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Original Research Article

The effectiveness of antibiotics against a major uropathogen-*Escherichia coli* and its biofilm assay by phenotypic methods

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ABSTRACT

Background: Urinary tract infection (UTI), an inflammatory disease occurs to a high multiplication of pathogenic microbes in the urinary system. *Escherichia coli*, a major uropathogen accounting for up to 80% UTI. Activity of antibiotics against *E.coli* is decreasing due to enzymatic, genetic and various other factors resulting in multidrug resistance. Biofilm is a great threat as it interferes antibiotic therapy, undergoes gene transformation, making the bacteria more virulent. Objectives of the study were to isolate the *E. coli* from the urine sample of symptomatic UTI patients, to study the effectiveness of antibiotics against *E.coli* by antibiotic susceptibility pattern, ESBL detection and to biofilm phenotype assay by four different methods.
Methods: A total of 400 urine samples from symptomatic UTI patients, October 2015 to March 2016 and processed. *E.coli* was isolated and antibiotic susceptibility was done as per CLSI guidelines. Biofilm detection by a) Congo red agar (CRA), b) Modified Congo red agar (MCRA), c) Tube adherence assay, d) Microtitre plate method.
Results: A total of 100 *E. coli* (69%) was obtained from 400 samples, 49% were ESBL producers and 84% shows multi drug resistant. Biofilm positive isolates shows 70% positivity by CRA, 91% by MCRA, 57% in tube adherence assay and 99% via microtitre plate method.
Conclusions: Resistance to antibiotics ladder is increasing and it's necessary to take actions to reduce its hindrance in the future. Advanced studies in biofilm will help to prevent the more virulence without any critical complications in therapy.

Keywords: Biofilm, *Escherichia coli*, ESBL, Multi drug resistance, UTI

INTRODUCTION

Urinary tract infection (UTI), an inflammatory disease occurs due to high multiplication of pathogenic microbes in the urinary system, altering the perfect function of urinary tract and kidney. The bacterial UTI is the second most serious health problem and causing morbidity.[1] About 50% - 80% of population endures UTI in their lifetime; 20% - 50% will have recrudescent infections.[2] Globally 150 million people are UTI diagnosed each year and $6 billion high expenditure in all age groups. This infection focuses towards young, sexually active women than men, though the prevalence in elderly men and women are similar. A study conducted in US has estimated that around 40% - 50% of healthy adult women will experience this infection.[3]

Escherichia coli commensal of the colon via symbiotic relationship causes intestinal infection opportunisticaly and a common cause of extra intestinal infection as UTI accounting for up to 80% of community acquired UTI's making it to consider as a major uropathogen. *E.coli* has

been classified into *Diarrhogenic E.coli* and *Extra intestinal E.coli* (ExpEC) which is divided into *Neonatal meningitis causing E.coli* (NMEC) and *Uropathogenic E.coli* (UPEC).[4] UPEC constitutes many virulence factors as adhesins, toxins, mainly targets for antimicrobial therapy due to the production of biofilm. Antibiotic resistance in *E.coli* is the major concern among Gram negative bacteria causing UTI.

Decreasing activity of antibiotics, especially the beta-lactam groups and cephalosporins because of the production β-lactamase and extended spectrum β-lactamase (ESBL) by *E.coli* and others factors leading to multi drug resistance. Antimicrobial drug resistance is rising worldwide with regional difference and the frequency of occurrence.[5,6]

Planktonic bacteria, characterized as freely suspended cells on the basis of their growth in nutritionally rich culture medium is different from intracellular bacterial communities (IBCs) is a micro ecosystem in which the pathogens grow along with hundreds of other bacteria, i.e., Biofilm - an assemblage of microbial cells of that are irreversibly associated with a surface and enclosed in an exopolysaccharide matrix. It's a distinct phenotype helps in a) altering the gene transcription and growth rate, b) increased resistance to chemical and physical therapy, c) survival of bacteria in hostile conditions, d) responsible for chronic, persistent, relapsing and the recurrence of infections, e) protecting the pathogens from host immune cells, f) increased resistance to antimicrobial agents.

As *E.coli* is frequently isolated from UTI than other bacteria showing resistance to available antimicrobial agents, this prospective study was undertaken to determine the prevalence of *E.coli* causing UTI, its antibiotic susceptibility pattern and ESBL detection by standard CLSI guidelines. Biofilm production of *E.coli* by different methods as it is the main source of multidrug resistance and recurrent urinary tract infection.

METHODS

It was a prospective study which was conducted at the Department of Microbiology in a tertiary care hospital from October 2015 to March 2016.

Inclusion criteria

Patients of all ages with symptomatic UTI (dysuria, loin pain, increased frequency and urgency of urination, burning micturition etc.,) attending the outpatient department (OPD) and inpatient department (IPD) of the hospital was included.

Exclusion criteria

Patients on antibiotic treatment prior to 1 week were excluded from the study.

Sample collection

A total of 400 urine samples collected from the patients as per the standard collection procedure of midstream urine sample (MSU) aseptically in a sterile, wide mouthed, screw capped container and catheterized urine samples received from inpatients of the hospital and were labelled appropriately and transported immediately to the Microbiology laboratory for processing.

Culture methods

The samples were cultured by bacteriological standards on 5% sheep blood agar, Mac conkey agar, Hicrome agar using standard inoculation loop and incubated at 37º C for 24 hours aerobically. Growth of the single morphotype colonies was considered and colony counts done for significant UTI.

Identification of E.coli

Basic biochemical tests (IMViC reactions) were done and identified after inoculation and incubation at 37º C for 18 hours. The results were read for detection of indole production (I), methyl red (MR) and Voges prauskauer (VP), citrate utilization (C), triple sugar iron agar (TSI) for fermentation of glucose, lactose, sucrose, gas and H_2S production, urease enzyme detection and mannitol motility medium (MMM).

Antibiotic susceptibility testing pattern

Inoculum was prepared by inoculating 2 to 3 colonies from culture media in nutrient broth and standardized with 0.5 MC Farland standard and swabbed onto a 90 mm Muller-Hinton agar plate using Kirby Bauer disc diffusion method were incubated at 37ºC overnight and zone of inhibition was measured as per CLSI guidelines. Antibiotic discs - Ampicillin 10µg, Amoxyclav 30µg, Cefuroxime 30µg, Cefepime 30µg, Cefpodoxime 30µg, Ceftazidime 30µg, Ceftriaxone 30µg, Gentamicin 10µg, Norfloxacin 10µg, Nitrofurantoin 300µg, Nalidixic acid 30µg, Co-trimoxazole 25µg, Imipenem 10µg, Piperacillin/ Tazobactum 100/10µg were used.

Screening method for ESBL production

Ceftazidime and Ceftazidime/clavulanic acid discs were kept 15-20 mm apart from each other (center to center), in addition another inducer Cefoxitin was placed at 15mm from Ceftazidime. An isolate was suspected to be an ESBL producer if the zone size increases with the addition of clavulanic acid inhibitor by ≥5mm and susceptibility to Cefoxitin as per CLSI guidelines.

Confirmation of ESBL

Cephotaxime and Cephotaxime/ clavulanic acid discs were kept 15-20 mm apart from each other on Mueller Hinton agar plates and incubated at 37º C overnight and

if the zone size increases by ≥5mm with the addition of β-lactamase inhibitor the isolate was confirmed as ESBL producer.

Detection of biofilm production

Early detection of biofilm might help in reducing the usage of antimicrobials which are resistant in vivo, enable to prescribe the higher level of antibiotics, which evades the biofilm and also in treating the long standing cases that may be damaging and may produce immune complex reactions. There are two assays, qualitative and quantitative assay constituting different methods for detection of biofilm.

Qualitative assay

Tube adherence method[7,8]

10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. Broths were incubated at 37ºC for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH7.3.The tubes were dried and stained with 0.1% crystal violet for 30 minutes.

Excess stain was washed with deionized water. Tubes were dried in an inverted position and observed for biofilm formation. Biofilm Production was considered positive when a visible film lined the wall and the bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong.

Congo red agar method (CRA)[9]

The medium composed of Brain heart infusion agar (52 gm/l); sucrose (36 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121° C for 15 minutes.

Then it was added to autoclaved Brain heart infusion agar with sucrose at 55° C. Plates were inoculated with test organism and incubated at 37ºC for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; non- biofilm producers usually remained pink.

Modified congo red agar method (MCRA)[10]

The medium composed of Blood agar base (40gm/l); glucose (10 gm/l) and Congo red dye (0.4gm/l). Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121 C for 15 minutes.

Then it was added to autoclaved blood agar base with glucose at 55° C. Plates were inoculated with test organism and incubated at 37ºC for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; non-biofilm producers usually remained pink.

Quantitative assay

Microtitre plate method[7,8]

Quantitative determination of biofilm was made using a microtitre plate adhesion assay in accordance with some modifications.[11] Three to five colonies were suspended in 5 ml of Trypticase soy broth (TSB) and incubated for 24 hrs. Then, diluted and adjusted to 0.5 McFarland turbidity standards to reach 10^5 CFU/ml.

An aliquot of 200µL of diluted bacterial suspension with 0.25% glucose was added to each well of 96-well flat-bottomed polystyrene microtitre plates and incubated for 18-24 hrs at 37°C. Media with suspended bacteria were then removed; the plates washed carefully 3-4 times with sterile distilled water and air-dried, then stained with 200 µL of 1% crystal violet for 30 minutes.

After washing the dye, the attached bacteria solubilized with 95% ethanol and the optical density of the adherent biofilm were determined twice with a filter of 450nm in ELISA reader.

In our experiment 200µL of TSB broth with 0.25% glucose were used as a negative control to obtain a background absorbance, which was then deducted from absorbance values obtained from the wells containing study isolates which gives the quantitative value of biofilm produced by the test organism.

All isolates were tested in triplicate. The absorbance value (OD) of the test organism is interpreted with the criteria given below[11,12] depending on standard calculations laid down by by which the study isolates were classified.[7] An optical density of 0.0541 was chosen as guideline to distinguish biofilm producers from the non-biofilm producers. These different phenotypic methods were used for biofilm detection and compared.

Table 1: Criteria for biofilm classification.

Non-biofilm producer	< OD Cut off
Weak biofilm	OD Cut off < ODT 450 < 2 OD Cut off
Moderate biofilm	2 OD Cut off < ODT 450 < 4 OD Cut off
Strong biofilm	ODT 450 > 4 OD NC

RESULTS

Out of 400 urine samples 145 culture positives in which 100 were *E.coli* isolates characterized by its colony morphology and confirmed by biochemical reactions. Of

the 145 bacterial isolates, 100 (69%) are confirmed as *E.coli* - the major group among UTI pathogens and other isolates - 45 (31%) includes Methicillin Sensitive *Staphylococcus aureus* (7%), *Methicillin Sensitive and Resistant Coagulase negative Staphylococcus*, *Proteus mirabilis* each of (3%), *Enterococcus faecalis*, *Pseudomonas aeruginosa* each of (2%), *Klebsiella pneumoniae* (7%), *Klebsiella oxytoca, Enterobacter species, Citrobacter freundii, Citrobacter diversus* each comprising of (1%).

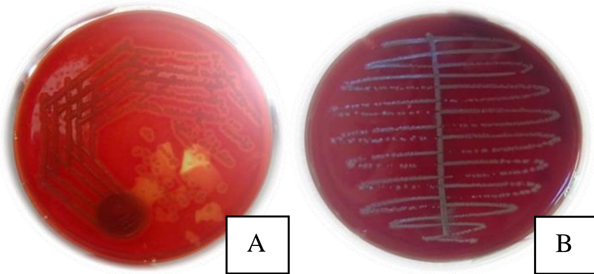


Figure 1: 5% Sheep blood agar showing a) beta haemolytic, b) few non-haemolytic colonies.

UTI with *E.coli* is higher in females (66%) than males (34%). Usually the younger, sexually active women are commonly affected with UTI in which 19 patients were 11 - 20 yrs then 21 -30 years, 51 - 60 yrs each of 15 patients were infected with *E.coli*.

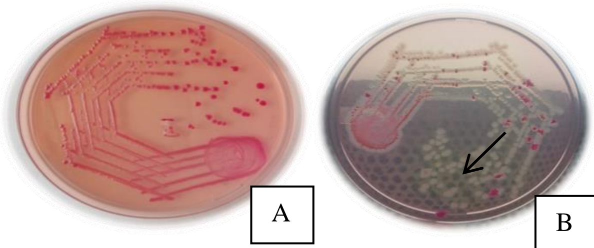


Figure 2: Mac conkey agar showing a) pink lactose fermenting, b) few pale coloured non-lactose fermenting colonies.

Figure 3: Hicrome UTI agar showing pink to purple colonies.

Antibiotic susceptibility showed increased resistance towards Nalidixic acid (94%) followed by betalactum group of antibiotics, increased sensitivity to Imipenem and Piperacillin/ Tazobactum (figure 8). Based on Ceftazidime and Ceftazidime/clavulanic acid zone of inhibition results (Figure 5) 49 isolates were reported as ESBL producing and remaining 51 as non-ESBL producers.

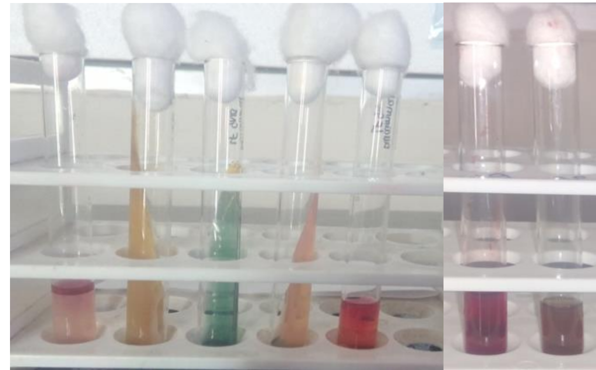


Figure 4: Biochemical identification.

Out of 99 biofilm producer, classification was done as Strong producer, Moderate producer, Weak producer and Non-producer – 3%, 25%, 72%, 1% respectively based on their OD values comparing to the criteria in Table 1. CRA method 70%, MCRA with 91% and Tube adherence assay showed the least about 57% comparing to other methods.

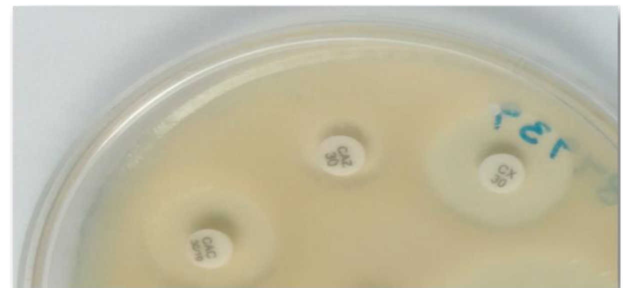


Figure 5: Detection of ESBL by ceftazidime and ceftazidime/clavulanic acid interpreting increase in > 5mm zone size by the addition of inhibitor.

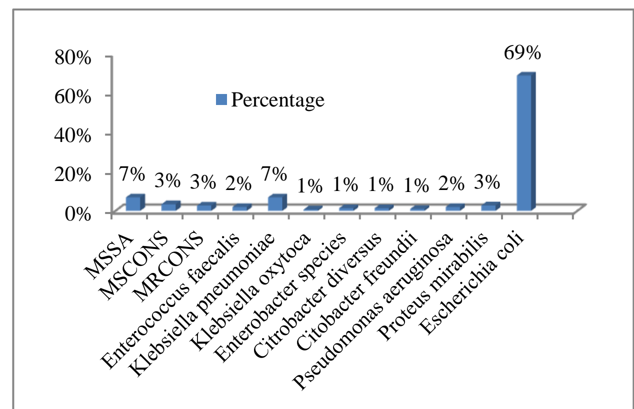


Figure 6: Different isolates obtained from symptomatic UTI patients.

Table 2: Biochemical identification.

Indole	TSI	Citrate	Urease	MMM	MR	VP
+	A+/A- gas+ No H_2S	-	-	Fermented Motile	+	-

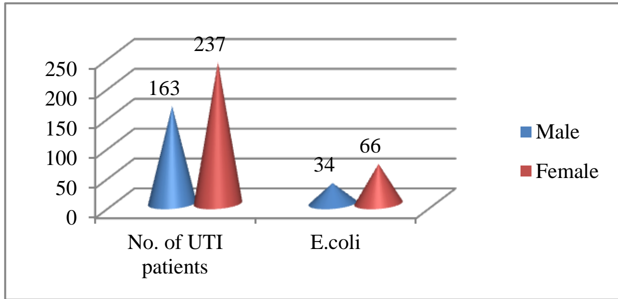


Figure 7: Gender distribution of patients with UTI and the isolated *Escherichia coli.*

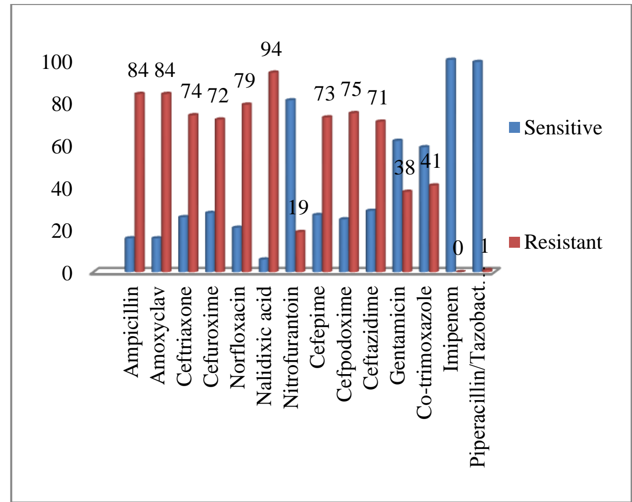


Figure 8: Antibiotic susceptibility pattern of *E. Coli* isolated from urine samples.

Table 3: Age and gender distribution of *E. coli* isolates among symptomatic UTI patients.

Age	Symptomatic UTI patients (N = 400)		Total	Culture positive *E. coli*		Total
	Male	Female		Male	Female	
≤1-10	30	33	63	1	9	**10**
11-20	22	26	48	**8**	**11**	***19***
21-30	22	46	68	5	10	**15**
31-40	12	32	44	1	6	**7**
41-50	18	43	69	4	9	**13**
51-60	16	33	49	5	10	**15**
61-70	26	15	41	6	8	**14**
71-80	14	8	22	3	3	**6**
81-90	3	1	4	1	0	**1**
91-100	0	0	0	0	0	**0**
Total	163	237	400	34	66	**100**

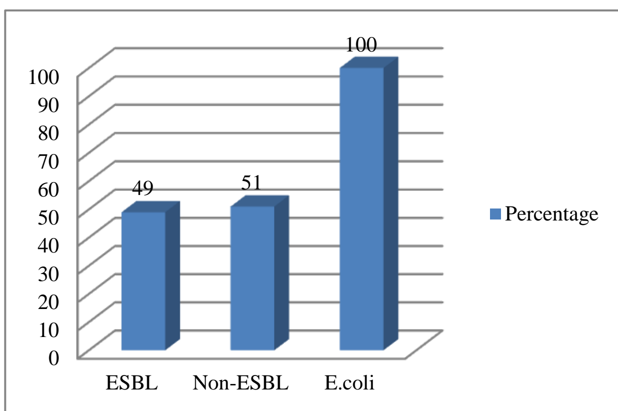


Figure 9: Distribution of ESBL and non-ESBL among E. coli isolates.

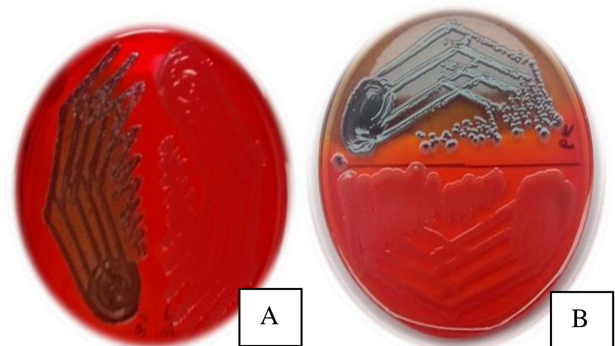
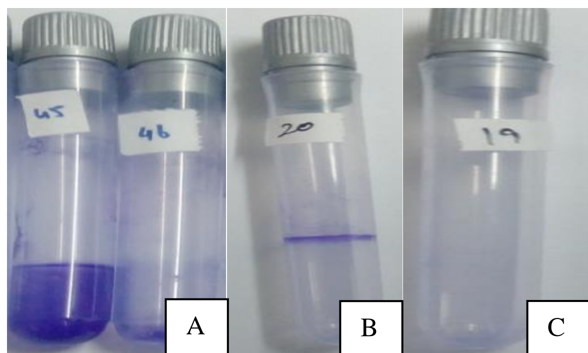


Figure 10: Congo red agar (CRA) and modified congo red agar (MCRA) - black dry crystalline colonies indicating biofilm production and pink to red colour colonies indicating non-biofilm production.

Table 4: The overall status of urine specimens processed.

Culture media	Findings
No. of urine specimens	400
No. of culture positive	145
No. of E.coli isolates	100
No. of isolates other than E.coli	45



A – Positive for biofilm; B & C – Negative for biofilm

Figure 11: Tube adherence assay.

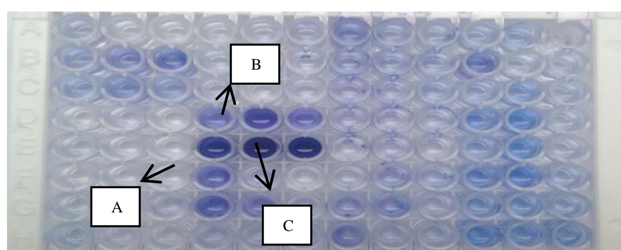


Figure 12: Microtitre plate assay: A – no colour, B & C – violet colour indicates biofilm production.

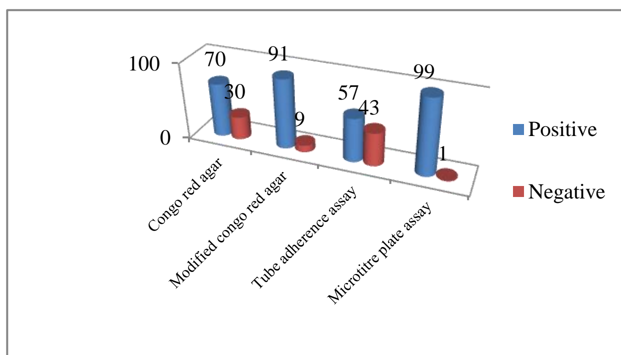


Figure 13: Biofilm positive *E. coli* by all four methods.

DISCUSSION

UTI by *E.coli* is substantially economic, social burden; formidable public health issue predisposes individuals to more infections and necessitates antibiotic therapy.[13] This study confirmed that UTI is more common in females than males agreeing with other previous studies. Out of 400 urine samples 145 were culture positive, in which 95 (65.6%) were females and 50 (34.4%) males.

Among the 145 bacterial isolates (Graph 1 & 2) *E.coli* 100 (69%) were found to be the major group of pathogens responsible for UTI and isolates other than *E.coli* 45 (31%) includes *Methicillin Sensitive Staphylococcus aureus* (7%), *Methicillin Sensitive and Resistant Coagulase negative Staphylococcus, Proteus mirabilis* each of (3%), *Enterococcus faecalis, Pseudomonas aeruginosa* each of (2%), *Klebsiella pneumoniae* (7%), *Klebsiella oxytoca, Enterobacter species, Citrobacter freundii, Citrobacter diversus* each comprising of (1%).

Table 1, summarizes the age and gender distribution of symptomatic UTI patients infected with *E.coli*. As females prone more to UTI in this study the percentage of *E.coli* is also more in females (66%) than males (34%). Usually the younger, sexually active women are commonly affected with UTI although the elderly adults were also similarly affected in this study 11 - 20 yrs was 19 then 21 -30 years, 51 - 60 yrs each of 15 patients were infected with *E.coli*.

In a study conducted in Bangladesh, the distribution of UTI infections 25.9% were males and 74.1% were females in which *E.coli* was more prevalent compared to other uropathogens. Of these *E.coli* showed 94% resistance towards ampicillin, resistance to cephalosporins were also above 60% in most cases.[14]

The pattern of isolation of organism in this study was also similar to the results from various regions of India and other countries which indicate that *Escherichia coli* is the commonest pathogen isolated in patients with UTIs and 84% isolates were resistant to 3 and more than 3 antibiotics showing multidrug resistance pattern.[1,15-22]

Similar to the study, out of 650 samples with highest prevalence of *E. coli* (54.6%) was the maximally isolated UTI causing bacterium (87%), ciprofloxacin (69.0%), norfloxacin (67.6%), ofloxacin (64.5%), gentamicin (62.5%), cefotaxime (58.0%), ceftazidime (54.7%) and ceftriaxone (47.0%) respectively. All isolated *E.coli* strains were multidrug resistant each to 4, 5, 6, 7 and 8 of the selected antibiotics used.[14] A retrospective study done at Bombay in which *E. coli* was isolated from 41.31% of UTI cases.[22]

Third generation cephalosporins were the other commonly used antibiotics in the treatment of UTIs. In the present study *E.coli* exhibited 71% resistance to ceftazidime and 74% resistance to ceftriaxone was higher to the reports (59.5%) and ceftrioxone resistance was also greater than the findings of (22.6%) and similar to a study (74.9%).[22-24] Therefore, it appears that cephalosporins were also becoming highly resistant to therapy.

E.coli showed a low resistance to nitrofurantoin (19%) in the present study. This is higher to the reports of (13%), (15%) and (9.3%).[14,23,24] The reason for low resistance may be because it was less frequently prescribed and

requirement of sixth hourly dosage and gastrointestinal disturbances following oral administration. Nitrofurontoin resistance in western countries was as low as 0.4 % in the USA and 5.7% in the Spain.[25]

The effectiveness of various antibiotics was studied for the common isolate *E.coli* from UTI based on the resistance patterns, the most effective antibiotic overall is Imipenem (99%) followed by piperacillin / tazobactum (99%) and nitrofurantoin (81%), Gentamicin (62%), Co-trimoxazole (59%). The high resistance pattern was noted against nalidixic acid (94%), ampicillin and amoxyclav each of (84%) followed by norfloxacin (79%), cefpodoxime (75%), ceftriaxone (74%), cefepime (73%), cefuroxime (72%), ceftazidime (71%).

Out of 100 isolates 49 *E.coli* were found to ESBL producing and remaining 51 were non-ESBL producers similar to the other studies.[22,26] The resistance exhibited by *E.coli* to aminoglycosides varies. In this study, 19% gentamicin resistance in non-ESBL producing and 1% resistant in ESBL producing *E.coli* was observed but 37.5% resistance to gentamicin was reported in another study.[14] Various other studies reported 5% resistance in non-ESBL producing *E.coli* and 72.6% resistance in ESBL producing *E. coli* to gentamicin.[27]

In a study out of 26 isolates from urine 7 were *E.coli* in which 6 isolates ESBL producers and highest incidence was found in the age group of 30 - 45 years.[28]

Intracellular Bacterial Communities (IBCs) defined as Biofilm which is the major cause of multidrug resistance against the common available antimicrobial agents and for chronic persistent, recurrent infections.[29] High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold.[30]

Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing. Availability of key nutrients, chemotaxis towards the surface, motility of bacteria, surface adhesins and the presence of surfactants are some factors which influence biofilm formation.[31]

In this study totally 41 isolates were found to be multidrug resistance comprising 34 resistant to more than three antibiotics and 9 were resistant to three antibiotics. Among 49 ESBLs and 51 non-ESBL producers all were found to produce biofilm (99%) and 1 non-ESBL isolate was also a non-biofilm producer. The microtitre plate assay has the advantage of being simple and can be easily modified to analyse the multiple strains and growth conditions within each experiment. In a study a total of 14 *Escherichia coli* isolates were assessed for their ability to produce biofilm in-vitro by slime production on Congo red agar medium (CRA) and microtitre plate assay. Out of 14 isolates tested, 12 were slime producing on CRA as indicated by black colonies.[32]

The different phenotypic methods – CRA, MCRA, Tube method, Microtitre method showed varied false positive and false negative in bacterial isolates. In a study done in *Pseudomonas aeruginosa*, 152 clinical isolates of Staphylococcus spp. were screened by tissue culture plate (TCP), Tube method (TM) and Congo red agar (CRA) method.[11,33]

88 (57.8%) displayed a biofilm-positive in the TCP method. Though TM correlated well with the TCP test for 18 (11.8%) strongly biofilm producing strains, weak producers were difficult to discriminate from biofilm negative isolates. Screening on CRA does not correlate well with either of the two methods for detecting biofilm formation in staphylococci, the microtitre plate method being the best one. As other studies, in this study biofilm was detected by three methods Congo red agar (CRA) and Modified Congo red agar (MCRA), tube adherence assay qualitatively and quantitatively by microtitre plate method. Among these methods microtitre plate assay showed higher percentage of detecting biofilm (99%).

Out of 99 *E.coli* biofilm producer was classified as Strong producer, Moderate producer, Weak producer and Non-producer – 3%, 25%, 72%, 1% respectively based on their OD values. CRA method 70%, MCRA with 91% and Tube adherence assay showed the least about 57% comparing to other methods. Certain modifications in the ingredients and concentrations in MCRA culture medium make the black pigment of the biofilm positive colonies stable compared to CRA medium.[10]

Some isolates which were biofilm positive in MCRA was shown as biofilm negative in CRA leading to false negative results and even tube adherence method showing lesser percentage than other methods may due to the greasiness of tubes and many undetermined factors. Microtitre plate method is the best and easiest method as it interprets quantitatively by measuring the optical density of isolate with the corrected cut-off OD control.

CONCLUSION

Resistance to antibiotics ladder is increasing and it's necessary to take actions to reduce its hindrance in the future. Advanced studies in biofilm and in the framework of its genes will help to prevent the more virulent factors which protect the bacteria from host immunity and to get rid of critical complications in therapy.

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