)
9/5	2	1-9
0	0	10-19
14/3	3	20-29
14/3	3	30-39
4/8	1	40-49
9/5	2	50-59
14/3	3	60-69
19	4	70-79
14/3	3	80-89
0	0	≥90
100	21	Total

Highest frequency was in the age group 70-79 related to females with 4 cases (Table 4-5 and Figure 4-3).

Table 4- Frequency of UPEC strains isolated from the urine of patients in terms of sex and age

≥90	80-89	70-97	60-69	50-59	40-49	30-39	20-29	10-19	1-9	Age group (in years) Sex
0	0	0	1 25	0	1 25	0	0	0	2 50	Males
0	3 17/6	4 23/5	2 11/8	2 11/8	0	3 17/6	3 17/6	0	0	Females
0	3 14/3	4 19	3 14/3	2 9/5	1 4/8	3 14/3	3 14/3	0	2 9/5	Total

In 21 UPEC strains isolated from the culture of patients infected with urinary infection, the serotypes O126, O157, O114, O26, O64, O55, O15, O44, O25, O20, and O1 were observed. O25 serotype with 4 cases (19.04%), and O126, O114, O55, O1, O44, and O20 with 2 cases (9.52%) followed by O157, O26, O64, and O15 with 1 case (4.77%) were identified. 1 strain was not able to be typed (Table 4-10 and Figure 4-6).

Table 5- Frequency of UPEC serotypes isolated from the urine of patients

Percentage	Number	Frequency Serotype
19.04	4	O25
9.52	2	O20
9.52	2	O44
9.52	2	O1
4.77	1	O15
9.52	2	O55
4.77	1	UT
4.77	1	O64
9.52	2	O114
4.77	1	O26
4.77	1	O157
9.52	2	O126
100	21	Total

Antimicrobial sensitivity pattern of UPEC strains isolated from patients' urine in the population under study

The isolated strains' resistance to the antibiotics ciprofloxacin, nitrofurantoin, nalidixic acid, tetracycline, cotrimoxazole, gentamicin, cephalexin, ampicillin, amikacin, cefotaxime, and cefixime was 28.5%, 0%, 42.8%, 38.9%, 61.9%, 19.1%, 52.38%, 100%, 4.76%, 47.61%, and 57.14%, respectively. Highest resistance was to ampicillin (100%) and lowest resistance was related to nitrofurantoin (0%).

Table 6-Number and percentage of antibiotic resistance in UPEC strains isolated form patients

%	Intermediate	%	Resistant	%	Sensitive	Sensitivity Antibiotic type
%0	0	%100	21	%0	0	Ampicillin
0	0	52/38	11	47/61	10	Cephalexin
0	0	19/1	4	80/9	17	Gentamicin
9/5	2	28/5	6	61/9	13	Ciprofloxacin
0	0	0	0	100	21	Nitrofurantoin
9/52	2	42/85	9	47/61	10	Nalidixic acid
4/78	1	4/76	1	90/47	19	Amikacin
9/52	2	47/61	10	42/8	9	Cefotaxime
0	0	61/90	13	38/09	8	Cotrimoxazole
0	0	57/14	12	42/85	9	Cefixime
9/52	2	38/09	8	52/38	11	Tetracycline

Table 7- Siderophore production in *E. coli* strains isolated from urine and feces samples of patients

Percentage (-)	Number (-)	Percentage (+)	Number (+)	Frequency Isolate
14/29	3	85/71	18	Clinical sample
90	18	10	2	Control sample (feces)

Table 8- Hemolysin production in UPEC samples isolated from urine samples of patients

	Percentage (-)	Number (+)	Frequency isolate
	71/42	15	Clinical sample

The present study is of analytical-observational type. *E. coli* and other coexisting flora of mammals' intestines often form a beneficial symbiotic relationship with their host, but some *E. coli* strains can leave the coexistence and adopt the ability to cause a serious disease inside the digestive system and other organs of the host. These pathogenic strains are widely categorized as diarrheal *E. coli* or extraintestinal pathogenic *E. coli* (EXPEC). In this classification, a number of pathotypes of diarrheal *E. coli* cause gastroenteritis but they rarely cause extraintestinal diseases. On the other hand, extraintestinal pathogens keep the ability to exist in intestines without causing complications but they have a capacity to be diffused and colonized in other host organs such as blood, central nervous system, and urinary system and they cause diseases. Among extraintestinal *E. coli*, uropathogenic *Escherichia coli* (UPEC) strains are often known as pathogenic factors in human. These strains are the cause of 70-95% of urinary infection cases in the society and 50% of nosocomial cases (14,15). In a prospective study done in Indian Health Center in 2009, among 404 isolates collected from 14 hospitals in 7 Asian countries which were all outpatients and their urine culture report confirmed UTI, *E. coli* and *K. pneumoniae* were the dominant pathogens, whereas *E. coli* was more prevalent in females and *Pseudomonas aeruginosa* and *Morganella morganii* were more prevalent in males. About half of *E. coli* isolates were resistant or intermediate towards ciprofloxacin and at least 30% of isolates were resistant to third-generation cephalosporins such as cefotaxime and ceftriaxone. In this research, also, the highest resistance to third- and fourth-generation cephalosporins was

shown. A similar study was carried out in Taiwan in 2000 on adults infected with UTI in the society and it was found that the predominant urinary pathogen was *E. coli* followed by *P. aeruginosa*, *K. pneumonia*, *Proteus* spp., *Enterobacter*. *Cloacae*, *S. aureus*, *S. saprophyticus*, and *Enterococcu*s spp, and these findings were partly compatible with the previous study (7). Additionally, in this country, *E. coli* strains had shown increasing resistance to cephalosporins and ciprofloxacin. However, a variety of effective antibiotics is applied in order to treat UTI in the society. In our research, the incidence of urinary infection has been obtained 81% for women and 19% for men. Sex proportionality in this study is consistent with other similar studies and this shows that women are more prone to urinary infection than men and this fact can be due to the shorter urethra in women than in men. Moreover, more susceptibility of women to urinary infections can also depend on their behavioral patterns, including sexual activity pattern, and the use of diaphragms and spermicides (these two facilitate the establishment of bacteria around the urethra). Urethra length, the dried environment around the urinary tract, and antibacterial properties of the prostate liquid are associated with fewer cases of infection in men. This is consistent with Abubakar (2009) which declared the incidence in females and males to be 54.3% and 45.7%, Lopez (2011) which reported the incidence in females and males to be 69% and 31%, respectively, and Emamghorashi et al. that reported the incidence level of 65.2% in females(1,4,8). In our study, from among 21 UPEC strains, the serotypes O126, O157, O114, O26, O64, O55, O15, O44, O25, O20, and O1 were obtained. O25 serotypes with 19.04% are considered the most frequent serotype of both genera followed by O126, O44, O55, O1, O44, and O20 with 9.52% and O157, O26, O64, and O15 with 4.77%. In the study of Blanco et al. (1995) in Spain on 103 *E. coli* strains isolated from urinary infections, 68% strains were identified in the serotypes O1, O2, O4, O6, O9, O18, O27, O73, O75, and O77. 32% of strains were not able to be typed. The most frequent serotypes were O2, O4, and O6 (2,3). In the study of Vranes et al. (2001) in Croatia on 160 *E. coli* strains isolated from urinary infections, 75.6% of the strains were identified in the serotypes O1, O2, O4, O5, O6, O7, O8, O9, O11, O15, O17, O18, O25, O50, and O75. 24.4% of strains could not be typed. O2, O4, and O6 were the most frequent strains (12). In our study, the most frequent serotype detected was O25. However, in European countries O2, O4, and O6, in Asian countries including China O1, and in India the serotypes O25, O131, O20, and O101 were the most frequent. O6, O18 and O2, O6 were the most frequent serotypes in the west and north of Iran, respectively. This suggests that many cases of urinary tract infections may be caused by a limited number of *E. coli* strains usually belonging to specific serotypes but the distribution of these serotypes is geographically different (3).

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