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ISOLATION AND CHARACTERIZATION OF MULTI DRUG RESISTANT UROPATHOGENIC *ESCHERICHIA COLI* FROM URINE SAMPLE OF URINARY TRACT INFECTED PATIENTS

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ABSTRACT

Abuse and improper prescribing policy of antibiotics causes remarkable increase of antibiotic resistance pattern among the *E. coli* isolates from Urinary Tract Infection (UTI). This study was conducted to isolate and identify multi drug resistant uropathogenic *Escherichia coli* from urine sample of urinary tract infected patients by convention and traditional techniques. Twenty two (22) UTI urine samples were collected from patients admitted to nearby hospitals. Characterization of isolated strains were determined by biochemical tests like Gram staining, indole, oxidase, catalase, methyl red, voges-proskauer, citrate utilization, hemolysis, motility; hydrolysis test of gelatin and urea; fermentation and utilization tests of glucose, lactose and sucrose. Sensitivity pattern of isolates were determined against some traditional and conventional antibiotics. From this study, it was revealed that 56.75% isolates were gram negative. Indole positivity was observed for 95.23% of Gram negative isolates. 100% of indole positive isolates were positive for catalse, methyl red, nitrate reduction, motility, hemolysis, carbohydrate fermentation on broth medium and carbohydrate utilization on Triple sugar iron agar. Negativity was seen for oxidase, Voges-Proskauer, urease, citrate, and gelatinase test. It was also observed that 100% of indole positive isolates gave positivity on differential media such as Mac Conkey agar and EMB agar. Antibiotic sensitivity indicates 100% of characterized strains were multi drug resistant. The findings of the study suggested that 20 uropathogenic *E. coli* were identified and all of them were multi drug resistant strains.

Keywords: Uropathogenic *Escherichia coli*, antibiotic susceptibility, disc agar diffusion, multi drug resistant.

1. INTRODUCTION

Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune,* isolated from the feces of newborns. It was later renamed *Escherichia coli,* and for many years the bacterium was considered as a commensal microorganism of the large intestine, provides benefit to their host by producing vitamin K_2 and giving a barrier against the attachment to other pathogenic bacteria in the intestine (Bentley R and Meganathan R, 1982; Hudault S, 2001; Reid G et al. 2001). It was not until 1935 that a strain of *E. coli* was shown to be the cause of an outbreak of diarrhea among infants.

Urinary tract infection (UTI) is one of the most common and life threatening infection present in

community practice (Stamm WE and Hooton TM, 1993; Warren JW et al. 1999). It occurs when a significant number of microorganisms ($>10^5$ cells/ml) present in urine from catheter specimen (Williams DN, 1996). Uropathogenic *E. coli* (UPEC) is responsible for approximately 90% of urinary tract infections (Todar K, 2007). UPEC utilizes P fimbrine to bind specifically to the P blood group antigen which contains a D-galactose-D-galactose residue. Binding of this P fimbriae not only specific to red blood cell but to a specific galactose disaccharide that is found on the surface of uroepithelial cells in approximately 99% of the population. UTI may associate both upper and lower tract. Lower tract UTI describes as cystitis. The major symptoms of cystitis are the urgency of urination, dysuria, irritation of urinary tract and tiredness. Most of the cases UTI occur as community acquired infection (Akram M et al. 2007). UTI very often treated with broad spectrum of antibiotics where as one narrower spectrum antibiotic may be effective. Fluoroquinolone are preferred as initial agents for empiric therapy of UTI for their high bacteriological and clinical cure rates. Abuse and improper prescribing policy of antibiotics causes remarkable increase of antibiotic resistance pattern among the *E. coli* isolates from UTI (Li Q et al. 2007). These types of resistance associated with genetic mutation and intra or inter species transfer of resistance gene through plasmid (Hughes M and Datta N, 1983). Microorganisms considered multidrug resistance (MDR) when it was resistance to at least three antibiotics (Santo E et al. 2007). Frequency of UTI cases caused by multidrug resistance *E. coli* required strong concern of medical practitioners and health agencies. Therefore regional studies on pattern of antibiotic sensitivity are very much necessary to overcome this problem.

Considering the majority of UTI cases caused by *E. coli* and increasing use of antibiotics followed by growing resistance in bacteria and emerging MDR strains, the present study was conducted to identify the UPEC and also investigate the drug resistance pattern of those *E. coli* strains collected from Midnapore Medical Collage and Hospital, West Midnapore, West Bengal, India. This will be useful for clinician in order to improve the empiric treatment.

2. MATERIALS AND METHODS

2.1 Culture media and Chemicals

Luria broth, Nutrient agar, Tryptic soy broth, agar powder, Beef extract, pancreatic digest of casein, blood agar base, Mueller-Hinton broth, Mac Conkey agar w/o crystal violet, EMB agar, Simmon's Citrate agar, Crystal violet, Lugol's iodine, Safranine N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride, antibiotic discs such as penicillin G, ampicillin, cephotaxime, gentamycin, streptomycin, tetracycline, erythromycine, chloramphenicol, kanamycine, oxacillin, norfloxacin, ciprofloxacin, amikacin and imipenem, were purchased from Himedia, India, Sodium chloride (Nacl), hydrogen peroxide (H_2O_2), sucrose, lactose, glucose, galactose, potassium dihydrogen phosphate (KH_2PO_4), di potassium hydrogen phosphate (K_2HPO_4), sodium hydroxide (NaOH), potassium chloride (Kcl), were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2 Collection and transport of sample

Twenty (22) UTI patient's urine samples were collected from patients admitted to nearby hospitals according to their infection history and treatment summary during a two month period from November 15, 2010 to January 15, 2011. These urine samples were the first 'clean catch' collected with sterile clean bottles. Urines were then transported to the laboratory in autoclaved Luria broth (LB) within 2 hours of collection (Chakraborty SP et al. 2011a).

2.3 Culture of microorganisms

Urine, kept in LB was incubated in a shaking incubator at 37°C for overnight. Bacterial cultures were grown on Nutrient agar (NA) media and purified by a single colony isolation technique on NA containing 10% sodium chloride (Chakraborty SP et al. 2011a). Isolates were sub cultured on fresh NA plates for characterization studies.

2.4 Quality control strains

S. aureus ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *Shigella flexneri* ATCC 12022 were obtained from Microbiology laboratory of Calcutta University and *S. epidermidis* ATCC 12228, *S.epidermidis* NCTC 5866, *E. coli* ATCC 23509, *E. coli* ATCC 25922, *E. faecalis* ATCC 51299 , *E. faecalis* ATCC 29212, *Pseudomonas aeruginosa 27853*, *Enterobactor aerogenes* ATCC 1304, *Proteus vulgaris* ATCC 13315, *Acinetobacter calcoaceticus* ATCC 19606 were obtained from Microbiology laboratory of Midnapore Medical College and Hospital. These strains were stored in agar slants at 4°C for further studies as reference strains.

2.5 Screening through Gram staining

Gram staining of isolates was performed according to standard method (Duguid JP, 1999). Briefly, on a glass slide bacterial smear was prepared from broth culture and heated gently to fix. The slide was flooded with 0.5% crystal violet and left for 30 sec. Then it was tilted and poured sufficient 1% Lugol's iodine to wash away the excess stain. The slide was covered with fresh iodine and allowed to act for 30 sec. The slide was tilted and washed off the iodine with 95 - 100% ethanol until colour ceases to run out of the smear. The slide was rinsed with water and 0.1% safranin was poured on it and left to act for 2 min. The slide was washed with water and blotted to dry and observed under microscope. *E. coli* ATCC 25922 was taken as positive control for this test.

2.6 Biochemical characterization tests

2.6.1 Catalase test

Both on tube and slide catalase test of isolates was performed according to MacFaddin (MacFaddin JF, 2000) using 3-6% hydrogen peroxide (H_2O_2). *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were taken as negative and positive control, respectively.

2.6.2 Oxidase test

Oxidase test of isolates was performed by filter paper method according Snell JJS et al. 1999 using 1% N, N, N', N'-tetramethyl-pphenylenediamine dihydrochloride solution. *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were taken as negative and positive control, respectively.

2.6.3 Indole test

Indole test was done by adding Kovac's reagent to culture media according to Cheesbrough, 1985**.** *E. coli ATCC 25922* and *Pseudomonas aeruginosa 27853* were taken as positive and negative control strains respectievely.

2.6.4 MR-VP test

Both MR and VP tests were done according to Cheesbrough, 1985 using 0.7 gm% Peptone mixture, 0.5 gm% Potassium Phosphate and 0.5 gm% Dextrose for culture media supplements. 0.4% Methyl red solution, 5% alpha napthanol in absolute alcohol and 40% sodium hydroxide solution were used as indicator for the said tests. *E. coli* ATCC 25922 and *Enterobactor aerogenes* ATCC 1304 were taken as positive and negative control strains for this test respectively.

2.6.5 Citrate Utilization

Citrate utilization test was performed according to the standard method of Simmons J, 1960 using, 0.1 gm% ammonium di-hydrogen phosphate, 0.5 gm% NaCl, 0.02 gm% $MgSO_4$, 0.008 gm% Bromothymol blue, 0.1 gm% di-potassium phosphate, 0.2 gm% Sodium citrate and 1.5 gm% Bacteriological agar. *E. coli* ATCC 25922 and *Enterobactor aerogenes* ATCC 1304 strains were taken as positive and negative control respectively for these tests.

2.6.6 Gelatinase test

Gelatin hydrolysis test was done according the method of Chakraborty SP et al. 2011b. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 strains were taken as positive control and negative control respectively.

2.6.7 Urease test

Urease test for bacterial isolates was done according to the method Chakraborty SP et al. 2011b. Media were prepared with urea 2 gm %, Agar 1.5 gm %, Nacl 0.5gm%, KH_2PO4 0.2 gm% and phenol red 0.0012 gm% in slant position. *E. coli* ATCC 25922 and *Proteus vulgaris* ATCC 13315 were taken as positive and negative control strains for the test.

2.6.8 Triple sugar Iron (TSI) tests

Triple sugar Iron test was performed according to Vanderzant C and Splitt stresser DF, 1992. All the bacterial isolates were inoculated on sterilized Triple Sugar Iron Agar tubes containing Peptone mixture 2 gm%, Sucrose 1 gm%, Beef extract 0.3 gm%, Dextrose 0.1 gm%, Sodium thiosulphate 0.03 gm%, Lactose 1.0 gm%, Sodium Chloride 0.5 gm%, Yeast Extract 0.3 gm%, Ferrous Ammonium Citrate 0.025 gm% and Bacteriological Agar 1.2 gm%. *E. coli* ATCC 25922 strains was considered as positive control.

2.6.9 Carbohydrate fermentation tests

Carbohydrate fermentation test of clinical isolates were performed according to the method of Cheesbrough, 1985. In brief, Isolates were inoculated on Phenol red Dextrose broth (Casein peptone 1.0 gm%, NaCl 0.5 gm%, Dextrose 0.5 gm%, Phenol red 0.0018 gm %) with Durham tubes for glucose fermentation study. For Sucrose and Lactose fermentation isolates were inoculated on Phenol red Sucrose broth(Casein peptone 1.0 gm%, NaCl 0.5 gm%, Sucrose 0.5 gm%, and Phenol red 0.0018 gm%) and Phenol red Lactose broth (Casein peptone 1.0 gm%, NaCl 0.5 gm%, Lactose 0.5 gm%, and Phenol red 0.0018 gm%). For all three fermentation tests broth medium were incubated for 48 hours in an aerobic incubator. Positive result was noted by change of media colour from red to yellow and production of gas on Durham's tube. *E. coli* ATCC 25922 and *Shigella flexneri* ATCC 12022 were taken as positive and negative control strains respectively.

2.6.10 Motility test

Motility test of isolates was performed according to Cheesbrough, 1985. Briefly, semi solid agar was prepared using beef extract-0.3%, pancreatic digest of casein-1.0%, Nacl 0.5% and agar-0.4%. *E. coli* ATCC 25922 *S. aureus* ATCC 25923 and were taken as positive and negative control, respectively.

2.6.11 Nitrate reduction test

Nitrate reduction tests were done according to Snell "Snell EE and Wright LD, 1941". In brief, Nitrate broth was prepared (casein Peptone 0.5 gm%, Beef Extract 0.3 gm%, Potassium Nitrate 0.1 gm%, Galactose 0.5 gm% and Disodium Phosphate 0.25 gm %) with Durham tubes (gas collector) in it. 0.8 gm% sulphanilic acid (dissolved in 5M acetic acid) and 0.6 gm% alpha naphthole (dissolved in 5M acetic acid) and Zinc dust were simultaneously added to those cultures. *Acinetobacter calcoaceticus* ATCC 19606 and *E. coli* ATCC 25922 were taken as negative and positive control strains respectively.

2.6.12 Hemolysis on blood agar

Hemolysis test of isolated bacteria was performed by inoculating them into blood agar plates (Nutrient agar 2.4 gm% and 5 % Sheep blood). Plates were incubated at 37°C for 24 hr. A clearing zone surround the bacterial colony was observed and recorded (Bannerman TL, 2003). *E. coli* ATCC 23509 and *E. coli* ATCC 25922 were taken as negative and positive control, respectively.

2.6.13 Screening agar media

Bacterial isolates were inoculated on Mac Conkey agar w/o Crystal violet (Gelatin peptone 1.7 gm%, Bile salts 0.5 gm%, Peptone mixture 0.3 gm%, Lactose 1.0 gm%, NaCl 0.5 gm%, Neutral red 0.003 gm% and Bacteriological agar 1.2 gm%) and incubated overnight at 37ºC. Pink-Red colour colony was considered as lactose fomenter organisms. Eosin Methylene Blue agar (EMB) was also prepared for screening test of isolated bacteria depending on the ability to ferment Lactose and Sucrose. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 10145 were taken as positive and negative control strains (Eaton AD et al. 1995).

2.7 Antibiotic susceptibility testing of isolated *E. coli* strains

2.7.1 Determination of Minimum Inhibitory Concentration

The MIC values of Penicillin, Ampicillin, Tetraclyclin, Gentamycin, Erythromycin, Chloramphenicol, Streptomycin, Kanamycin, Oxacillin, Norfloxacillin, Ciprofloxacin, Amikacin, and Imipenem, were determined by a broth dilution method using Mueller–Hinton broth (MHB), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). About 5 x 10^4 cells in MHB were treated with different concentrations of antibiotics and

shaken for 16 h at 37°C. The minimum concentration at which there was no visible turbidity was taken as the MIC of that antibiotic (Chakraborty SP et al. 2011b).

2.7.2 Determination of Minimum Bactericidal Concentration

The MBC value of antibiotics was determined according to Chakraborty SP et al. 2011b. This is an extension of the MIC Procedure. Antibiotics treated bacterial culture showing growth or no growth in the MIC tests were used for this test. Bacterial culture used for the MIC test were inoculated onto the Mueller–Hinton agar and incubated at 37°C for 24 hr. Microbial growth or death were ascertained via no growth on Mueller–Hinton agar plate. The minimal concentration of the antibiotic that produced total cell death is the MBC.

2.7.3 Susceptibility testing by disc agar diffusion

Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method (Bauer AW et al. 1966) and according to the method of Chakraborty SP et al. 2011b. The tested bacterium was from an overnight culture (inoculated from a single colony) and freshly grown for 4 hours at approximately 10^6 CFU/ml. With this culture, a bacterial lawn was prepared on Mueller-Hinton agar. Filter paper discs of 6-mm size were used to observe antibiotic susceptibility patterns against 14 antibiotics [amount of antibiotic per disc in microgram (µg); Penicillin G (10 Unit), Ampicillin (10 µg), Tetraclyclin (30 µg), Gentamycin (10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg), Streptomycin (10 µg), Kanamycin (30 µg), Oxacillin (1 µg), Norfloxacillin (10 µg), Ciprofloxacin (10 µg), Amikacin (30 µg), and Imipenem (10 µg)]. Antibiotic discs were obtained commercially from Himedia. The diameter of zone of bacterial growth inhibition surrounding the disc (including the disc) was measured and compared with the standard for each drug. This gave a profile of drug susceptibility vis- à - vis antibiotic resistance. Quality control was performed using *E. coli* ATCC 25922.

3. RESULT

3.1 Biochemical tests of isolates

The clinical isolates were identified using standard biochemical tests. Purification of bacterial culture were done using single colony isolation technique on Nutrient agar containing 10% sodium chloride, exhibited several types of colonies. From the study it was observed that, 56.75% isolates were Gram negative and 43.25% isolates were Gram positive; 95.23% of gram negative isolates are indole positive. 100% of indole positive isolates were Catalase positive, MR positive, Nitrate reduction test positive, Carbohydrate fermentation (Glucose, Lactose, Sucrose) test positive and also positive for carbohydrate utilization with gas production on TSI. All Indole positive isolates were negative for Oxidase test, VP test, Urease test, Citrate test and Gelatinase test. It was also revealed that 100% of Indole positive isolates were uropathogenic for giving hemolytic activity (60 % beta hemolytic and 40 % gamma hemolytic) on blood agar and are all motile (Table 1a and Table 1b). On MacConkey agar w/o Crystal violet and EMB agar 100% of Indole positive isolates gave red-pink colour colony and green with metallic shine colony respectievely. Thus among 37 clinical isolates 20 (54.05%) isolates were confirmed to be uropathogenic *Escherichia coli* (UPEC).

Table 1a: ***Standard biochemical tests of clinical isolates, collected from urine sample of UTI patient. ND = Tests are not done, + ve = tests are positive, - ve = tests are negative.***

Sample	Isolates	Gram Staining	Indole	Oxidase	Catalase	MR	VP	Urease	Citrate utilization
S1	S1a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S1b	+ ve	ND	ND	ND	ND	ND	ND	ND
S2	S2a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S3	S3a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S3b	+ ve	ND	ND	ND	ND	ND	ND	ND
S4	S4a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S4b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S5	S5a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S5b	+ ve	ND	ND	ND	ND	ND	ND	ND
S6	S6a	- ve	- ve	ND	ND	ND	ND	ND	ND
	S6b	+ ve	ND	ND	ND	ND	ND	ND	ND
S7	S7a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S8	S8a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S8b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S9	S9a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S9b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S10	S10a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S11	S11a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S11b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S12	S12a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S12b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S13	S13a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S13b	+ ve	ND	ND	ND	ND	ND	ND	ND
S14	S14a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S14b	+ ve	ND	ND	ND	ND	ND	ND	ND
S15	S15a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S16	S16a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S16b	+ ve	ND	ND	ND	ND	ND	ND	ND
S17	S17a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S18	S18a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S18b	+ ve	ND	ND	ND	ND	ND	ND	ND
S19	S19a	+ ve	ND	ND	ND	ND	ND	ND	ND
	S19b	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
S20	S20a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve
	S20b	+ ve	ND	ND	ND	ND	ND	ND	ND
S21	S21a	+ ve	ND	ND	ND	ND	ND	ND	ND
S22	S22a	- ve	+ ve	- ve	+ ve	+ ve	- ve	- ve	- ve

Table 1b: ***Standard biochemical tests of clinical isolates, collected from urine sample of UTI patient. ND = Tests are not done, + ve = tests are positive, - ve = tests are negative. A/AG = Acid slain and acid butt with gas production.***

Sample	Isolates	Nitrate reduction	Gelatinase	TSI	Carbohydrate fermentation			Hemolysis	Motility
					G	L	S		
S1	S1a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S1b	ND	ND	ND	ND	ND	ND	ND	ND
S2	S2a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S3	S3a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S3b	ND	ND	ND	ND	ND	ND	ND	ND
S4	S4a	ND	ND	ND	ND	ND	ND	ND	ND
	S4b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S5	S5a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S5b	ND	ND	ND	ND	ND	ND	ND	ND
S6	S6a	ND	ND	ND	ND	ND	ND	ND	ND
	S6b	ND	ND	ND	ND	ND	ND	ND	ND
S7	S7a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S8	S8a	ND	ND	ND	ND	ND	ND	ND	ND
	S8b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S9	S9a	ND	ND	ND	ND	ND	ND	ND	ND
	S9b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S10	S10a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S11	S11a	ND	ND	ND	ND	ND	ND	ND	ND
	S11b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S12	S12a	ND	ND	ND	ND	ND	ND	ND	ND
	S12b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S13	S13a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S13b	ND	ND	ND	ND	ND	ND	ND	ND
S14	S14a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S14b	ND	ND	ND	ND	ND	ND	ND	ND
S15	S15a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S16	S16a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S16b	ND	ND	ND	ND	ND	ND	ND	ND
S17	S17a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S18	S18a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S18b	ND	ND	ND	ND	ND	ND	ND	ND
S19	S19a	ND	ND	ND	ND	ND	ND	ND	ND
	S19b	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
S20	S20a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile
	S20b	ND	ND	ND	ND	ND	ND	ND	ND
S21	S21a	ND	ND	ND	ND	ND	ND	ND	ND
S22	S22a	+ ve	- ve	A/AG	+ ve	+ ve	+ ve	+ ve	Motile

3.2 Antibiotic susceptibility testing

3.2.1 MIC of antibiotics

The MIC values of Penicillin, Ampicillin, Tetraclyclin, Gentamycin, Erythromycin, Chloramphenicol, Streptomycin, Kanamycin, Oxacillin, Norfloxacillin, Ciprofloxacin, Amikacin, and Imipenem, for UPEC isolates were determined. In each set of experiment, bacterial control tubes showed no growth inhibitory effect of antibiotics. These MIC values were compared with the NCCLS breakpoints of Minimum Inhibitory concentration for UPEC. It was observed that MIC values of penicillin G, erythromycin, streptomycin and oxacillin for 100% of isolated UPEC strains; ampicillin and kanamycin for 90% of isolates; gentamycin and ciprofloxacin for 70% isolates; tetraclyclin and norfloxacin for 60% isolates; cephotaxime, amikacin and chloramphenicol for 40% UPEC strains was beyond the sensitive range (Figure 1a, Figure 1b).

Figure 1a: ***Determination of MIC value of antibiotics for E. coli isolates. MIC of Ciprofloxacin for MC 17 isolate is 2 µg/ml . Here, -ve : negative control, + ve : positive control, A : 0.5 µg/ml, B :2 µg/ml, C: 4 µg/ml, D : 8 µg/ml, E: 16 µg/ml, F: 32 µg/ml, G: 64 µg/ml, H: 128 µg/ml, I: 256 µg/ml.***

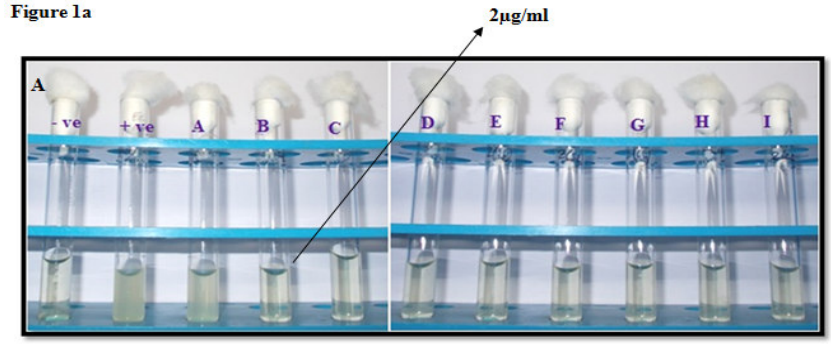
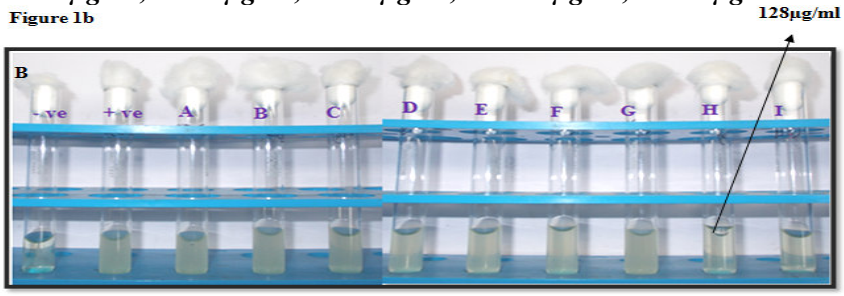


Figure 1b: ***Determination of MIC value of antibiotics for E. coli isolates. MIC of Ciprofloxacin for MC 4 is >256 µg/ml. Here, -ve : negative control, + ve : positive control, A : 0.5 µg/ml, B :2 µg/ml, C: 4 µg/ml, D : 8 µg/ml, E: 16 µg/ml, F: 32 µg/ml, G: 64 µg/ml, H: 128 µg/ml, I: 256 µg/ml.***



3.2.2 MBC of antibiotics

The MBC values of all said antibiotics for UPEC isolates were determined. In each set of experiment, bacterial control plates showed no growth. It was observed that MBC values of penicillin G, erythromycin, streptomycin and oxacillin for 100% of isolated UPEC strains; ampicillin and kanamycin for 90% of isolates; gentamycin and ciprofloxacin for 70% isolates; tetraclyclin and norfloxacin for 60% isolates; cephotaxime, amikacin and chloramphenicol for 40% UPEC strains was beyond next two concentrations of MIC values (Figure 2a and Figure 2b).

Figure 2a: ***Determination of MBC value of antibiotics for E. coli isolates. MBC of Ciprofloxacin for MC 17 isolate is 4µg/ml (A). Here, -ve : negative control, + ve : positive control, A : 0.5 µg/ml, B :2 µg/ml, C: 4 µg/ml, D : 8 µg/ml, E: 16 µg/ml, F: 32 µg/ml, G: 64 µg/ml, H: 128 µg/ml, I: 256 µg/ml.***

Figure 2a

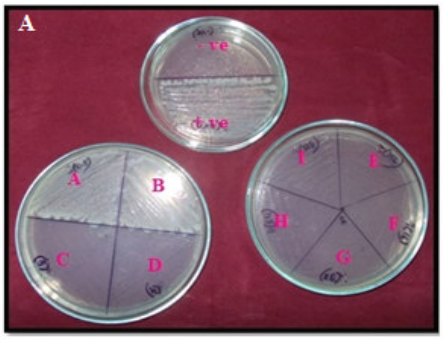


Figure 2b: ***Determination of MBC value of antibiotics for E. coli isolates. MBC of Ciprofloxacin for MC 4 is >256 µg/ml (B). Here, -ve : negative control, + ve : positive control, A : 0.5 µg/ml, B :2 µg/ml, C: 4 µg/ml, D : 8 µg/ml, E: 16 µg/ml, F: 32 µg/ml, G: 64 µg/ml, H: 128 µg/ml, I: 256 µg/ml.***

Figure 2b

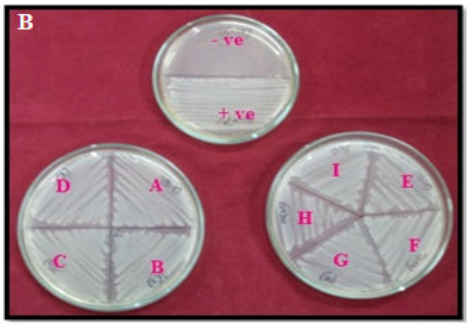


Figure 3: ***Disc agar diffusion test of MC 4 against 14 antibiotic discs. Here P: Penicillin G, A: Ampicillin, T: tetraclyclin, G: Gentamycin, E: Erythromycin, C: Chloramphenicol, S: Streptomycin, K: Kanamycin, Ox: Oxacillin, Nx: Norfloxacillin, Cf: Ciprofloxacin, Ak: Amikacin, and I: Imipenem.***

Figure 3

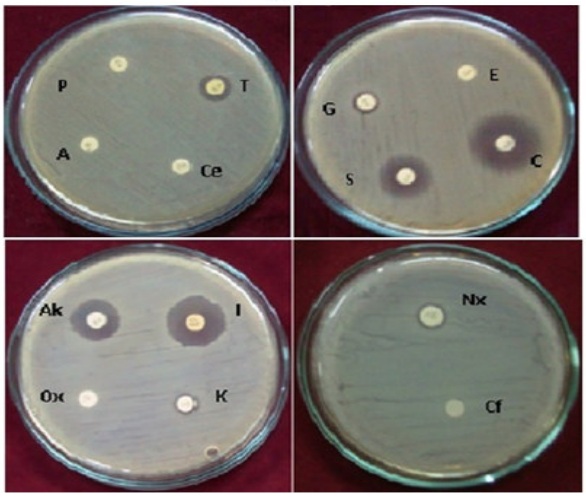
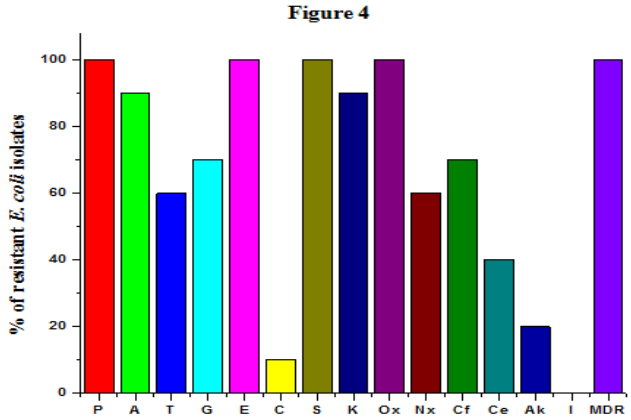


Figure 4: ***Antibiotic sensitivity pattern of 20 uropathogenic E. coli strains isolated from urine sample of UTI patient.***



3.2.3 DAD test

Antibiotic resistance profile and pattern of multi drug resistance by DAD reveled that out of 20 UPEC isolates 100% resistance property were found to penicillin G, erythromycin, streptomycin and oxacillin; 90% resistance found to ampicillin and kanamycin; 70% resistance to gentamycin and ciprofloxacin; 60% resistance to tetraclyclin and norfloxacin; 40% or less resistance pattern was identified to cephotaxime, amikacin and chloramphenicol. All 20 isolates were sensitive to imipenem (Figure 3-4). Isolated strains those are resistant to 3 or more antibiotics are considered as multi drug resistance strains (MDR). It was found that 100% of *E. coli* isolates were multidrug resistant. There are eight types of resistance pattern were recognized among MDR strains. Among the 20 MDR strains 10% were resistant to five antibiotics, 30% to seven antibiotics, 20% to eight antibiotics, and 40% to nine or more antibiotics (Table 2).

Table 2: ***Pattern of multidrug resistance among uropathogenic E. coli isolates.***

Antibiotic sensitivity pattern	Number (%)
P, G, E, S, Ox	2 (10%)
P,A T, E, S, K, Ox, Ce	2 (10%)
P, A, G, E, S, K, Ox, Nx, Cf	4 (20%)
P, A, T, E, S, K, Ox, Nx, Cf	4 (20%)
P, A, T, G, E, S, K, Ox, Ce	2 (10%)
P, A, T, G, E, C, S, K, Ox, Ce	2 (10%)
P, A, G, E, S, K, Ox, Nx, Cf, Ak	2 (10%)
P, A, T, G, E, S, K, Ox, Nx, Cf, Ce	2 (10%)

4. DISCUSSION

Antibiotic resistance is a major clinical problem in treating infections caused by *E. coli.* The resistance to the antimicrobials has increased over the years

and normal intestinal microbial flora became a reservoir for resistant genes (Okeke IN et al. 2000).This may be due to an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a vital role in the evolution of antibiotic resistance among bacteria. These bacteria then pass the plasmid containing resistant gene among other bacterial cells and species (Chakraborty SP et al. 2011a).

Throughout the study, twenty two UTI infected patient's urine samples were collected from nearby hospitals, samples were transported to the laboratory within 2 hours of collection and species identification was carried out by Gram staining and standard biochemical characterization tests. In this study, 56.75% of clinical isolates were Gram negative and 25.93% isolates were Gram positive. Gram positive clinical isolates were not involved in this study as it is commonly known that *E. coli* is gram negative. Clinical isolates were gram negative, which may be due to having a thinner peptidoglycan layer and presence of outer lipid membrane separated by periplasmic space. Crystal violet-iodine complex (CV-I) binds both inner and outer wall; but during decolorization this outer lipopolysaccharide layer looses its integrity and inner membrane become exposed. Washing of CV-I complex and counter stain Safranine presented the cell wall as red-pink appearance (Okeke IN et al. 2000).

Enterobacteriaeae (enterics) are Gram-negative bacteria that grow in the intestinal tract of humans and other animals. The IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests are frequently employed for identification of this group of microbes which includes such organisms as *Klebsiella*, *Enterobacter*, and *Escherichia coli* (Barnes JH et al. 2003). Our study shows 95.23 % of gram negative isolates were indole positive this is due to production of the tryptophanase enzyme by those isolates that can break down the amino acid tryptophan to indole. When indole reacts with para dimethylaminobenzaldehye (Kovac's reagent) a pink-colored complex is produced. Indole positivity of those isolates differentiates them from most *Klebsiella sp* and *Enterobacter sp* (MacFaddin JF, 2000). Table 1a shows 100% of indole positive isolates were MR test positive and VP test negative. MR-VP media contains glucose and peptone. All enterics oxidize glucose for energy; however the end products vary depending on bacterial enzymes. Our clinical isolates can ferments the glucose in MR-VP media that decreases pH of the media below 4.4, detected by methyl red indicator which turns the media colour cherry red. Voges-Proskauer was negative for all indole positive isolates that may be due to lacking of production of acetyl methyl carbinol. MR positivity and VP negativity gives strong support in favors of *E. coli* (MacFaddin JF, 2000). In our study all indole positive isolates gave negative citrate utilization test. This is due to non metabolism of citrate compound as a only source of carbon in the media. So under basic condition Bromthymol blue can not change the media colour from green to blue. This finding is highly correlated with the finding of Kanungo S, 2009. Isolates were oxidase negative that may due non availability of cytochrome c oxidase and therefore cannot utilize oxygen for energy production with an electron transfer chain. Oxidase negativity supports that isolates were in Enterobacteriaeae family (Prescott LM et al. 1999). Urease test was done for the detection of urea hydrolysis ability of those clinical isolates. Negative results were observed for all indole positive isolates. Most of the urinary tract isolates are urease positive. In our study all clinical isolates were urease negative; this may be due to lack of urease enzyme required for hydrolysis of urea to ammonia (MacFaddin JF, 2000). It was observed in this study that all isolated strains showed a positive result in nitrate reduction and negative result in gelatin liquefaction. Nitrate positivity of isolates may be due to use of nitrate ($NO3^-$) as an external terminal electron acceptor in exchange of nitrite ($NO2^-$) during anaerobic respiration (Finegold SM et al. 1974). Negative result for gelatin liquefaction suggests absence of gelatinase enzyme required for breaking down of gelatin (Farmer JJ et al. 1981). Reactions in TSI agar slant revealed that all isolates showed yellow slant and butt with gas production but no production of hydrogen sulphide gas. This indicates the glucose, lactose and sucrose fermentation ability of those clinical isolates (Ewing, 1986). In the present

study all the isolates fermented dextrose, lactose and sucrose with the production of both acid and gas. This indicates another supports for carbohydrate fermentation ability of clinical isolates (Buxton A and Fraser G, 1977). Our results also demonstrated that, 100% of indole positive clinical isolates have haemolytic activity (α-haemolysis-70% and γ-haemolysis-60%) and are all motile. Hemolytic activity of isolates reveled that those isolates were pathogenic due to production of haemolysin, which binds with the haemolysin receptor present on the surface of RBC, that favor haemolysis (Zinnah MA et al. 2007). Isolates were motile due to presence of flagellum, supports for colonization (Ghadir S et al, 2010). All 20 were grown on EMB agar and Mac Conkey agar w/o crystal violet for confirmation of *E. coli* as differential media. Green with metallic shine on EMB agar and pink-red colonies on Mac Conkey agar considered as positive identification of *E. coli*. Satisfactory result on all biochemical tests and colony characteristic on differential agar it was confirmed that all twenty (20) isolates were uropathogenic *E. coli*. The clinically isolated *E. coli* strains were newly named as MC, from MC 1 to MC 20.

From the last few years antibiotic resistance property of pathogenic *E. coli* became a serious threat for human health. Multi drug resistance pattern became a great challenge for medical practitioners to formulate antibiotic prescribing policies.

In this study, the result of MIC, MBC and DAD tests revealed that 100% isolated *E. coli* (MC 1, MC 2, MC 3, MC 4, MC 5, MC 6, MC 7, MC 8, MC 9, MC 10, MC 11, MC 12, MC 13, MC 14, MC 15, MC 16, MC 17, MC 18, MC 19, MC 20) were resistance to penicillin G, erythromycin, streptomycin and oxacillin; 90% of isolated *E. coli* (MC 1, MC 2, MC 3, MC 4, MC 5, MC 6, MC 7, MC 8, MC 10, MC 11, MC 12, MC 14, MC 15, MC 16, MC 17, MC 18, MC 19, MC 20) were resistance to ampicillin and kanamycin; 70% (MC 1, MC 2, MC 3, MC 4, MC 5, MC 7, MC 14, MC 15, MC 16, MC 17, MC 18, MC 19, MC 20) were resistance to gentamycin and ciprofloxacin; 60% isolates were (MC 4, MC 5, MC 6, MC 7, MC 8, MC 10, MC 11, MC 12, MC 15, MC 16, MC 17, MC 19) resistance to tetraclyclin and norfloxacin; 40% of isolated strains (MC 4, MC 5, MC 6, MC 7, MC 15, MC 16, MC 17, MC 19) were resistant to cephotaxime. It was also observed that in case of amikacin MC 3 and MC 20 were resistant and in case of chloramphenicol MC 7 and MC 16 were resistant. Here from our study it was confirmed that all the isolates were multi drug resistant (MDR). The rate MDR phenotype was higher than many of the study. A study in Aligarh, India showed that 90% isolates were resistant against ampicilin, 60-79% isolates were resistant against chloramphinicol, erythromycin, tetracycline (Khan AU and Zaman MS, 2006). In our study the same drug resistance pattern was identified for ampicillin and tetracycline where as higher resistance pattern was identified against erythromycin and too much less resistance pattern for chloramphenicol. The same study showed most effective antibiotic against *E. coli* were found to be kanamycin and streptomycin, are not supported by our study due to high level of resistance pattern among those antibiotics. Our study revealed, resistance to fluoroquinolones (ciprofloxacin, norfloxacin etc.) among clinical isolates was great alarming to all medical professionals as ciprofloxacin is a common and most effective drug for complicated UTIs. This finding is nearly similar to the findings of Rani et al (Rani H et al. 2011). This same study also showed a lower level of resistance in compare to present study among parenterally administered antibiotics, cefotaxime, gentamicin, and amikacin to be 58.8%, 18.7% and 4.6% respectively. No drug resistance was seen with imipenem supports the findings of our present study.

Chloramphenicol, Cephotaxime, amikacin and imipenem can be still prescribed for UTI as their resistance rate still under control. The high level of antibiotic resistance among *E. coli* isolates may be due to self prescription policy, comparatively cheaper antibiotics intake, lack of dependency on laboratory guidance and in adequate doses of antibiotics intake. In many areas of India antibiotics can be easily available over the counter without

prescription of registered medical practitioner. This is the main cause of misuse of antibiotics.

It has been understood that the there is a co-relation between antibiotic misuse and antibiotic resistance. So, there is urgency for establishment of a new antibiotic intake policy that should be strictly followed by all the concerned authorities. Only this may be helpful for the effective control of antibiotic resistance.

5. CONCLUSION

From our study it can be concluded that twenty uropathogenic *E. coli* strains was successfully characterized by biochemical techniques. High level of antibiotic resistance pattern was found among those uropathogens. It is quite alarming to note that almost all of the isolates included in this study were found resistant to five or more antibiotics. Antibiotic resistance is becoming a big problem for the individuals admitted to health care centers with chronic conditions as well as for medical professionals. All isolates showed multiple antibiotic resistance property, maxi-mum resistance was found against penicillin G, erythromycin, streptomycin and oxacillin whereas least resistance was detected against cephotaxime, amikacin and chloramphenicol. All twenty isolates were sensitive to imipenem and hence those might be the drugs of choice to treat UPEC.

Abbreviations:

CFU : Colony formation unit
DAD : Disc agar diffusion
E. coli : *Escherichia coli*
E. faecalis : *Enterococcus faecalis*
H_2O_2 : Hydrogen peroxide
K_2HPO_4 : Di potassium hydrogen phosphate
KCl : Potassium chloride
KH_2PO_4 : Potassium dihydrogen phosphate
LB : Luria broth
MBC : Minimum bactericidal concentration
MDR : Multi drug resistant
MHB : Mueller-Hinton broth
MIC : Minimum inhibitory concentration
NA : Nutrient agar
NaCl : Sodium chloride
NaOH : Sodium hydroxide
NB : Nutrient broth
NCCLS: National Committee for Clinical Laboratory Standards
S. aureus : *Staphylococcus aureus*
S. epidermidis : *Staphylococcus epidermidis*
TSI : Triple sugar iron agar
UPEC : Uropathogenic *Escherichia coli*

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DECLARATION OF INTEREST

Authors declare that there are no conflicts of interests.

REFERENCES

1. Akram M, Shahid M, Khan AU. Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in J N M C Hospital Aligarh, India. *Annals of Clinical* Microbiology and Antimicrobials. 2007:6:4.
2. Bannerman TL. Staphylococcus, Micrococcus, and other catalasepositive cocci that grow aerobically, In Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (ed.), Manual of clinical microbiology, 8th ed. ASM Press, 2003; 8: 384–404.
3. Barnes JH Vaillancourt JP, Gross WB. chapter 18. colibacillosis, In: Saif Y.M. et al. (eds.) : disease of Poultry, 11th edn. Lowa state university press; 2003; Ames, lowa, USA. pp. 631-652.

4. Bauer AW, Kirby WMM, Sherris JC, Turch M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol.1966:45:493-6.
5. Bentley R, Meganathan R. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol. Rev. 1982;46; 241–80.
6. Buxton A, Fraser G. Animal Microbiology. Vol. 1. *Escherichia coli*, Blackwell Scientific Publications, Oxford, London, Edinburg, Melbourne. 1977; pp. 92-102.
7. Chakraborty SP, Kar Mahapatra S, Roy S. Biochemical characters and antibiotic susceptibility of *Staphylococcus aureus* isolates. Asian Pacific Journal of Tropical Biomedicine. 2011b; 1(3): 192-196.
8. Chakraborty SP, KarMahapatra S, Bal M, Somenath Roy. Isolation and Identification of Vancomycin Resistant *Staphylococcus aureus* from Post Operative Pus Sample. Al Ameen J Med Sci. 2011:4: 152-168.
9. Cheesbrough M. Medical laboratory manual for tropical countries. Vol. II. Microbiology. 1985;400-480.
10. Duguid, J.P. Staining methods. In: Collee JG, Fraser AG, Marmion BP, Simmons A, editors. Mackie & McCartney Practical Medical Microbiology. 14th ed. Edinburgh: Churchill Livingstone; 1996; pp 793-812.
11. Eaton AD, Clesceri LS, Greenberg AE (). Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association; 1995. Washington, DC.
12. Ewing. Edwards and Ewing's identification of Enterobacteriaceae, 4th ed. Elsevier Science Publishing Co; 1986. Inc.,
13. Farmer JJ, Fanning GR, Huntley-Carter GP, *et al*. Kluyvera, a new (redefined) genus in the family Enterobacteriaceae: identification of Kluyvera ascorbata sp. nov. and Kluyvera cryocrescens sp. nov. in clinical specimens. J Clin Microbiol. 1981;13(5):919–33.
14. Finegold, SM.,Sutter VL, Attebery HR, and Rosenblatt JE. Isolation of Anaerobic Bacteria. Man of Clin Micr Biol, 2nd. ed. American Society for Microbiology, 1974, Washington, D.C. pp. 365-375.
15. Ghadir S, El-Housseiny, Mohammad M, Aboulwafa, Nadia A, Hassouna. Cytotoxic Activities of Some *Escherichia coli* Isolates: Possible Mechanisms and Approaches for Inhibition. Journal of American Science. 2010;6(10):260-268.
16. Hudault S, Guignot J, Servin AL. *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. Gut. 2001:49: 47–55.
17. Hughes M, Datta N. Conjugative plasmid in bacteria of the pre-antibiotic era. Nature. 1983;302:725-726.
18. Kanungo S. A Simplified Analysis of Different *Escherichia coli* Strains by Using RAPD Technique. Not. Bot. Hort. Agrobot. Cluj.2009;37:257-260.
19. Khan AU, Zaman MS. Multiple drug resistance pattern in Urinary Tract Infection patients in Aligarh. Biomedical Research. 2006;3:179-181.
20. Li Q, Sherwood JS, Logue CM. Characterization of antimicrobial resistant *Escherichia coli* isolated from processed bison carcasses. J Appl Microbiol. 2007;103:2361-2369.
21. MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Philadelphia; 2000. Lippincott Williams & Wilkins, pp 451-453.
22. NCCLS. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, 5th edn, 17, no. 2. Approved standard M7-A5; 2000. Wayne, PA: NCCLS.
23. Okeke IN, Fayinka ST, Lamikanra A. Antibiotic Resistance in Escherichia coli from Nigerian Students, 1986-1998. Emerging Infectious Diseases. 2000;6:393–396.
24. Prescott LM, Harley JP, Klein DA. Isolation of pure bacterial cultures from specimens: Microbiology International (4th edition) WCB McGraw's Hill Companies Boston; 1999. Pp. 714-796.
25. Rani H, Kaistha N, Gupta V, Chander J. Choice of Antibiotics in Community Acquired UTI due to *Escherichia Coli* in Adult Age group. Journal of Clinical and Diagnostic Research. 2011;3:483-485.

26. Reid G, Howard J, Gan BS "Can bacterial interference prevent infection? Trends Microbiol. 2001;9: 424–8.
27. Santo E, Salvador MM and Marin JM. Multidrug-Resistant Urinary Tract Isolates of Escherichia coli from Ribeirão Preto, Sao Paulo, Brazil. The Brazilian Journal of Infectious Diseases. 2007;11:575-578.
28. Simmons J. Standard Methods for the Examination of Water and Wastewater. Eleventh Edition. APHA Inc; 1960. New York, pp 626.
29. Snell EE, Wright LD. A microbiological method for the determination of nicotinic acid. J Biol Chem. 1941; 139: 675-686.
30. Snell JJS, Brown DFJ, Roberts C. editors. Quality Assurance Principles and Practice in the Microbiology Laboratory; 1999. Public Health Laboratory Service. London: pp. 147-148.
31. Stamm WE, Hooton TM. The management of urinary tract intection. N Engl J Med. 1993;329:1328-1334.
32. Todar K Pathogenic *E. coli*. Online Textbook of Bacteriology. University of Wisconsin–Madison Department of Bacteriology. 2007.
33. Vanderzant, C, Splitt stresser DF. Conpendium of methods for the microbiological examination of foods. 3rd ed. American Public Health Association; 1992. Washington DC, pp. 331-338.
34. Warren JW, Abrutyn E, Hebel JR, Johnson JR, Schae-ffer AJ, Stamm WE. Guidelines for antimicrobial treatment of uncomplicated acute bacterial cystis and acute pyeloephritis in women. Clin Infect Dis. 1999:29:745-758.
35. Williams DN. Urinary Tract Infection: Emerging insights into appropriate Management. Postgrad Med. 1996;99;189-99.
36. Zinnah MA, Bari MR, Islam MT, Hossain MT, Rahman MT, Haque MH, Babu SAM, Ruma RP Islam MA. Characterization Of *Escherichia Coli* Isolated From Samples Of Different Biological And Environmental Sources. Bangl J Vet Med. 2007;5:25–32.