

Incidence of β -lactamases Among Novel Multidrug Resistant Clinical Isolates of UTI Patients: An Antimicrobial Resistance Surveillance Study

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Abstract: Community or Nosocomial Urinary Tract Infections (UTI) are increasing worldwide speedily by creating problem to currently available β -lactam antibiotic therapies. Thus it is an urgent need of research to find the effective therapeutic options to treat / manage the UTI. Therefore, the present work is designed to determine prevalence of β -lactam resistance in seven novel bacterial isolates from the community UTI as well as to identify and characterize the types of β -lactamases involved in UTI. Total Five Hundred and Forty Nine (n= 549) clinical strains isolated from Urine samples and identified as *E. coli*, *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter* spp., *Escherichia* spp., *Enterococcus* spp., *A. haemolyticus*, *Micrococcus*, *Serratia* spp., *Shigella* spp., *Staph. Aureus* etc. Antimicrobial susceptibility profile of these strains revealed high level resistance to Ampicillin (99%), Ceftazidime (98.99%), Amoxicillin (98%), Cefotaxime (99%), Cefaclor (98%) and Penicillin (99%). Thus out of 549 only seven were detected as novel Extended Spectrum β -lactamase producers. All the seven isolates were identified at 16 S r RNA level and submitted to NCBI with following accession numbers such as: JX827388.1, JX827385, JX827384.1, JX827383.1, JX827382, JX827386 and JX827387. Minimum inhibitory concentration of each novel strain was determined against 25 different β -lactam antibiotics i.e. Ampicillin, Amoxiclav, Oxacillin, Vancomycin, Gentamycin, Ciprofloxacin, Nitrofurantoin, Ceftazidime, Cotrimaxazole, Trimethoprim, Imipenem, Amikacin, Amoxicillin, Cephalexin, Cefuroxime, Nalidixic acid, Cefazoline, Cefdinir, Cefexime, Ceftriaxone, Cefuroxime, Cefotaxime, Aztreonam, Cefaclor, Penicillin etc. All these strains revealed to possess virulence markers such as α -hemolysin, Cell surface hydrophobicity, Aerobactin production, Serum Resistance, Colicine production etc. In addition to this an antimicrobial surveillance of the same strain was tested for multiple antibiotic resistance scale with the help of WHONET software designed by World Health Organization.

Keywords: β -lactamases, Urinary Tract Infection, WHONET Software, Virulence Markers

1. Introduction

Urinary Tract Infection is the most common nosocomial bacterial infection encountered in human population from neonate to geriatric age groups [1]. The incidence of UTI is more in women as compared to men and prevalence increases substantially with increasing age (i.e. 5-10% UTI for the age of 70 years and ~10% UTI for the age of 80 years and older). In many parts of the world near about 80-85% Gram-negative enteric and 15-20% Gram-positive microorganisms constitutes a serious problem in development of Urinary Tract Infection. Enteric bacteria (in particular *E.coli*) is the most frequent cause of UTI.

Multidrug resistant pathogens travel not only locally but also globally and hence spread rapidly in susceptible host [2]. For better decision- making of treatment, physicians need more information about local susceptibility pattern of Uropathogens. UTI can only be ascertained on the basis of microscopy and microbial culture of urine therefore it is rational to do bacteriological examination of urine sample along with their antibiogram.

β -lactamases which hydrolyze the amide bonds of the, β -lactam ring of sensitive Penicillins and Cephalosporins are widely distributed among microorganisms [3] and play important role in microbial resistance to β -lactam antibiotics. Extended spectrum β -lactamases (ESBLs) are capable of hydrolyzing Penicillins, Cephalosporins (including wide

spectrum ones) and Monobactams, except for the Cefamicines and the Carbapenemics [4]. β -lactamase producing bacteria can cause serious therapeutic failure if not detected in time. Classical ESBLs evolved from Class A TEM (from TEM-1 or TEM-2) and SHV (from SHV-1) enzymes and these remain the most prevalent types of ESBLs though class D ESBLs (i.e. of the OXA family) have also been known for some time. Still over the past several years a plethora of non-TEM, non-SHV, non-OXA ESBLs have been reported in several organisms all over the world (eg. Families BES, GES, PER, TLA, VEB and CTX-M) with CTX-M type ESBLs [5]. In contrast to the TEM-,SHV- and OXA- derived ESBLs which result for mutation of their narrow-spectrum counterparts many of the other ESBLs which are typically plasmid encoded or otherwise mobile originated from naturally occurring (i.e. chromosomal) enzymes are also considered. Variety of other β -lactamases which are plasmid mediated or integron –associated class A enzyme have been recently discovered. They are not simple point mutations of any known β -lactamases but they are characterized by their geographic diversity such as- VIM (Verona Integron-Encoded Metallo- β -Lactamase), KPC (*K. pneumoniae* Carbapenemase) (Class A), NDM-1 (New Delhi metallo- β -lactamase), Amp-C, Inhibitor Resistant β -Lactamases, Chromosome-Mediated β -Lactamases, Extended Spectrum β -lactamases etc.

The emergence and dissemination of resistant bacteria is a natural process in which bacteria get adapted to a hostile environment rich in antibacterial agents. The multidrug resistance of the bacteria contributes to their survival and virulence. These virulence factors can act independently or their actions may be complementary to each other [6]. Virulence factors are very often responsible for causing diseases in the host as they inhibit certain host functions. Pathogen possesses a wide array of virulence factors. Some are intrinsic to the bacteria (eg. Capsule and endotoxin) whereas others are obtained from Plasmids (eg. some toxins). Besides bacterial adherence, several virulence factors may contribute to the pathogenicity, including the production of α -hemolysin, Colicin, Aerobactin and Serum resistance [7]. *Enterobacteriaceae* uropathogens present in the gastro intestinal tract initiates the UTI. These isolates express chromosomally encoded virulence markers. These markers of Uropathogenic *E.coli* (UPEC) are expressed with different frequencies in different disease states ranging from asymptomatic bacteriuria to chronic pyelonephritis. It is now recognized that there are a subset of fecal *E.coli* having the above mentioned factors which can colonize periurethral area, enter urinary tract and cause symptomatic disease.

The treatment with an antimicrobial is decided either on the basis of antibiogram of the individual isolate or if sensitivity testing data is available on the basis of % Resistant (%R) and / or % Sensitive (%S) bacterial population. Sensitivity Index of antimicrobial, (SI) calculated from percent Resistant Intermediate Sensitive (%RIS) results generated using standard NCCLS., 2000, testing method [8] and uniform analysis by WHONET 5.6, might be a uniform

and better criterion for rational use of antimicrobials. To satisfy the urgent need for an efficient surveillance system to monitor the possible impact of this policy, and to study the epidemiology of antimicrobial resistance, we used WHONET 5.6 software (project 2009-2011) to establish a regional network for continuous monitoring of such resistance. This study is based on the assumption that the routine results of the antimicrobial sensitivity tests performed daily in clinical laboratory should be considered to be a major resource for resistance surveillance. To do this we studied our isolates with the help of WHONET software. The World Health Organization has established a program to tackle the problem of antimicrobial resistance which is known as Antimicrobial Resistance Monitoring (ARM) program. It requires accurate and easily accessible data on antimicrobial resistance to support decision making and to take action from local to the global level.

Hence, The present study was therefore undertaken to find out the presence of different β -lactamases from hospitalized patients in Nanded District using standard methods presently available for their detection as well as an attempt was made to correlate the pathogenicity of all isolated pathogens from UTI patient with different virulent factors i.e. Haemolys in production, Cell surface hydrophobicity, Serum resistance, Colicin and Aerobactin in production. Realizing the high prevalence of drug resistance in bacterial population, the study was aimed to find out the surveillance of drug resistance. This data was analyzed with the help of WHONET 5.6 software. The data presented cover the period June 2009-December 2011.

2. Materials and Methods

2.1. Study Population / Cases

The study population was drawn from patients attending Government Medical College and Hospital, Nanded for a period of six month from July 2009 - January 2010. Urine samples from 360 cases (both male and female of age 5-70 years) of UTI and 30 normal controls from department of Urology, Nephrology, Medicine, Pediatrics and few other departments of hospital were studied. Patients on antibiotic therapy were excluded from study.

2.2. Collection of Urine Sample

Early morning mid-stream urine samples were collected using Sterile (dry heat at 160°C for 1h) wide mouthed sterile plastic container (HiMedia) with screw cap tops with name, age, sex, time of collection of the patients prior to antimicrobial treatment and transported immediately to laboratory in an ice-cold condition by adding boric acid (1.8%) [9, 10]. The samples were analyzed bacteriologically [11].

2.3. Sample Processing

2.3.1. Culture

- a) A calibrated sterile platinum wire loop (0.4 mm diameter) for the semi-quantitative method was used for

the plating. A loopful (0.01ml) of well mixed each urine sample was inoculated into duplicate plates of Blood and Mac Conkeys agar. All plates were then incubated at 37°C aerobically for 24 h. The plates were examined microscopically for bacterial growth and colonies were counted and multiplied by 100 to give an estimate of the number of bacteria present per milliliter of Urine. A significant bacterial count was taken as any count equal to or in excess of 10,000 CFU/ml [12].

- b) A fresh voided urine sample provided by the patient were used for Dipstick and/or Leukocyte esterase test. Subsequently, a Dipslide (Uriline, 56508; BioMeurex, Plainview, NY) was prepared according the manufacturer's instructions. On the day of sample collection the Dipslide was incubated at 37°C for 16-20 h. Thereafter, growth on the Dipslides was compared with a chart provided by the manufacture and recorded from no growth to $\geq 10^7$ colony-forming units (CFU/ml). Bacterial growth on the dipslides of $\geq 10^3$ CFU/ml was identified using standard biochemical tests [13].

2.3.2. Microscopy

All Urine samples were centrifuged at 5000 rpm for 5 min separately. A 0.1 ml volume of Urine was applied to a glass microscope slide, allowed to air dry, stained with gram stain and examined microscopically. The deposits were examined using both $\times 10$ and $\times 40$ objectives. Samples with ≥ 10 white blood cells/mm³ were regarded as pyuric [14]. Bacterial isolates were identified generally using a battery of tests [15]. All urine samples having growth of single morphotype of colony counts of 10^4 - 10^5 colonies/ml was considered probably significant and a count of $<10^4$ colonies /ml as significant growth of Perennial or Uretral flora [16].

2.4. Isolation and Identification of UTI Isolates

For isolation of almost all uropathogens a loopful of urine sample (n=360) was streaked on 7 different selected and differential growth media viz. Mac Conkey's agar, Blood agar, CLED (Cysteine Lactose Electrolyte Deficient) agar, Nutrient agar, EMB (Eosin Methylene Blue) Agar, UTI agar and combinational agar (CLED+ Mac Conkey's + Blood) and incubated at 37°C for 24 h [17]. After incubation colonies were selected and characterized on the basis of morphological, cultural, Typical and Atypical biochemical tests and differentiated according to bacterial molecular identification procedure of 16S rRNA.

2.5. Screening of Uropathogens for MAR Antibigram with Respect to β -lactam Class

All identified uropathogens were subjected to antimicrobial sensitivity test (AST) on Muller-Hinton agar by Kirby-Bauer Disc Diffusion method [18]. About 0.5 Mac Farland cell density adjusted microbial suspension was swab inoculated on pre-sterilized Muller-Hinton agar plates. 25 different antibiotics specified for uropathogens, β -lactam class (HiMedia) were used for the test. Plates were incubated

at 37°C for 24 h. The sensitivity of isolate towards each antimicrobial agent was evaluated by comparing the zone of inhibition with standard interpretative chart as recommended by NCCLS and results were categorized in to Resistant, Sensitive and Intermediate (NCCLS, 2000). The *E. coli* (ATCC 25922) was used as a positive control. Antimicrobial discs with potency used for Gram negative bacteria were Ampicillin (30 μ g); Amoxiclav (30/10 μ g); Oxacillin (30 μ g); Vancomycin (30 μ g); Gentamycin (10 μ g); Ciprofloxacin (30 μ g); Nitrofurantoin (10 μ g); Ceftazidime (30 μ g); Cotrimaxazole (30 μ g); Trimethoprim (30 μ g); Imipenem (10 μ g); Amikacin (10 μ g); Amoxicillin (30 μ g); Cephalixin (30 μ g); Cefuroxime (30 μ g); Nalidixic acid (30 μ g); Cefazoline (30 μ g); Cefdinir (30 μ g); Cefexime (30 μ g); Ceftriaxone (30 μ g); Cefuroxime (30 μ g); Cefotaxime (30 μ g); Aztreonam (30 μ g); Cefaclor (30 μ g) and Penicillin (10 μ g) (CLSI 2010).

The Multiple Antibiotic Resistance Indices (MARi) were calculated as follows:

MAR index for isolates

$$= \frac{\text{Number of antibiotics to which the isolate is resistance}}{\text{Number of antibiotics tested}}$$

2.6. Extended Spectrum β -lactamase Test

Presence of Extended spectrum β -lactamases were determined by modified Double Disc Synergy Test (DDST) [19]. The 0.5 Mac Farland turbidity adjusted bacterial test strains were swab inoculated on Muller-Hinton agar plates (HiMedia) and 3G Cephalosporin i.e. Cefotaxime (30 μ g), Ceftazidime (30 μ g) were placed 15 mm apart from a disc of Amoxicillin plus Clavulanic acid (augmentin 20 μ g + 10 μ g) at the center of a Petri-dish and incubated at 37°C for 18-24 h. An enhanced zone of inhibition between any one of the β -lactam disc towards the augmentin disc was interpreted as presumptive evidence for the presence of ESBLs [20].

2.7. Metallo β -lactamase Test

Presence of Metallo β -lactamase was determined by modified Combined Imipenem-EDTA (IMP-EDTA Combined Disc Test) [21]. Screened ESBL positive isolates were swab inoculated on Muller-Hinton agar (HiMedia) plates as per the CLSI guidelines. Two Imipenem disc 10 μ g (HiMedia) were placed on plate and appropriate amount of 10 μ l of EDTA solution was added to one of them so as to make the final concentration 750 μ g. Inhibition zones were compared after 16-18 h of incubation at 37°C. Isolates were considered as MBL positive when inhibition zone increases with the imipenem and EDTA disc ≥ 7 mm than the Imipenem disc alone.

2.8. Amp C β -lactamases Test

Presence of Amp C β -lactamases was detected by Modified Three Dimensional Extract Method (TDEM) [22]. A 0.5 Mac Farland (50 μ l) resistant bacterial suspension was inoculated into 12 ml of Trypticase Soy Broth and was

incubated at 37°C for 24 hrs. The MHA plate surface was inoculated with *E.coli* ATCC25922 (Sensitive to Cefoxitin) as described standard Disk diffusion method. A 30 μ g Cefoxitin disk was placed at the center of the inoculated plate. With a sterile scalpel blade, a liner slit beginning 5 mm from the edge of the disk was cut in agar in an outward radial direction. Overnight incubated fresh bacterial suspension was picked with sterile wire loop and the slit was inoculated at depth, beginning near the disc and moving outwards with a heavy inoculums. The plates were incubated at 37°C for overnight. Enhanced growth of the surface organism (*E.coli*) at the point where slit intersected the zone of inhibition was considered a positive result and was interpreted as evidence for the presence of Amp C β -lactamase.

2.9. β -lactamase Iodometric Test

Existence of β -lactamase enzyme in each isolate was quantitatively determined by modified iodometric method [23]. Briefly, the starch indicator solution containing 1.0g of soluble starch (Merk) in 100 ml of Distilled water was prepared fresh each day or two. The iodine reagent contained a mixture of 2.03g of iodine and 53.2 g of potassium iodide which dissolved in 100 ml of distilled water and stored in a brown glass bottle. The 0.05 M of Phosphate buffer of pH 5.8,7,8 was made. A solution containing 10,000U of Penicillin G per ml of phosphate buffer was freshly prepared and dispensed in 0.5 ml volumes in small vials. A 0.5 Mac Farland freshly prepared test bacterial solution was added in to vial and incubated for 1 hr. After 1 hr or longer at room temperature, two small drops of starch indicator were added to the suspension, followed after mixing by one small drop of iodine reagent. A blue color was immediately developed due to the reaction of iodine with starch. The reaction mixture was further rotated for up to 1 min. Persistence of blue color for longer than 10 min constituted a negative test and indicated that the Penicillin molecules had not undergone β -lactam ring cleavage. Rapid decolorization is considered as positive test. Two tubes of Penicillin solution without bacteria served as controls, one tube received drops of starch and iodine at the beginning of the hour, and the second control tube was held until the end of the hour when the reagents were added to the tests. Retention of the blue color by both controls for the duration of the test period testified to be the absence of spontaneous Penicillin hydrolysis or possible hydrolysis by enzymes of microorganisms that might contaminate the starch or buffer solutions.

2.10. β -lactamase Nitrocefin Test

β -lactamase Nitrocefin Test [24] is the gold standard method for rapid determination of β -lactamase activity. A working solution of Nitrocefin was prepared by adding 0.5 ml of Dimethyl Sulfoxide to 5 mg of Nitrocefin powder (Calbiochem, UK). A 0.5 ml of 0.1 M Phosphate buffer (pH 7.0) is added to make a final Nitrocefin concentration of 500 μ g/ml (frozen at -20°C). A loopful of test organism was removed from colonial growth and emulsified in one drop of

the above prepared Nitrocefin solution (500 μ g/ml) on a clean glass test tube. A change in color from yellow to red within 15 min indicates a positive reaction.

2.11. Determination of Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC)

MIC of all confirmed β -lactamase producers were determined by Tube Dilution method [25]. About 25 different antibiotics i.e. Ampicillin, Amoxiclav, Oxacillin, Vancomycin, Gentamycin, Ciprofloxacin, Nitrofurantoin, Ceftazidime, Cotrimaxazole, Trimethoprim, Imipenam, Amikacin, Amoxicillin, Cephalixin, Cefuroxime, Nalidixic acid; Cefazoline, Cefdinir, Cefexime, Ceftriaxone, Cefuroxime, Cefotaxime, Aztreonam, Cefaclor, Penicillin specified for uropathogens, β -lactam class (HiMedia) in the range from 240-0.01 mg/ml were prepared in 3 ml nutrient broth, 0.5 Mac Farland turbidity adjusted confirmed β -lactamase positive culture was added into each test tube individually. Similar test tube set containing all above cited antibiotics were treated as control group. Test tubes were incubated at 37°C for 24 h and examined for turbidity. After incubation, tube containing least concentration of drugs showing no visible sign of growth was considered as the minimum inhibitory concentration (MIC). Tubes showing positive MIC were further continued for determination of MBC on sterile Muller-Hinton Agar plates.

2.12. Virulence Characterizations

2.12.1. Hemolysin Production

Production of hemolysin was assayed on Sheep blood agar plates [26]. All isolates were grown overnight at 37°C in LB medium. 50 μ l of 0.5 Mac Farland turbidity adjusted culture was streaked on sheep blood agar plate and incubated overnight at 37°C. Hemolysin production was verified by the presence of a clear hemolytic halo around the colonies.

2.12.2. Colicin Production

Colicin production was evaluated by the Agar overlay method [27]. All isolates were cultured in tryptic soy broth at 37°C overnight and plated onto tryptic soy agar. The colonies obtained after overnight incubation at 37°C were killed with chloroform and overlaid with 3 ml soft Tryptic Soy agar containing Colicin indicator strain (*E.coli* K₁₂ ATCC 23559). After overnight incubation at 37°C, the capacity of colicin production was detected by the presence of a halo around the producer colonies.

2.12.3. Cell Surface Hydrophobicity

Cell surface hydrophobicity was tested by Salt aggregation test [28]. Bacteria were tested for their hydrophobic property by using different molar concentrations of ammonium sulfate. Those bacteria which aggregated with salt particles and formed the clumps were considered to be hydrophobic. A strain of *E.coli* which was haemolytic, MRHA positive and consistently positive for cell surface hydrophobicity was used as positive control whereas a strain which was non-hemolytic, negative for MRHA and consistently negative for

cell surface hydrophobicity was used as negative control. All isolates grown on MHA plates were inoculated into 1ml of PBS pH 6.8 and 0.5 Mc Farland turbidity matched tubes to get a colony count of 5×10^9 colonies/mL. Different molar concentrations of ammonium sulfate namely 1M, 1.4M and 2M were prepared. About 100 μ l of 0.2 M PBS (pH 6.8) was taken in clean sterilized petri plate and 100 μ l of 1M, 1.4M and 2M concentrations of ammonium sulphate were taken in another petri plates. Then 100 μ l of each isolate suspension was added to each of these plates. The clumps formed in different molar concentrations of ammonium sulphate was observed under Leights Wetzlar inverted microscope-binocular (0.32) at $20\times$ magnification and scored positive on one to four scale (1+ to 4+). Strains were considered hydrophobic if they aggregated in concentrations of 1.4M. Isolates which gave doubtful results were retested.

2.12.4. Aerobactin Production

Production of aerobactin was assayed by growing the strains isolated in a LB medium containing 200 μ M of α, α dipyridyl for 24 h at 37°C, without shaking. The grown mass was spun for 3min at 12000g, the supernatants filtered through a 0.22 μ m nitrocellulose membrane and 50 μ l aliquots added to orifices made in a LA medium [26], previously seeded with strain LG 1522 [29]. Petri dishes were kept at 37°C for 48h and the production of aerobactin visualized by the growth of strain LG 1522 around the orifices.

2.12.5. Serum Inactivation Assay

Serum inactivation assay was performed by the standard method [30]. All the isolates were inoculated at 0.1%(v/v) inoculums level into glucose phosphate broth containing bromothymol blue and 2% human serum and incubated at 37°C for 24 h. The serum resistance was observed by change in color from green to yellow and extinction of yellow color was measured at 500 nm.

2.13. WHONET Antimicrobial Surveillance Study

2.13.1. Participating Hospitals

The present study was conducted in the School of Life Sciences (Biotech Lab), SLS, S.R.T.M.U. Nanded (MS), India from June 2009 to June 2010 of five different pathology laboratories from Nanded city. To participate in the network the main prerequisite is the availability of a PC installed with WHONET software. All data were entered into the program, if possible on a daily basis. Data entry was done either manually (for hospitals using the disc diffusion method for sensitivity testing) or by automatic download with the aid of BACLINK software (available upon request from Dr J. Stelling, Communicable Disease Surveillance and Response Programme, World Health Organization, Geneva). Each laboratory was asked to agree to make available all electronic files for purposes of preparing multicentre reports.

2.13.2. Isolation and Identification of Pathogenic Bacteria

Isolation of pathogenic bacteria from clinical specimens and identification to the species level was performed by standard methods as mentioned above.

2.13.3. Sensitivity Testing

The sensitivity testing methods used were as follows: Kirby-Bauer disc diffusion. The zone diameters or minimum inhibitory concentrations (MICs). Antibiotics were selected according to WHO model list of essential drugs. β -lactamase production was assessed by Nitrocefin test method. NCCLS breakpoints for third generation Cephalosporins have recently been revised to detect ESBL production.

2.13.4. Quality Control

Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) strains were used as internal quality controls. Moreover, the External Quality assurance scheme was organized by the WHO Collaborating Centre for monitoring International Bacterial Resistance to Antimicrobial Agents, Centers for Disease Control and Prevention, Atlanta, GA, USA.

2.13.5. Data Reporting

Main prerequisite for compilation of data was a PC installed with WHONET software. All data was entered into the program manually on a daily basis. A large number of permutation combinations were available in the program for analysis.

3. Results and Discussion

Identification of uropathogen and their susceptibility pattern is an important criteria to treat Urinary Tract Infections (UTIs). In the present study, Urine specimens were cultured to see pattern of uropathogens. Prior antibiotic therapy clinical conditions like non-gonococcal urethritis or others UTI could be the factors responsible for insignificant bacteriuria. No growth indicates the need for educating the patients about the method of collection of clean catch mid-stream urine specimens.

Table 1. Urine culture test.

Diagnostic Tests	Urine culture	
	$\geq 10^3$ CFU/mL	10^3 CFU/mL
Nitrite test in all patient		
Positive	204	7
Negative	156	126
Leukocyte esterase test in cases of nitrite negative tests		
Positive	132	93
Negative	24	33

n=360

Out of 360 collected urine samples 336 (82.44%) urine samples revealed significant culture positive urine test (Table 1). In case of the performance characteristic of the diagnostic tests out of 360 UTI positive samples 204 specimen showed positive nitrite with $\geq 10^3$ CFU/mL, whereas 132 positive specimens showed Leukocyte esterase positive in cases of nitrite negative tests (Table 1). These tests can reveals prevalence of UTI in relation to age and sex of patients. Age and Size distribution of the culture-positive cases, 36 (10.71%) were children aged 10 years or less, and 46 (12.79%) were aged 60 years and above. The age group

41-60 exhibited highest growth of uropathogens i.e.113 (33.63%). In all age groups, except those aged 0-10 years, females were frequently affected than males (Table 2). The age and Sex distribution of the patients followed the natural epidemiologic pattern of UTI. There was a predominance of young and middle aged females, whereas in the older age groups of more than 60 years 1:2 proportions of male and female had UTI.

Table 2. Age and Size distribution of the culture positive Urine samples.

Age groups (years)	No of samples		Total
	Male	Female	
0-10	15	21	36 (10.71%)
11-20	22	34	56 (16.66%)
21-40	23	62	85(25.29%)
41-60	26	87	113 (33.63%)
Above 60	15	31	46 (12.79%)
Total	101	235	336 (82.44%)

n=360

Table 3. The Microscopic examination of Urine samples.

Bacteriuria and Pyuria	No	Percentage (%)
Significant Pyuria	130	38.69%
In Significant Pyuria	170	50.59%
Significant pyuria with Growth	125	37.20%
Significant pyuria without growth	12	3.57%
Significant pyuria with insignificant growth	17	5.05%

n=336

Microscopic examination of the centrifuged urine revealed that out of 336 culture positive urine samples 130 (46.93%) specimen showed significant pyuria (pus cells of 5 cell/hpf) while 170 (61.37%) showed insignificant pyuria (pus cells of 1-2 cells / hpf). Out of 130 significant pyuritic samples, 125 (37.20%) yielded significant bacterial growth, 17(5.05%) insignificant bacterial growth while 12(3.57%) yielded no bacterial growth (Table 3). The presence of simple bacteriuria particularly in females should not be neglected; it may act as a risk factor for urinary tract infection. The prevalence of bacteriuria in female population increases gradually with time reaching 5% in women of child bearing age and 10-20% in post menopausal age. In our study 130 out of 336 female patients (38.69%) showed significant bacteriuria. The presence of pus cells in urine (pyuria) is an indication of the Urinary Tract Infection. In one of the reported study it has been observed that approximately 75% of the patients with urinary tract infection have pyuria which is in close conformity with our study [31].

In case of uropathogen isolation, of the 549 isolates obtained, Gram-negative bacteria had a higher frequency of occurrence than Gram-positive isolates. These includes: *E. coli* 262 (47.72%); *Pseudomonas aeruginosa* 45 (8.19%); *Proteus mirabilis* 47(8.56%) *Klebsiella pneumoniae* 44 (8.01%), *Enterobacter* spp. 33(6.01%), *Escherichia* spp. 12(2.18%), *Acinetobacter haemolyticus* 18(3.27%) *Serratia* spp.11 (2%), *Micrococcus* spp. 5(0.91%), *Shigella* spp 18(3.27%) and *Enterococcus* spp. 25(4.60%). Gram positive bacteria accounted for 40 (22.22%) of the isolates like

Staphylococcus aureus 28 (5.10%). It was also found that the rate of isolates of *E. coli* and *P. aeruginosa* were higher in isolates exclusively from females (Table 4). The most common organism isolated in these patients were *E. coli* (47.72%), *P. aeruginosa* (8.19%), *P. mirabilis* (8.56%) and *S. aureus* (5.10%). This finding is similar to other reports [15] which indicate that a Gram-negative bacterium, particularly *E. coli*, is the commonest pathogen isolated in patients with UTI. In this study, the prevalence of UTI in females is more than in males. Of the 549 isolates obtained, 394 were from female patients while 154 were from male. This is in agreement with reports (Cheesbrough, 2000) which states that UTI is more frequent in females than in males, during youth and adulthood. We isolated total 549 uropathogens from 130 significant culture positive urine samples and differentiated according to bacterial identification procedure of Bergey's Manual of Determinative Bacteriology as well as molecular identification system. Results revealed wide number of different uropathogens which indicate the urine samples were severely infected by number of bacteria hence the patient need quick and proper treatment.

Table 4. Isolation of uropathogens among 130 significant positive Urine culture Test.

Isolates	Sex				Among all subjects	
	Female		Male			
	No	%	No	%	No	%
<i>E.coli</i>	222	56.34	40	25.97	262	47.72
<i>Proteus mirabilis</i>	31	7.9	16	10.38	47	8.56
<i>Klebsiella pneumoniae</i>	33	8.37	11	7.14	44	8.01
<i>Pseudomonas aeruginosa</i>	24	6.09	21	13.05	45	8.19
<i>Enterobacter spp</i>	21	5.32	12	7.79	33	6.01
<i>Escherichia spp.</i>	5	1.26	7	4.54	12	2.18
<i>Staphylococcus aureus</i>	9	2.28	19	12.33	28	5.10
<i>Acinetobacter haemolyticus</i>	11	2.83	7	4.54	18	3.27
<i>Serratia spp</i>	5	1.26	6	3.89	11	2.00
<i>Micrococcus</i>	2	0.50	3	1.94	5	0.91
<i>Shigella spp.</i>	17	4.31	1	0.64	18	3.27
<i>Enterococcus spp</i>	13	3.29	12	7.79	25	4.60
Total	394	100.00	154	100.00	549	100.00

No. reflects the number of isolates. P-value = 0.03

The *in-vitro* antibiotic sensitivity pattern of the isolates to common anti-microbial agents is shown in Fig 1. Results indicated that Ampicillin, Ceftazidime, Amoxicillin, Ceftriaxone, Cefazoline, Cefdinir are the most ineffective antibiotics against isolated MDR pathogens. *E.coli* was the principle pathogen isolated showing a high susceptibility to Nitrofurantoin (85%), Imipenem (80%), Amikacin (80%), Amoxicillin (75%) but showed variable sensitivity to other commonly used antibiotics. *E.coli* having MARi Index 0.9 revealed highest drug resistance to Ampicillin (99%), Ceftazidime (98.99%), Amoxicillin (98%), Ceftriaxone (99%), Cefuroxime (98%), Cefotaxime (99%), Cefaclor (98%), and Penicillin (99%). In the present study, *Klebsiella* species (MARi index 0.8) showed high susceptibility to

Imipenem (100%) and Amikacin (85%) but were relatively resistant to commonly used β -lactam antibiotics. *Proteus* species were 100% susceptible to Imipenem, 50% to Amikacin, Nitrofurantoin, Ceftriaxone and Amoxiclav each as per the earlier findings [32]. *Pseudomonas* species (MARI index 0.84), a common cause of hospital-acquired UTI was found resistant to Imipenem (100%), Amikacin (75%), Ceftazidime (95%). *Pseudomonas* species were relatively

susceptible to the second line of anti-*Pseudomonas* drugs and most of these were associated with high-level resistance to the first line antibiotics investigated namely Amoxiclav, Ceftriaxone, Ciprofloxacin and Gentamicin. This may be due to widespread use of common antibiotics in the hospitals and cross-resistance among different bacteria. Similar results were reported by other investigators [33, 34].

Table 5. Molecular Identification of β -lactamase producing uropathogens.

NCBI Submission (2012): *Escherichia fergusonii* strain GCR -1, *Shigella flexneri* strain DMB- 1, *Enterococcus faecalis* strain ADR-1, *Shigella flexneri* strain SMB-11, *Enterobacter cancerogenus* strain MAB-1, *Shigella dysenteriae* strain CNK-77, *Enterobacter cowanii* strain RMB-3.

• *Escherichia fergusonii* strain GCR -1; GenBank: JX827388.1

Agcatatctaccgtggggggggtccgaaggttaacacactactctttgtctaccactccatgggggtgacggcggtgtgtacaaggccgggaacgtattaccgtggcattctgatccacgattactagcgattccgacttcacgagtcgagttgcagactccaatccggactacgacgactttatgaggtccggttctctcgcgaggtcgtctctttgtatgtccattgtagcacgtgtgtagccctgggtcataaggggcatgatgactgacgtcatcccacttctccggttatcactggcagttcctttgagttcccgccggacgctggcaacaaagataagggttgcgtctgttgcgggacttaaccaacatttcacacacagctgacaacagccatgcagcacctgtctcaggttcccgaaggcaaatctcatctctgaaaacttccgtggatgtcaagaacaggtaagggttctccgttgcacccaattaaacacatgctccaccgttgcggggcccgctcaattcaatttaacattgcggacgtactccccagcggttagacttaacgcgttagctccgggaagccagcctcagagacacacatcccagtcgacatcgttaccgggtgtactacccgggatctaatacgtgtgttccccactttctacctgaccgtcagttctctgtaacggagacgccttctccccggatgcctccccatctcagcatttaccgctacacgtggattctaccctctctctatacgaagcttgccactatcacagcgactccccacgtgagccggggttctcactgacactaaaaagagcgcgctgcgtttacgcccgttaatttgcattacgttgcgcctccacattatagcgctgctgtgacgaatttccgggcttctctgtagtaacgtcatgacagacagctactcctgcctcccgctgcaagtaagttacagatcaagacctctctacacccgcatgctgcctcacgttggccattgccaataatcatgcgctccgtaggacggagcggttcgagtcagtggtgaatcttcaacgtagactgccatggaagcgtaccagccatgcctggaactcatgacagccttagccctctatgctcgcccgcttatgacgaatgact

• *Shigella flexneri* strain DMB-1 ; GenBank: JX827385

gctacacatg cagtcgaacc gagtgcagc cgcaagcaag ctgtctgctt tgcgtgacgag tggcgagcgg gtgagtaatg tctgggaac tgcctgatgg agggggataa ctactggaaa cggtagctaa taccgataa cgtcgaaga ccaagagggg ggaccttcgg gcctcttgcacatcgatgtg cccagatggg attagctagt aggtggggta acggtcacc taggcgacgacccctagctg gtctgagagg atgaccagcc acactggaac tgagacacgg tccagactc ctacgggagggc agcagtgggg aatattgcac aatggcgcca agcctgatgc agccatgccg cgtgtatgaa gaaggccttc ggggtgtaaa gtactttcag cggggaggaag gggagtaaaag ttaatacctt tgcctatga cgflaccgcg agaagaagca ccggtcaact cctgtccagc agccgcggta ataccgaggg tgaagcggtt aatcggaatt actggcgta aagcgcacgc aggcggttg ttaagtca gttgaaatcc cgggctcaa cctgggaact gcactgata ctggcaagt tgagtc

• *Enterococcus faecalis* strain ADR-1 ; GenBank: JX827384.1

tacacatgca gtgcgaacgt gtgctatcca tcccagatgc ttgcactcaa tgaagaagaggacggcgga cgggggagta acactgtgggt aacctacca tcagaggggg ataactctg gaaacaggtg ctaataccgc ataacagttt atgccgcatg gcataagagt gaaaggcgt ttcgggtgct gctgatggat ggaccgcgg tgcattagct agttgggtgag gtaacggctc accaaggcca cgtatcatag ccgacctgag aggggtgatg gccacactgg gactgaga cagggccagac tctacggga ggcagcagta gggaaatctc ggcaatggac gaaagtctga ccgagcaacg ccgcgtgagt gaagaaggtt ttggatcgt aaaaactctg ttgtagagaa gaacaaggac gtttagtaac gaactgcce tgcaggtatc taaccagaaa gccacggcta actacgtgcc agcagccgcg gtaataccta ggtggcaagc gttgtccgga ttattggcgtaaaagcag cgcagcgcg

• *Shigella flexneri* strain SMB-11 ; GenBank: JX827383.1

ctacacatgc agtcgaacgg tagcagcgca aacagcttgc ttttctgctg acgagtggcgacgggtgag taatgtctgg gaaactccct gatggagggg gataactact ggaacggtagtaataaccg cataactgc caagacaaa gaggggggacc ttggggctc ttgcatcgg atgtgccag atgggattag ctgttgggt gggtaacggc tcacctagcg gacgatccct agctggtctg agaggatgac cagccacact ggaactgaga caggttccag actcctacgggagcgacgag tggggaatat tgcacaatgg gcgcaagcct gatgcagcca tggcgctgtatgaagaagg ccttcgggtt gtaaaact ttcagcgggg aggaaggag taaagttaac accttctc attgacgtta cccgagaag aagcaccgc taactccgt ccagacggc cggtaatagc gagggtgcaa gcgttaatc gaattactgg gcgtaaagc cagcagcgcggttgaag tcagatgtga aatccccggg ctcaactgg gaactgcac tgatactggcaagcttgagtc

• *Enterobacter cancerogenus* strain MAB-1; GenBank: JX827382.

ggggagtagc gccgaaggt taaaactcaa atgaattgac gggggccgc acaagcggtggagcatgtgg ttaattcga tgaacgcga agaacttac ctactctga catccagagaacttagcaga gatgctttgg tgcctcggg aactctgaga caggtgtgc atgctgtcgtcagctcgtg ttgtgaaatg ttgggttaag tcccgaacg agcgcaacc ttatccttg ttccagcgg tccggccgg aactcaagg agactgccag tgataaactg gaggaaggtg gggatgact caagtcatc tggcccttac gtagtaggct acacacgtc tacaatggcgatacaaga gaagcgact cgcgagagca agcgacactc ataaagtgcg tctgtatccggttgagtc tgaactcga ctcatgaagtcggaatcgtatgactg gtagatg

• *Shigella dysenteriae* strain CNK-77 ; GenBank: JX827386

tacacatgca gtcgtgcaag gtaccaagac tttgtctt tcttctgag tgggggaggg gtgagtaatg tttgggaac tgcctgatgg aggggattaa ctactggacc ggtacttaataccgataac gtcttaagac caaggagggg gaccttcggg cctcttcca tccgagtgtgccgaatggga ttcgtatgta tttgggttaa cggctcact aggcacat cctaccaggt ctgacagga tgaccagcca cattggaact gatacaggt ccacactcct acggcacgcaggggtgggga atattgcata ttggcgcaaa tctgaagca cccgtccgc gtgtatgacg aaggccttca ggttttctag atcttcaactgggatgaag ggaaaaaagt tattaacttctgtgtg ttccccga gaacaatct agaataact cgtgccatc ccgcgcgagta cggagggt gcaagcgtga atcggaatta ctggacttat agcgacgca ggcggctgtgctgtctat gttgaatcc cgagctccccctgtaactg caggagatac tttcaagctt gtagtc

• *Enterobacter cowanii* strain RMB-3 ; GenBank: JX827387.1

catacatgcatgctgataagagtaacgcaagcagcttgcgttttgcgtgacgagtgccggaggggtgagtaattgtctggga aactcgtgatggaggagataactactggaacggtagtaataacgataacgtcgcaagaccgaagagggggaccttc gggcctcttgcacatcgatgtgccagatgggattagctagtggtgggtaacggctacactagggacgacatccctagctgg tctgagaggatgaccagccacactggaactgaacacggtccagactcctacgggagggcagcagtggggaattatgcaca tggcgcaagcctgatgacgacatgccgctgtatgaagaaggccttcgggttgaagtacttctacgaggagggaaggcg ataaggtaaa taacctgtc gattgacttt acccgagaa gaagcaccgg ctaactccgt gccagc

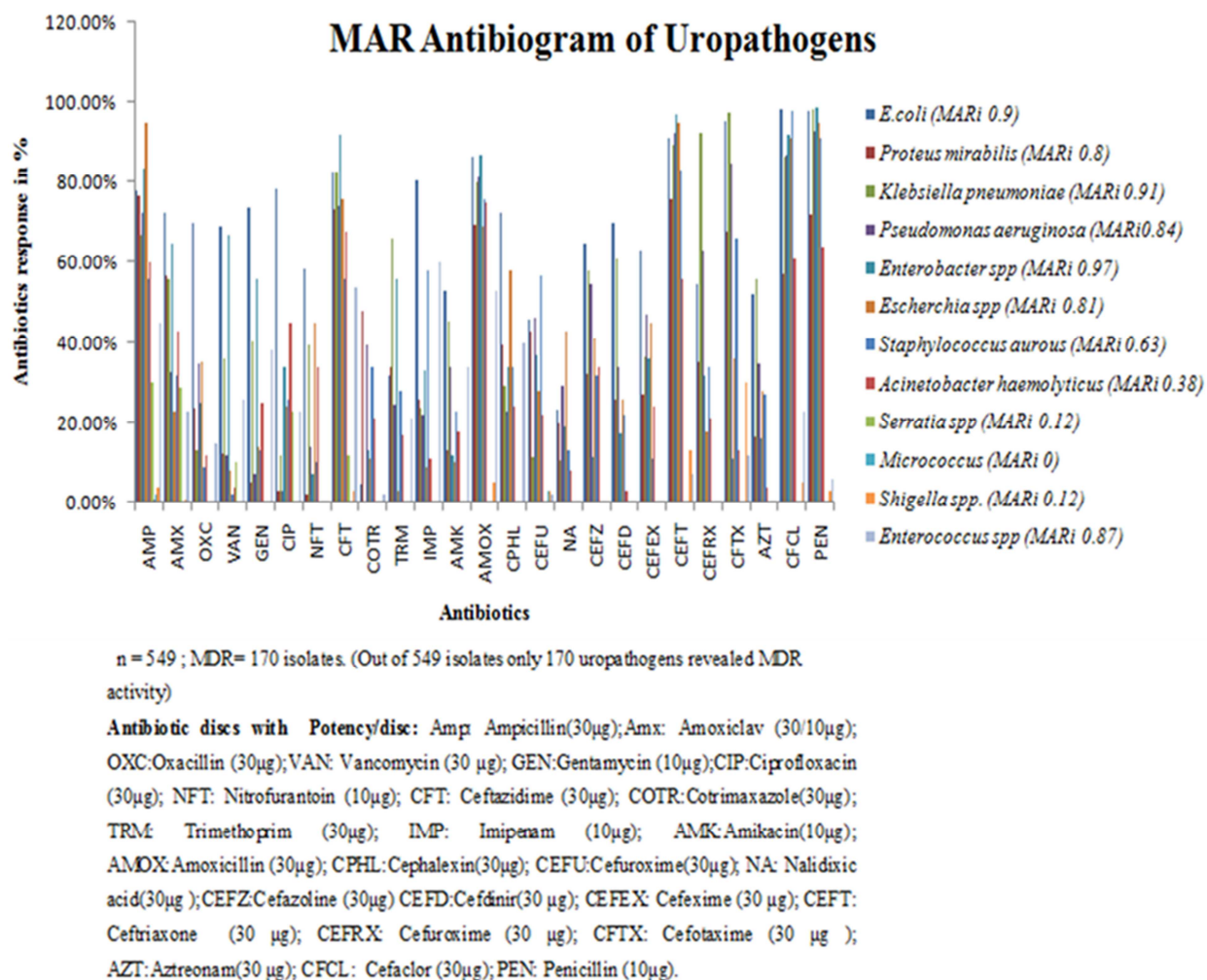


Fig. 1. Screening of uropathogens for MAR antibiogram and MAR Index with respect to β -lactam class.

S.aureus, MARi index 0.63 were found 100% sensitive to Vancomycin, 83% to Amikacin and Gentamicin each but were less susceptible to other common antibiotics. Coagulase-negative *Staphylococcus* were 80% sensitive to Vancomycin and Imipenem but relatively resistant to other antibiotics Jones R. *et al.*, 1999 reported similar study. As regards to *Enterococci* (MARi index 0.87), 83% of the isolates were resistant to Imipenem, 67% to Ciprofloxacin, 50% to each of Vancomycin, Gentamicin and Ceftriaxone. A report from SENTRY antimicrobial surveillance program, USA also reported the similar results. The *Staphylococcus*, *Acinetobacter*, *Serratia* spp and *Shigella* spp were exhibited high drug resistance towards third generation β -lactam antibiotics with MARi index of 0.63, 0.38, 0.12 and 0.12 respectively. If MAR index of isolated bacteria is greater than 0.2 then it implies that strains of such bacteria originate from an environment where several antibiotics are used. MAR index of isolates in present study indicate large portion of bacteria were exposed to the antibiotics showing MAR index more than 0.2. It may be due to large portion of the bacterial isolate being previously exposed to several antibiotics. The present data gives idea about the common trend of increased antibiotic resistance of uropathogens in this region (Nanded),

which may be due to geographic variation or indiscriminate or sublethal use of antibiotics. This data not only help in proper treatment of UTI patients but helps to prevent further development of bacterial drug resistance by limiting the use of antibiotics. Finally, since the hospital environment is a sort of collection agency for many pathogenic microorganisms by virtue of many seriously ill patient who passes through it therefore it is extremely important for the hospital management to do everything possible to minimize the spread of these organisms to other patients. A high isolation rate of pathogens from urine samples of clinically suspected UTI shows a good correlation between clinical findings and microbiological methods. Gram-negative bacteria from *Enterobacteriaceae* family were the commonest organisms isolated.

Out of screened drug resistant uropathogens 7 prominent β -lactamase producers such as, Extended spectrum β -lactamases, Metallo- β -lactamases, Amp C β -lactamases etc. were identified by molecular identification method (16 S r RNA). All these novel isolates revealed to be having all β -lactamases in a single strain. We submitted this data on www.ncbi.com to GENBANK for sequence submission (Table 5) having accession numbers: *Escherichia fergusonii*

strain GCR -1; GenBank: JX827388.1, *Shigella flexneri* strain DMB-1; GenBank: JX827385, *Enterococcus faecalis* strain ADR-1; GenBank: JX827384.1, *Shigella flexneri* strain SMB-11; GenBank: JX827383.1, *Enterobacter cowanii* strain RMB-3; GenBank: JX827387.1, *Shigella dysenteriae* strain CNK-77; GenBank: JX827386, *Enterobacter cancerogenus* strain MAB-1; GenBank: JX827382.

In the Double disc synergy test, the combinations of Cefotaxime against Amoxicillin / Clavulanic acid with Ceftazidime of all isolates were found to be positive. We agree with observations of previous studies considering ceftazidime to be most effective in detecting ESBL producers among the 3GCs. The strength of our study is that, in our study population the percentage of ESBL producers detected by the phenotypic confirmatory method also produced MBL by Imipenem-EDTA method and Amp C β -lactamase by Three dimensional extract method.

Methods of detecting ESBL producing bacteria have been evolving for more than a decade, beginning with the description of the disc approximation test [35] and subsequently described by Jarlier V. *et al.* 1988 [36]. The three-dimensional test is a more sensitive procedure for the detection of ESBLs. However, this technique had its own limitations. Subsequently, the test was modified with the use of a slit technique with bacterial suspensions and enzyme extracts, but filling these slits was a fastidious procedure. Despite different modifications tried by various researchers, to date, no satisfactory technique has been established. Bearing all these points in mind, we tried some modifications in search of a simple and more user-friendly procedure than existing conventional phenotypic tests. In the modifications for enzymic extracts, all AmpC and ESBL producing bacterial isolates were detected correctly by the designed modifications (trenches/wells). Comparing the modifications with the conventional slit test, the enhanced growth of surface organisms was interpreted more easily in the designed modifications [Figure .2, 3, 4, 5, 6]. All the ESBL and AmpC producing isolates were detected correctly by adopting this method.

MBL production has been previously reported from India [37-39]. Most studies have used IPM-EDTA combined disc, Double Disc Synergy test using IPM-EDTA and modified With the Imipenem and EDTA combined disc test with a cut-off >7 mm, the positive and negative results were more clearly discriminated. The combined disc method using Imipenem + EDTA [Fig.3] was found to be superior to DDST (using imipenem - EDTA) and EDTA disc potentiation with Cefotaxime, Cefotaxime, Cefizoxime and Cefepime [40]. From this study, it was apparent that various different mechanisms exist for production of multiple β -Lactamases, especially in places where newer β -Lactams were being routinely prescribed. In case of MBC, in some cases we got similar values as like MIC and total killing of bacteria took place at the same MIC concentration [Table 6]. All novel isolates were further analyzed by phylogenetic tree analysis by CLUSTAL W 2 [Fig.7].

Bacterial virulence factors are responsible for

pathogenicity and can be effective screening criteria to find out pathogenic nature of UTI pathogens. Thus all novel 7 MDR isolates were continued further for the determination of Virulence factors. All isolates were serum resistant, Colicin producers, haemolytic, Aerobactin producers and having Cell surface hydrophobic [Fig. 8, 9, 10, 11, 12].

The haemolysin, especially α -haemolysin, also known as cytotoxic necrotizing factor, is strongly proinflammatory leading to secretion of IL - 6 sets pace for pathogenesis of renal disease. The capacity to cause MRHA is due to various adhesins mainly P fimbriae, P associated fimbriae, and FIC fimbriae as seen in pyelonephritis cases. These adhere to fibronectin on uroepithelial cells contributing to persistence. The cytolytic protein toxin secreted by most haemolytic *E.coli* strains is α -haemolysin. These seven isolates produces cell associated lysis on blood agar plates and hence haemolysin cause a clear zone of lysis [41] [Fig.8]. In the present study, though the nature of haemolysin was not further characterized it can be considered as cytotoxic necrotising factor (haemolysin). The difference between cases and controls for production of haemolysin was highly significant $p < 0.001$ [42]. Hemolysin production was found to be broadly distributed, being the second most detected virulence factor among vaginal isolates [43].

All isolates were Colicin producers [Fig.9]. All seven isolates gave 1+, 3+, 4+ grade clumping in 1 M, 1.4 M and 2 M ammonium sulphate concentrations respectively [Fig.10]. The role of cell surface hydrophobicity (CSH) in mediating bacterial adherence to mammalian cells is reported elsewhere [44]. Crystalline surface layers "S" present on both Gram-negative and Gram-positive organisms, play a vital role [45]. Hydrophobicity is a recently described novel virulence mechanism by *E.coli*.

All investigated strains were aerobactin positive [Fig.11]. About 90% of urinary strains isolated from UTI patients produced aerobactin [28] which confers an advantage to bacterial strains for the colonization of the urinary tract. During parturition, the physical barriers of the cervix, vagina and vulva are compromised providing an opportunity for environmental bacteria to ascend in the genital tract causing uterine infection. This situation is in a dynamic flux, of regular contamination, followed by its clearing up and by spontaneous re-contamination of bacteria during the first weeks following parturition [47].

All isolates were revealed Human serum bactericidal activity at 2% concentration [Fig.12]. Moreover, serum resistance also supports the bacterial resistance to different drugs. As human body is a rich source of bacteria but due to the presence of such inherent antibiotic resistant pathogens the opportunists would be converted into virulent and drug resistant. Serum and antibiotic resistance are one of the survival strategies of the disease causing organisms to overcome the human immune defense mechanism, which is a matter of concern as far as the treatment of the disease. It is accepted fact that bacteria are killed by normal human serum through lytic activity of alternative complement system [48]. Bacterial resistance to killing by serum, results in individual

or combined effects of capsular polysaccharide, O polysaccharide and surface proteins [49].

To our knowledge the methods of detection of the above mentioned virulence markers are reasonably easy and screening them in a clinical microbiology laboratory is a worthwhile exercise. Bacterial adherence to host cells is a crucial step in the initiation of various infectious diseases. Adherence factors facilitate the colonization of the urinary tract for ascending infection

Surveillance of resistance is essential to monitor and control the spread of antimicrobial resistance. The WHONET program provides uniform guidelines for performing antimicrobial susceptibility tests. It also provides software through which data from each laboratory is entered into a common code and file format for that laboratory. This data can be shared with another laboratory working on WHONET program for further collaboration and for implementation of various control measures.

All seven novel multiple β -lactamase producers were studied for sensitivity pattern against various antibiotics by WHONET 5.6 Software. *Escherichia fergusonii* GCR-1,

Shigella flexneri DMB-1, *Enterococcus faecalis* ADR-1, *Enterobacter cowanii* RMB-3, *Shigella dysenteriae* CNK-77, *Enterobacter cancerogenus* MAB-1, revealed highest percentage of ESBL production. All isolates were 100% resistant to antibiotics according to histogram study [Fig.13]

The WHONET study shows that highest resistance was against ampicillin (94%) followed by Nalidixic acid and Cotrimoxazole (86.4 and 80.8%, respectively). Resistance rate to majority of drugs was 42-59%. These include Clindamycin, Erythromycin, Netilmicin, Gentamicin, Ciprofloxacin, Norfloxacin, Piperacillin, Nitrofurantoin, Amikacin and Coamoxyclav. Resistance to Ceftazidime and Cefaperazone was 38.5 and 20.7%, respectively. All data was entered into the program manually on a daily basis. A large number of permutation combinations were available in the program for analysis. Hence the species isolated, resistance rates and resistance phenotypes can be compared between hospitals, resistance problems can be recognized, and priorities for further research can be set based on solid epidemiological data by WHONET 5.6 software.

Table 6. Determination of Minimum Inhibitory Concentration (MIC).

Strains	Minimum Inhibitory Concentrations (μ g/mL) Antibiotics									
	AMP	AMX	OXC	VAN	GEN	CIP	NFT	CFT	COTR	TRM
<i>E. fergusonii</i> GCR 1	0.001	256	10	0.05	0.1	256	4	1	0.5	2
<i>Shigella flexneri</i> DMB-1	32	30	0.1	0.01	240	1	0.5	1	32	16
<i>Enterococcus faecalis</i> ADR-1	5	0.05	8	2	0.1	0.05	8	0.5	1	1
<i>Shigella flexneri</i> SMB-11	<0.2	0.01	0.25	32	8	0.001	0.01	4	240	32
<i>Enterobacter cancerogenus</i> MAB-1	0.1	30	64	0.01	<0.2	0.01	32	16	0.5	1
<i>Shigella dysenteriae</i> CNK-77	0.064	0.01	1	2	120	4	0.1	0.001	32	32
<i>Enterobacter cowanii</i> RMB-3	5	1	1	5	2	0.25	30	0.1	240	1

AMP: Ampicillin; AMX: Amoxiclav; OXC: Oxacillin; VAN: Vancomycin; GEN: Gentamycin ;CIP: Ciprofloxacin; NFT: Nitrofurantoin; CFT: Ceftazidime; COTR: Cotrimaxazole; TRM: Trimethoprim

Table 6. Contd...

Strains	Minimum Inhibitory Concentrations (μ g/mL) Antibiotics									
	IMP	AMK	AMOX	CPHL	CEFU	NA	CEFZ	CEFD	CEFEX	CEFT
<i>E. fergusonii</i> GCR 1	>256	128	<0.2	1	30	64	16	8	1	64
<i>Shigella flexneri</i> DMB-1	32	0.1	8	4	0.5	>256	32	0.001	128	1
<i>Enterococcus faecalis</i> ADR-1	1	64	32	>240	14	64	8	1	0.001	8
<i>Shigella flexneri</i> SMB-11	0.5	1	0.1	5	32	1	8	8	32	0.001
<i>Enterobacter cancerogenus</i> MAB-1	0.1	2	128	128	1	0.5	<0.2	64	1	0.1
<i>Shigella dysenteriae</i> CNK-77	4	0.5	32	64	<0.2	2	5	8	<0.001	0.001
<i>Enterobacter cowanii</i> RMB-3	1	2	64	30	128	32	8	64	0.4	64

IMP: Imipenam; AMK: Amikacin; AMOX: Amoxicillin; CPHL: Cephalixin; CEFU: Cefuroxime; NA: Nalidixic acid; CEFZ: Cefazoline; CEFD: Cefdinir; CEFEX: Cefexime; CEFT: Ceftriaxone

Table 6. Contd...

Strains	Minimum Inhibitory Concentrations (μ g/mL) Antibiotics				
	CEFRX	CFTX	AZT	CFCL	PEN
<i>E. fergusonii</i> GCR 1	10	32	1	240	4
<i>Shigella flexneri</i> DMB-1	4	0.01	0.12	5	128
<i>Enterococcus faecalis</i> ADR-1	32	10	1	1	8
<i>Shigella flexneri</i> SMB-11	8	8	4	32	16
<i>Enterobacter cancerogenus</i> MAB-1	1	0.032	0.01	480	240
<i>Shigella dysenteriae</i> CNK-77	5	30	128	16	2
<i>Enterobacter cowanii</i> RMB-3	30	0.01	1	0.5	0.5

CEFRX: Cefuroxime; CFTX: Cefotaxime; AZT: Aztreonam; CFCL: Cefaclor; PEN: Penicillin.

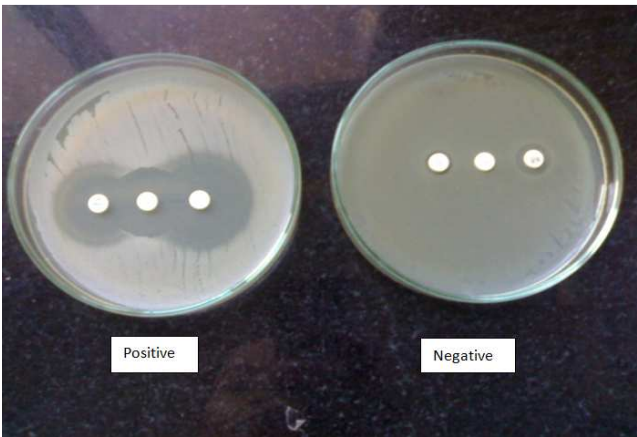


Fig. 2. Extended Spectrum β -lactamase Test.



Fig. 3. Metallo β -lactamase Test.

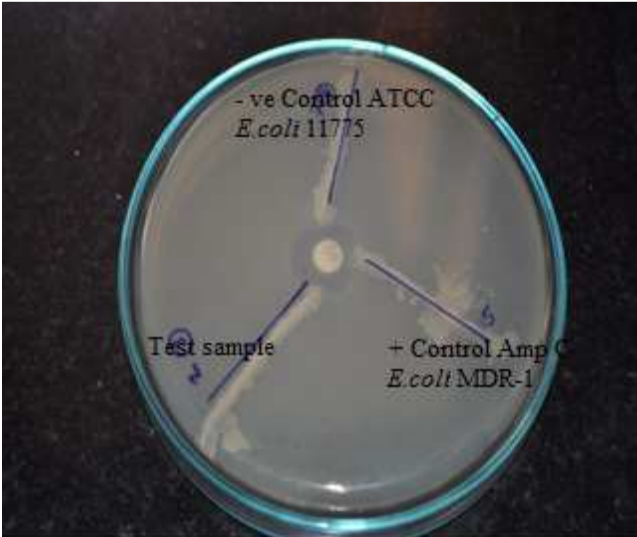


Fig. 4. Amp C β -lactamase Test.



Fig. 5. Iodometric Test.



Fig. 6. Nitrocefin Test.

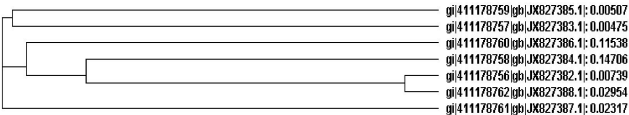


Fig. 7. Phylogenetic analysis of β -lactamase producers (Clustal w2).



a) Production of α -haemolysine



b) Production of β -haemolysine

Fig. 8. Hemolysin Production Test.



Fig. 9. Colicin Production Test.

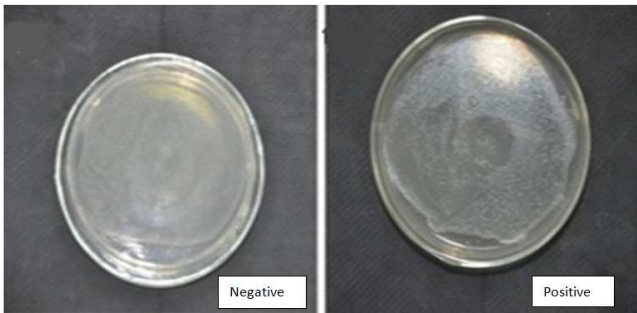


Fig. 10. Determination of Cell surface hydrophobicity.

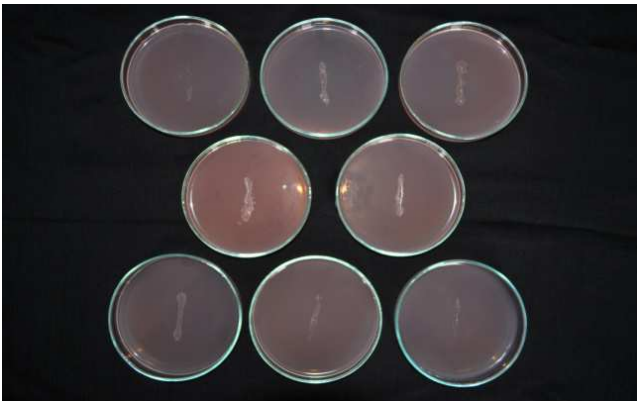
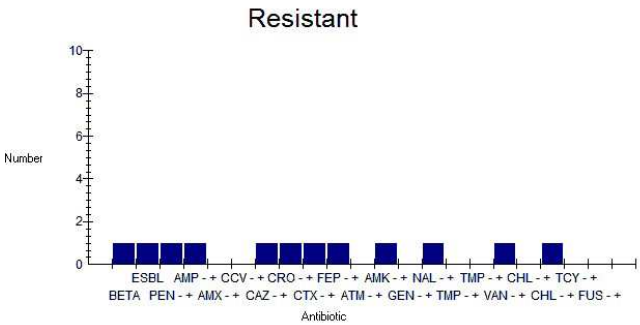


Fig. 11. Aerobactin Production Test.



Fig. 12. Serum Resistance Test.



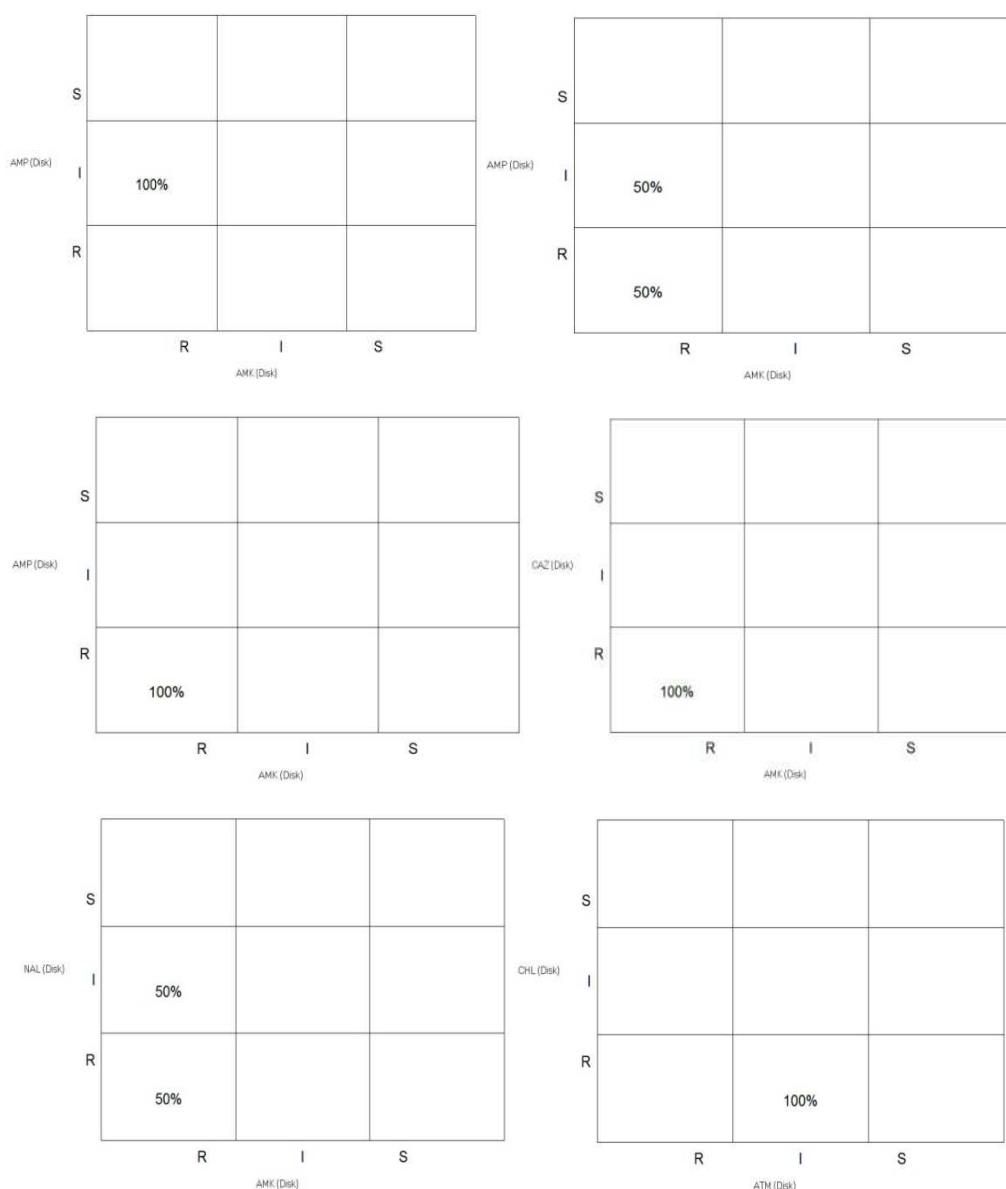


Fig. 13. Histograms.

4. Conclusion

The distribution of pathogens that cause UTI is challenging. The antibiotic resistance is on the increase state globally especially among Gram-negative bacteria. Therefore, there is a need for periodic antibiotic resistance survey to help physicians and the local population on the best treatment strategies. NCCLS should come up with a standard technique that will be easy and fast in detecting ESBL from both *Enterobacteriaceae* and other organism. They should also create awareness to both the clinician and the local population about the existence of ESBL and we suggest that more research should be embarked on search of an effective alternative antibiotic that can be used in treating infections caused by ESBL producing organisms. We can say the potential of this electronic network for studying the patterns

and trends in the epidemiology of antimicrobial resistance in the various participating hospitals will help to contribute to identification of the main factors for the emergence of resistance, as well as the priorities for further investigating the genetic and molecular mechanisms responsible and thus for the development of a strategy to confront this major public health problem.

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