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RESEARCH ARTICLE

URINARY TRACT INFECTION: ETIOLOGY AND ANTIMICROBIAL RESISTANCE WITH REFERENCE TO ADHESIVE ORGANELLES

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ABSTRACT

Background: Urinary tract infection (UTI) is a serious health problem, affecting millions of people each year.
Aim: The present study was undertaken to study the profile of uropathogenic bacterial flora in adult, their antibiotic resistant pattern, adherence factors of uropathogens and *in vitro* adherence capabilities of Uropathogens.
Material and Methods: The samples were tested microbiologically by standard procedure. Antibiotic susceptibility of the isolated pathogens was tested for commonly used antibiotics by Kirby-Bauer technique according to CLSI guidelines. Adherence assay were done by hemagglutination test and Giemsa staining technique. Detection of hemolysin production was done on 5% washed sheep blood agar. Motility test was done by stabbing the isolates into SIM biochemical media.
Results: Significant bacteriuria was present in 40% of samples. The most common pathogens isolated were *Escherichia coli* (52%), followed by *Klebsiella pneumoniae* (16%), *Acinetobacter anitratus* (11%), *Proteus mirabilis* (6%), *Enterobacter* species (5%), *Citrobacter* species (2%), *Pseudomonas aeruginosa* (2%), *Morganella morganii* (1%), *Enterococcus* species (3%) and *Staphylococcus aureus* (2%). The mean susceptibility of uropathogens was for amikacin (Ak-81%), nitrofurantoin (Nf-60%), cefotaxime (52%), ceftriaxone (Ci-47%), ciprofloxacin (Cf-45%), norfloxacin (Nx-33%), cotrimoxazole (Co-18%) and nalidixic acid (Na-17%). Mean adherence of pathogens to epithelial cells was 70 bacteria cell^{-1}. Of all, 52% isolates were hemagglutinating, 34% were alpha-hemolytic, 31% were beta-hemolytic and 68% were motile.
Conclusion: *Escherichia coli* are the commonest cause of UTI. Adherence is one of the essential pre-requisites to establish UTI. Majority of UTI in men are mono-microbial. Most of uropathogens are susceptible to amikacin (81%) and nitrofurantoin (60%).
Key words: UTI, uropathogens, adherence, antimicrobials.

INTRODUCTION

Urinary tract is the second most common site of bacterial infections in humans.[1] The prevalence of Urinary tract infection (UTI) depends on age, sex, race and predisposing factors.[2] Predisposing factors in the development of UTI are anatomical, physiological, infective, social and environmental.[1,3] UTI occurs in any age and in sex.[4] Mostly it is common in women than men. Female to male ratio is 4.6:1.[5]

Bacteria responsible for UTI, often originate from the faecal and perineal flora.[6,7] UTIs are almost always caused by bacteria, although some viruses, fungi and parasites can infect the urinary tract as well. More than 85% of UTIs are caused by bacteria. *Escherichia coli* is the commonest cause of UTI followed by *Klebsiella* species, *P. mirabilis, A. anitratus, Enterococcus faecalis,* coagulase negative *Staphylococcus, Streptococcus* species.[1,8,9]

Under normal circumstances, these bacteria are cleared from the urinary system by effective protective mechanisms. If, however, they over come these mechanisms, subsequent progress is determined by host susceptibility and bacterial virulence factors.[7] A significant factor for the pathogenesis of UTI is bacterial adhesion.[10, 11] Uropathogens achieve adherence to mucosal surface of urinary tract by fimbrial and non-fimbrial adhesions.[9] According to hemagglutination (HA) patterns bacterial adhesions have been classified into two types, i.e., mannose sensitive hemagglutination (MSHA) and mannose resistant hemagglutination (MRHA).[12]

Bacteriological examination of the urine is the major aid to the diagnosis of infections. Culture technique is employed to detect bacteria in urine.[13] The organisms are identified and their susceptibility to antimicrobial agents is determined.[14] Adherence assay is done by HA test and Giemsa staining technique.[15,16] Detection of hemolysin production is done on 5% washed sheep blood agar.[17,18] Motility test is done by stabbing the isolates into SIM biochemical media.[17]

MATERIALS AND METHODS

This is prospective study conducted in Department of Microbiology, Yuvaraja's College (Autonomous), University of Mysore, Mysore, India; from December 2010 to November 2012. Urine specimens obtained from adult patients (above 18 years) attending to K.R. hospital and Mission Hospital Mysore, clinically diagnosed as UTI and submitted to Microbiology Department of Clinical Laboratory Service (CLS) for bacteriological culture and sensitivity constitute the subject for study. Informed consent was taken from each subject included in the study. For this study, the inclusion criteria were dysuria, fever due to UTI, frequency, painful micturation and exclusion criteria were malignancy, diabetes, tuberculosis, metabolic disorder, subject on antibiotic treatment, dialysis, HIV seropositive on ART and catheterized urine specimen.

Microscopic study was done for each specimen. The microscopic findings e.g., pus cells, erythrocytes, epithelial cells, casts and crystals were recorded.[19,20]

Urine culture of the un-centrifuged urine was done by semi-quantitative method using standard wire loop. Sterilized inoculation loop was dipped in urine pot at 90°, a loopful of urine holding 0.001 ml was taken. It was inoculated on cystein lactose electrolytes deficient agar (CLED) media, 5% sheep blood agar and MacConkey agar respectively. The inoculated plates were incubated aerobically in bacteriological incubator set at 37°C for 24-48 h.[21,22] Identification was done on the basis of colony morphology, grams stain, catalase test, oxidase test and standard biochemical tests that include triple sugar iron agar (TSI) media, Simmons' citrate agar media, sulphide indole motility (SIM) media, and Christensens' urease medium .[23]

Antibiotic sensitivity test (AST)

It was done by Kirby-Bauer disk diffusion test method on Muller-Hinton agar (MHA) plate and interpreted according to CLSI guidelines. *Escherichia coli* ATCC 25922 were used as control and tested along with the test strains daily as described for *Enterobacteriaceae*.[14]

Adherence assay

Adherence assay was done by HA test[16, 17, 24] and Giemsa staining technique.

A) Hemagglutination (HA) test

It was done by Slide agglutination method and microtiter plate agglutination method.

a) Slide agglutination method

Eight glass slide (3×1 inch) was taken and bacterial isolate number on each glass slide plus human (H), sheep (S), rabbit (Rb), rat (Ra) in first 4 slide respectively; similarly H+, S+, Rb+, Ra+ in next 4 slide respectively for red blood cell suspension with 1% mannose were marked. For each slide 50 µl of PBS with pH 7.2 was added; bacterial colony was taken from 5% sheep blood agar plate and emulsion was made on each glass slide. A total of 50 µl of 4% red cell suspension with 1% mannose and without mannose of all 4 species was added on respective marked slide with bacterial milky white emulsion (approximately 1×10^9 bacteria/ml); then mixed with sterilized wooden tooth pick separately on each glass slide. The slide was rotated for 1 minute.[16] Hemagglutination was observed, initially macroscopically and then microscopically using 10× objective and interpreted as +1, +2, +3 according to agglutination pattern. The results were recorded.

b) Microtiter hemagglutination method

With the help of sterilized inoculation loop, from bacterial stock vial subculture was done in BHI broth (3-5 ml) and incubated aerobically for 4 h at 37°C in bacteriological incubator.[1] Following incubation, subculture was done in 10 ml Brain Heart Infusion (BHI) broth in McCartney bottle and incubated statically aerobically overnight at 37°C in bacteriological incubator.[25] In test tube rack 16 clean, sterilized (25 ml) test tubes were kept and marked 1-16 respectively. Each tube was filled with 9 ml PBS; pH 7.2. Using 1 ml delivering micropipette, the overnight bacterial broth in McCartney bottle was taken in and out 3-4 times, then 1 ml broth was pipetted out, any excess drop was removed with tissue paper from outside of the tips. Carefully the broth in pipette was delivered into the tube number 1, by touching the wall of tube but not dipping into the diluents. With fresh sterile tips, the first dilution was done by filling in and out 3-4 times, and then 1 ml suspension was transferred to next tube (tube number 2). In the same way remaining dilutions were done. Surface viable count was done by Miles and Misra method.[26] Using 20 ml micropipette, from each tube 20 µl suspension was allowed to fall from a height 2.5 cm on a well dried nutrient agar plate in duplicate, where it spreads over 1.5-2 cm in diameter.

The plates were incubated aerobically over night at 37°C. Colony without confluence was counted and calculated bacteria per ml using formula: Bacteria per ml in BHI broth = Number of colony × 50. After that the broth culture was compared with McFarland 0.5 standards. The turbidity was adjusted by diluting with PBS pH 7.2. The tube containing 2×10^8 bacteria per ml in PBS pH 7.2 were used for HA test.[27] A 96 well micro titer plate was taken. For 1 bacterial isolates 8 wells were selected, number isolated and marked M+ and M-. 100 micro liter 4% red blood cells suspension of human, sheep, rabbit, and rat without mannose in PBS pH 7.2 and with 1% mannose in PBS pH 7.2 were added in respective well. Bacterial inoculum (100 µl) was inoculated to BHI broth (2×10^8 bacteria ml^{-1}) in each well and mixed by shaking for 1 minute. Well containing only the suspension of 4% erythrocytes were used as negative control. Using Parafilm, the micro titer plate was covered. A small pellet of erythrocytes at the bottom after 1 hour incubation at 37°C were considered negative, and those containing an even sheet of erythrocytes across the well were considered positive.[27] Following incubation the first reading (HA) was recorded. Again applying parafilm microtiter plate was covered and kept in 4°C for overnight and second reading was recorded.

B) Giemsa staining

Adherence assay of uropathogenic bacteria was done on buccal epithelial cells by Giemsa staining as described by Foresteir et al.[15] With certain modifications for selected uropathogenic clinical isolates. Detection of hemolysin production and motility testing were done by standard microbiological procedure.[17]

RESULTS

Out of 250 test urine specimen, 150 (60%) were either insignificant growth or sterile and 100 (40%) culture positive (significant bacteriuria), 98% were monomicrobial and 2% were polymicrobial (Table-1). The 100% of control specimen were either insignificant growth or sterile. Out of 102 isolates 95 (93.13%) were gram negative bacteria and 7 (6.87%) gram positive bacteria. (Table 1, 2 & 3).

Table 1: Distribution of organisms isolated in UTI patients

Bacteria	IP* n = 125 (%)	OPD** n = 125 (%)	Total n = 250
Escherichia coli	30 (48.4)	22 (55)	52 (51)
Klebsiella pneumonia	10 (16.12)	6 (15)	16 (15.7)
Acinetobacter anitratus	6 (9.7)	5 (12.5)	11 (10.8)
Enterobacter species	3 (4.83)	2 (5)	5 (4.9)
Proteus species	3 (4.83)	3 (7.5)	6 (5.9)
Citrobacter species	2 (3.22)	--	2 (1.96)
Pseudomonas aeruginosa	2 (3.22)	--	2 (1.96)
Morganella morgani	1 (1.61)	--	1 (1)
Enterococcus species	3 (4.83)	1 (2.5)	4 (3.9)
Staphylococcus aureus	2 (3.22)	1 (2.5)	3 (2.94)
Total	**62 (49.6%)**	**40 (32%)**	**102 (100%)**

**** IP: inpatient; **OPD: outpatient department***

Table 2: Incidence of UTI in age group divided in 10 year interval

Age (year)	Significant bacteriuria (%)	Sterile (culture negative) (%)	Total (%)
18-27	17 (32)	36 (68)	53 (21.2)
28-37	13 (27)	35 (73)	48 (19.2)
38-47	12 (41.4)	17 (58.6)	29 (11.6)
48-57	16 (47)	18 (53)	34 (13.6)
58-67	15 (50)	15 (50)	30 (12)
68-77	12 (40)	18 (60)	30 (12)
78-87	15 (57.7)	11 (42.3)	26 (10.4)
Total	**100(40%)**	**150(60%)**	**250 (100%)**

Table 3: Antimicrobial resistance pattern

Bacteria (No.)	A (%)	Ak (%)	Ce (%)	Ci (%)	Cf (%)	Co (%)	Na (%)	Nf (%)	Nx (%)
Escherichia coli (n=52)	100	17.3	47	52	63.5	85	80.8	21.2	71.2
Klebsiella pneumoniae (n=16)	100	12.5	75	75	75	93.7	87.5	68.7	75
Acinetobacter anitratus (n=11)	100	18.2	27.3	27.3	18.2	81.8	81.8	72.7	45.5
Enterobacter species (n=5)	100	60	80	100	40	60	100	60	80
Proteus mirabilis (n=6)	100	0	0	33.3	50	66.7	100	0	50
Citrobacter species (n= 2)	100	50	100	100	50	100	100	100	100
Pseudomonas aeruginosa (n=2)	100	50	100	100	50	100	100	50	100
Morganella morgani (n=1)	100	0	0	0	0	100	0	0	0
Enterococcus species (n=4)	100	0	0	0	0	25	100	100	25
Staphylococcus aureus (n=3)	100	33.3	33.3	0	33.3	33.3	100	33.3	33.3
Total No. (n = 102)	**100**	**19.6**	**47**	**52**	**54**	**80.4**	**82.4**	**40.2**	**65.7**

Hemagglutination properties

There are 23 different agglutination patterns in 52 hemagglutinating uropathogens. (Table-4, 5 & 6)

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Table 4: Distribution of hemagglutination and non-hemagglutination properties of uropathogens isolated from patients with various forms of UTI

Bacteria	Total No.	Only MRHA No. (%)	Only MSHA No. (%)	Both MRHA and MSHA No. (%)	Non-hemagglutination (%)
Escherichia coli	52	4 (12.5)	3 (9.37)	25 (78.12)	20 (38.5)
Klebsiella pneumonia	16	1 (14.28)	4 (57.14)	2 (28.57)	9 (46.25)
Acinetobacter anitratus	11	2 (28.57)	2 (28.57)	3 (42.85)	4 (36.4)
Enterobacter species	5	--	1 (50)	1 (50)	3 (60)
Proteus species	6	--	1 (50)	1 (50)	4 (67.7)
Citrobacter species	2	--	--	1 (100)	1 (50)
Pseudomonas aeruginosa	2	--	--	1 (100)	1 (50)
Morganella morgani	1				1 (100)
Total (%)	**95**	**7 (13.46)**	**11 (21.11)**	**34 (65.38)**	**43 (45.27)**

Table 5: Pattern of adherence to human buccal epithelial cells with hemagglutination property of uropathogens

Bacteria	Mean adhesion in only MRHA	Mean adhesion in only MSHA	Mean adhesion in MRHA and MSHA
Escherichia coli	45	62	71
Klebsiella pneumonia	40	54	60
Acinetobacter anitratus	42	55	65
Enterobacter species	-	50	55
Proteus species	-	44	65
Citrobacter species	-	-	54
Pseudomonas aeruginosa	-	-	50

Table 6: Distribution of hemolysin production by gram negative uropathogenic isolates

Bacteria	Total No.	Alpha hemolysis (%)	Beta hemolysis (%)	Gamma hemolysis (%)
Escherichia coli	52	24 (46.15)	14 (27)	14 (27)
Klebsiella pneumonia	16	4 (25)	3 (18.75)	9 (56.25)
Acinetobacter anitratus	11	1 (9)	5 (45.45)	5 (45.45)
Enterobacter species	5	-	2 (40)	3 (60)
Proteus species	6	1 (16.67)	2 (33.33)	3 (50)
Citrobacter species	2	1 (50)	1 (50)	-
Pseudomonas aeruginosa	2	-	2 (100)	-
Morganella morgani	1	1 (100)	-	-
Total	**95**	**32 (33.7)**	**29 (30.5)**	**34 (35.8)**

DISCUSSION

In present study, *E. coli* was the most common organism isolated in 51% of patient. This finding is in between result of study by Raco *et al.*[28] (1998) (49.37%), Bonadio *et al.*[29] (2001) (54.7%), *K. pneumoniae* was the second most common organism isolated in 16% of patient. The similar result was seen in study by Das *et al*. (2006) (15.7%)[30] and Neto *et al.*[31] (2003) (15%). *Acinetobacter anitratus* was the third most common organism isolated in 11% of patient. This result is higher than the other study.[30,31] *Proteus mirabilis* was the fourth most common organism isolated in 6% of patient. The similar findings was studied by Villar *et al.*[32] (1996) (6%) and Raco *et al.*[28] (1998) (8.86%). *Pseudomonas aeruginosa* was isolated 2% of patient. The similar finding was seen in study by Das *et al.* [30] (2006) (2.9%), 4 isolates of *Enterococcus* species

(3.9%) and *S. aureus* of 2.94%. The similar finding was seen in study by Das *et al.*[30] (2006) (3.4%).

Significant bacteriuria was highest 57.7% in age group 78-87, followed by 50% in age group 58-67, 47% in age group 48-57, 41.4% in age group 38-47, 40% in age group 68-77, 32% in age group 18-27 and 27% in age group 28-37.The present results indicate that incidence of UTIs is more in older male patients. It might be due to prostate enlargement, because 42 patients included in present study were having BPH as an underlying condition. This result was supported by previous study.[33]

Antimicrobial resistance

The problem of bacterial antibiotic resistance emerged as soon as the first antibiotics became available for clinical use. Area-specific monitoring studies aimed to gain knowledge about the type of pathogens responsible for UTIs and their resistance patterns may help the clinician to choose the correct treatment.[34] In present study, the antimicrobial resistance pattern of bacteria isolated from patients with UTI shows that 100% to ampicillin, 82.4% to nalidixic acid, 80.4% to cotrimoxazole, 65.7% to norfloxacin, 54% to ciprofloxacin, 52% to ceftriaxone, 47% to cefotaxime, 40.2% to nitrofurantoin and 19.6% to amikacin.

Most of isolated uropathogens showed multiple antibiotics resistance in this area. It may be due to large proportion of the bacterial isolate being previously exposed to several antibiotics. The present study data gives an idea about the common trend of increased antibiotics resistance of uropathogens in this region, which may be due to geographic variation or indiscriminate or sub lethal use of antibiotics. These results not only help in proper treatment of UTI patients but also discourage the indiscriminate use of antibiotics and prevent further development of bacterial drug resistance. This result will also help the clinicians to give proper treatment and prescription of most sensitive antibiotic to the patient and avoid use of resistant antibiotics.

Hemagglutination properties

In the present study, 95 gram negative uropathogenic bacteria have been tested for HA by using 4% RBCs suspension of human, sheep, rabbit and rat in absence and in presence of 1% mannose, 52 (54.73%) were hemagglutinating and 43 (45.27%) non-hemagglutinating. *Escherichia coli* and *A. anitratus* were maximum hemagglutinating 61.5% and 63.5% respectively, followed by *Citrobacter* species and *P. aeruginosa* 50%, *K. pneumoniae* 43.7% and *Enterobacter* species 40%. There were 23 different agglutination patterns in 52 hemagglutinating uropathogens. Thirty-seven (71.15%) hemagglutinating bacteria were both MRHA and MSHA of different species of erythrocytes, 10 (19.23%) were only MRHA of different species of erythrocytes and only 7 (13.46%) were MSHA of different species of erythrocytes.

Fifty-two hemagglutinating gram negative uropathogenic bacteria have been tested for adherence to human buccal epithelial cells. Adherence was higher in gram negative uropathogenic bacteria which give both MSHA and MRHA, in comparison to only MRHA or MSHA respectively. Maximum adherence capability was seen in *E. coli* (71/ cell) giving both MSHA and MRHA, followed by *A. anitratus* and *Proteus* species (65/cell), *K. pneumoniae* (60/cell), *Enterobacter* species (55/cell) and *P. aeruginosa* (50/cell). Similarly adherence of uropathogens giving only MRHA, higher adherence capability was seen in *E. coli* (62/cell), followed by *A. anitratus* (55/cell), *K. pneumoniae* (54/cell), *Enterobacter* species (50/cell) and *Proteus* species (44/cell). There was no only MRHA in other uropathogens. Adherence of uropathogens giving only MSHA, higher adherence capability was seen in *E. coli* (45/cell), followed by *A. anitratus* (42/cell) and *K. pneumoniae* (40/cell). There was no only MSHA in other uropathogens.

The present results show that *E. coli* has higher adherence capability then other uropathogens, regardless of type of HA. The present study describes an *in vitro* model for the study of bacterial adhesion, using buccal epithelial cells of healthy individuals and uropathogens isolated from patients with UTI. The results confirm that adherence of microorganisms to uroepithelial cells is important for the establishment of infections of urinary tract. In present study 95 gram negative uropathogenic bacteria have been tested for hemolysin production; 32 (33.7%) were α-hemolytic, 29 (30.5%) were β-hemolytic and 34 (35.8%) were γ-hemolytic.The present results indicate that there is no significant correlation in the hemolytic properties in case of hemagglutinating and non-hemagglutinating gram-negative uropathogens.

CONCLUSION

Escherichia coli are the commonest cause of UTI. Adherence is one of the essential pre-requisites to establish UTI. Majority of UTI in men are mono-microbial. Most of uropathogens are susceptible to amikacin (81%) and nitrofurantoin (60%). Most of the Gram-negative bacteria showed hemagglutinating to both MRHA and MSHA of different species of erythrocytes. Maximum adherence capability was seen in *E. coli* (71/ cell) giving both MSHA and MRHA.

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