ISOLATION, ESBL SCREENING, MOLECULAR IDENTIFICATION OF BACTERIAL STRAINS OBTAINED FROM URINARY TRACT INFECTION

Haseera. N[1], Baskaran. K[2]*, Shalet Varghese[3], Nirmala Devi. N[4], Arifa P. P[5]. Rathi, MA[6].

Abstract

Background: Urinary tract infections (UTIs) are the most common type of illness in both elaborate and refined the world. Anyway the infections are more familiar in men, women and children; they are at a significant rate in men and of all age groups. A distinctive dare for clinical culture and infection control regular is to deal with extended-spectrum beta-lactamase (ESBL), which now frequently causes CAI, including UTIs, and indicate a challenge for exponent in choosing actual antibiotics. This study aimed to isolate ESBL-yielding bacteria from UTI, patients and analyse their phenotypic attribution.

Materials and Method: For the study total numbers of 123 urine samples from UTI patients were collected from Kerala's different Medical College Hospitals; between year 2021- 2022. The isolates and identification of positive bacteria were retrieved and screened for ESBL production by gram staining, biofilm, and Double Disk Diffusion Synergy Test (DDST). Isolates with ESBL phenotype were further characterized by antibiotic resistance testing, sequencing, and phylogenetic trees of ESBL genes.

Results: Total number of UTI patient urine sample for sixty three positive isolates were screened as E. coli on EMB agar plates and an IMViC biochemical test was conducted. MAR was determined using twelve different antibiotics using microtiter plate assay. Biofilm formation, weak, moderate, strong. The isolates tested, zone of inhibition ceftazidime, cefotaxime, ceftriaxone, were observed for isolates E. coli which showed resistance to third-generation cephalosporins were subjected to phenotypic confirmatory test by modified DDST to detect the ESBL production. Based on the confirmatory test, eight of the isolates were confirmed as ESBL producers. E.coli strain 16S rDNA U65 sample yield amplification products of approximately 1499bp.

Conclusion: In our results highlight the challenge for a culture of a mid-stream urine specimen earlier inaugurate antibacterial.

Keywords: Urinary tract infections, Extended-spectrum beta-lactamase, phenotypic, Double Disk Diffusion Synergy Test, Biofilm

[1,2]*,[3,4,5]Department of Biochemistry, Sree Narayana Guru College, Coimbatore, Tamilnadu, India.
[6]Department of Biochemistry, Karpagam Academy of Higher Education, Coimbatore, Tamilnadu, India.

***Correspondence Author:** Dr. K. Baskaran, MSC., M.Phil., Ph.D.
Assistant Professor Department of Biochemistry, Sree Narayana Guru College, Coimbatore-641105 Tamil Nādu, India. E-mail: baskar.bio86@gmail.com, Cell number: 91+8760302579

I. INTRODUCTION

Urinary tract infections (UTIs) are one of the most disregarded diseases in the globe and are obligated for one-fourth of healthcare-similar infections [1]. They are the most usual infectious disease relate serious straight medications. The diagnosis and treatment policy are quite a challenge behind various infections are subclinical, and the connection of similar symptoms with the infections is unknown. Antibiotic resistance considered bacteria lead to common infections is expanding in all regions of the world [2]. Intriguing, the pattern of resistance mind different from hospital to community, great hospital to small hospital, state to state, and even extend from country to country [3]. The appearances of resistance to antibiotics clarify the gravity of using observed plan for treatment [4]. In UTI patients, antibiotic treatment is frequently started experimental now the results of urine culture and sensitivity testing are available. Suitable antibiotic use in patients with UTI appears to lower the length of hospital stay and consequently privilege patient outcomes and healthcare expense [5]. Hence, it becomes major too often monitor the resistance or susceptibility patterns of Uropathogenic, through the guidelines for observed antibiotic therapy can be enhance to include antibiotics with low resistance, supporting

clinicians in correct management of UTIs with slightest therapeutic failures [6], [7].

The main explanation for the production of ESBLs in bacteria is the growth of the multi-drug resistant (MDR) effect. Among the microbes, bacteria are the most preponderant microbes to bring flexible behavioural character among a diversity of mechanisms. Let gramnegative bacteria and their feature nature is more acceptable to develop resistant effects among many antibiotic families mid genetic mechanisms. The antibiotics alone or in mixture with any other antibiotics are build co-resistant enzymes and it locates out very hard such as reduce membrane permeability, diffusion, reflection of excess of flow pump, and continual moderation of penicillin-binding proteins. Regularly, MDR bacteria are a worldwide risk and are familiar to increase high disease and death in health care. The generality of infection by MDR bacteria bring severe infection and extend stay in the human body [8], [9]. Exploration of bacteria causing infections at prematurely stages has been the major direction in health care and medical portion [10], [11]. A delay in finding at the beginnings stages would result in harmful effects [12]. Hence, different methodologies have been greeted for the detection of Uropathogenic from many biological samples [13]. The finding of new antibiotics is being correct by arising antibiotic resistance mechanisms by the bacteria [14]. Unfortunately, the treatment and precluding of several UTIs are becoming frantic day by day. The aim of the present work is (i) to isolate and identify bacteria from UTI-infected patients urine samples (ii) to evaluate the presence of ESBL gene, (iii) grams staining, virulence factors (iv) molecular identification (16s rDNA) and phylogenic trees and antibiotic resistance patterns of isolated bacteria.

II. MATERIALS AND METHODS

A. Collection of Urinary Samples

UTIs patients urine samples 123 collected from several hospitals in Kerala from year 2021-2022 were included in this study. Urine samples were collected in a wide-mouthed sterile screw-capped container. The early morning midstream clean catch urine was collected for all the UTIs patients. The samples were analysed in the lab within 2 hours. Urine samples were taken from collected containers using a sterile calibrated bacteriological loop. A loopful of well-mixed UTIs urine samples were inoculated onto Eosin Methylene Blue (EMB) broth. The tubes were then incubated in the incubator at 37C for 18-24 hours. After the incubation period, the tubes were examined for turbidity. The isolates were characterized for the identification of E. coli following standard microbiological techniques.

B. Isolation, Identification of bacterial

The quadrant streaking method helps to form discrete CFU. The samples after incubation in EMB broth were then streaked and cultured on EMB agar plates using a sterile loop through intermittent heating. EMB plates were incubated overnight at 37ºC. Metallic green sheen colonies in EMB plates were selected as presumptive E. coli strains [15]. The nutrient agar medium was prepared and then poured aseptically into Petri dishes in a biological safety cabinet. The primary cultures which were observed with the growth of E. coli were then purified using a single colony isolation technique on nutrient agar (NA). The NA plates were then kept for 24 hours of incubation in an inverted position [16].

C. Biochemical investigation

The presumptive E. coli isolates were further subjected to the IMViC test, Indole test (to determine the ability of an organism to produce the enzyme tryptophanase, which can be hydrolysed the amino acid tryptophan to form indole and pyruvic acid), Methyl-red test (to determine enteric based on their ability to produce an acidic end product of glucose metabolism), Voges-Proskauer test (to test for evidence of an enteric bacterium that produced non-acidic end product during the metabolism of glucose). In 1898, German bacteriologists Daniel Wilhelm Otto Voges and Bernhard Proskauer developed this reaction at Institute for Infectious Disease, and Citrate utilization test (to test the ability of an organism to use citrate as a sole carbon and energy source). Colonies were confirmed as E. coli using these biochemical tests [17], [18].

D. Morphological Identification Gram's staining

Gram staining is a common technique that differentiates Gram-positive and Gramnegative bacteria based on the stain the bacterial cells take up and the morphology of the bacterial cells. A drop of normal saline was taken on the centre of a clear glass slide and a colony was taken by a sterilized inoculating loop to make a thin emulsion. A very thin layer was prepared by spreading the emulsion uniformly. This film was fixed by passing it over the flame two or three times. Smear was covered by a crystal violet stain for 30 -60 seconds. Then the stain was washed with distilled

water thoroughly and covered with Gram's iodine for 30-60- seconds. Again, the stain was washed with distilled water and decolorized with acetone alcohol, and washed with distilled water. The smear was stained with safranin for one minute and washed with water. The back of the slide was wiped and placed in a draining rack, for the smear to air dry. Test specimens were examined and compared with positive and negative controls under a microscope [19].

E. Antibiotic Resistance by Resazurin Microtiter Plate Method

The screening for antibiotic resistance was carried out against Fifteen antibiotics
(Amikacin, Imipenem, Meropenem, Gentamicin, Ciprofloxacin, Cefoxitin, Sulfamethoxazole, Ceftriaxone, Fosfomycin, Piperacillin, Nitro furantoin, Ceftazidime, Cefuroxime, Cefixime, and Ampicillin) by micro broth dilution method and interpreted as per CLSI (Clinical and Laboratory Standards Institute) breakpoint. A 96-well microtiter plate was used. An exponential culture of a test strain in Muller-Hinton (MH) broth (HiMedia) was suitably diluted with a normal saline solution (0.9% NaCl) to obtain an equivalent to the 0.5 McFarland standard solutions. To an aliquot of 20 µL overnight grown test culture, an aliquot of 100 µL of the antibiotic stock solution and an aliquot of 100 µL of MH broth was added to the fourth to the ninth well of the microtiter plate so that the final concentration of antibiotics in the well were 1, 2, 3, 4, 5 and 6 µg/ml. Finally, an aliquot of 20 µL 0.5% resazurin was added to all wells and the microtiter plate was incubated at 37°C for 2 hours. Resazurin was used as an indicator of bacterial growth. Wells were then examined for the development of pink color, which indicates the growth of the bacteria and the absence of the pink color represents the inhibition of bacterial growth [20], [21], [22].

F. Virulence Factors

Haemolysin Production E. coli isolates were screened for hemolysis on blood agar. The blood agar plates were prepared according to the manufacturer's instructions. The isolates were inoculated into a chemically defined medium and incubated for 14 h at 37^{o}C. The bacteria from broth cultures were streaked on the plates and incubated at 37^{o}C for 24 h, and bacteria producing a clear zone of hemolysis were recorded as hemolysis-positive [23].

G. Detection of biofilm formation

The E. coli confirmed cultures were inoculated in the NA and kept for overnight incubation at 37^{o}C. About 100µl of the dilutions per well was added in a 96-well dish. The microtiter plate was then incubated for 4-24 hrs at 37^{o}C. After incubation, the cells were dumped out by turning the plate over and shaking off the liquid. The plate was then gently submerged in a small tub of water. After shaking out the plates, the process was repeated for the second time. This step helps to remove the unattached cells and media components which can significantly lower the background staining. 125µl of 0.1% solution of crystal violet (CV) was mixed in water and then added to each well of the microtiter plate. The plates were then incubated at room temperature for 10 – 15 minutes. It was then submerged in a tub of water, shaken out, and blotted vigorously on a stack of paper towels to remove all excess cells and dye in the plates. Turn the microtiter plate upside down and dry for a few hours or overnight. For qualitative assays, the wells can be photographed when dry. For the quantification of the biofilm, 125µl of 30% acetic acid was added to each well of the microtiter plate to solubilize the CV. The microtiter plate was incubated at room temperature for 10 – 15 minutes. The 125µl of solubilized CV was then transferred to a new flat-bottomed microtiter dish. The quantitative absorbance in a plate reader was read, with 30% acetic acid in water as the blank [24], [25], [26].

H. Phenotypic MH agar plates method

MH agar plates were prepared from a commercially available dehydrated base following the manufacturer's instruction. Immediately after autoclaving, the media was cooled in a water bath from 45°C to 50°C. The cooled medium was poured into petri dishes to a depth of approximately 4 mm which corresponds to 25 to 30 ml of media for plates. The agar medium was allowed to cool to room temperature and then stored in a refrigerator (2°C to 8°C). pH of the medium was adjusted to 7.2-7.4.

H (a). Phenotypic Initial Screening of ESBL

Antibiotic filter paper discs with 10µg/ml of ceftazidime, cefotaxime, and ceftriaxone were used for the initial screening of ESBL production. An Inoculum of 0.5 McFarland standards was prepared from colonies on agar plates. MH agar plates were inoculated by the lawn culture method using a germ-free cotton swab. In the case of gram-negative bacteria, excess liquid was removed from the swab by gently pressing or rotating it against the inner wall of the test tube. The swab is then streaked across the MH agar plate to form a bacterial lawn. Using flame-sterilized forceps; the

filter papers are then placed on the plates. About 1µl of each antibiotic was suspended into filter paper discs. The plates were then incubated overnight at 35°C for 18– 24 hours (CLSI 2012). Using the published CLSI guidelines, the susceptibility or resistance of the organism to each drug tested was determined. For each drug, on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) is indicated based on the interpretation chart. All the strains which showed a diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone were selected for checking the ESBL production [27].

H (b). Phenotypic Confirmatory Test for ESBLs and MDDST

The ESBL production was tested MDDST by using Piperacillin/ Tazobactam along with cefotaxime. A lawn culture of the organisms was made on a MH agar plate, as was recommended by CLSI. Using flame-sterilized forceps, the filter paper discs are then placed on the plates. About 1µl of Piperacillin/ Tazobactam and Cefotaxime antibiotic was suspended to filter paper disc. The filter paper discs for Cefotaxime were placed 15mm apart from that of the Piperacillin/ Tazobactam. Any distortion or increase in the zone towards the disc of Piperacillin/ Tazobactam was considered positive for ESBL production [28].

I. Genomic DNA, Phenol Chloroform method

The E. coli were cultured overnight (new) and were centrifuged at 13,000 rpm for 10 minutes, and the supernatant was disposed of deliberately. The pellet was resuspended in 1 ml of 0.85% NaCl solution and centrifuged at 10,000 rpm for 20 minutes. About 600 µl of cell lysis buffer was included and vortexed, at that point, 7µl of proteinase K (50 µg/ml) was included and marginally vortexed. The tubes were stored at 65°C for an hour in the water bath (microtubes were fixed with parafilm). About equal volume of PCI solution (saturated Phenol: Chloroform: Isoamyl alcohol; 25:24:1) was included and after that blended by rolling the tubes between the palms. The tubes were centrifuged at 13,000 rpm for 15 minutes and the upper layer was taken in new microtubes. The upper layer was taken in a new microtube. To this collected aqueous phase 1/10th volume of 3M sodium acetate was added. An equal volume of ice-cold isopropanol was added and tubes were inverted 10 times so that DNA gets precipitated. The tubes were centrifuged at room temperature for 10 minutes at 10,000 rpm. The supernatant was discarded and the pellet was washed twice with 70% isopropanol. The desiccated DNA was collected and 100 µl of hydration buffer (1x MilliQ TE) was added and permitted to rehydrate at room temperature for 10 minutes and afterward put away in a 4°C cooler [29], [30].

J. Sequencing of 16S rRNA

16S rDNA is a well-established universal primer for all bacterial species which is the conserved region in the whole bacterial genome and was used as an endogenous control or internal control to validate the DNA for bacterial genomic study. The primer selection was done from a literature review and cross-verified using NCBI-Primer-BLAST search (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The selected positive E. coli strains were confirmed by 16S ribosomal DNA (rDNA) analysis. PCR amplification was performed using the 16S rRNA. Specific primers for E. coli were designed including the variable region of the 16S rRNA for the identification of various strains of E. coli and to discriminate among other bacterial species. It is a reporter gene for the detection of E. coli that was amplified [31], [32].

K. Phylogenetic trees analysis

The sequence obtained was subjected to BLAST analysis using NCBI-BLAST software. This is carried out for sequence similarity search. Representative sequences of similar neighbours in BLAST analysis were retrieved and aligned using a multiple alignment program. The multiple alignment file was used to create the tree using MEGA-X [33].

III. RESULTS

A. Primary Screening on EMB Agar

Isolation of bacterial colonies from the collected for UTIs patient urine samples was performed on an EMB medium, and bacterial colonies with understood growth characteristics were selected. Bacterial isolates producing appearance sheen due to the meta-chromatic effects of the dye was selected as presumed [U25, U27, U28, U37, U40, U61] E. coli for further study. No colonies were observed for control samples. The E. coli isolates were further pure cultured on NA. Gram-negative bacilli, motile which ferment sugars with the production of acid were presumptuously considered as E. coli [Fig 1, 2]. These bacterial strains showing visible growth were further used for analysis.

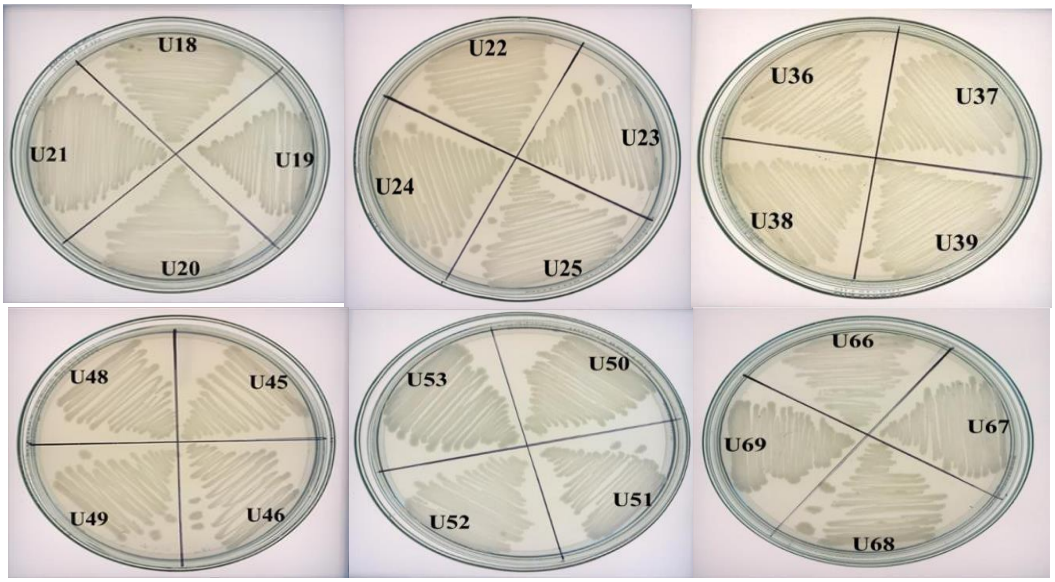


Fig. 1. Primary Screening on EMB Agar.

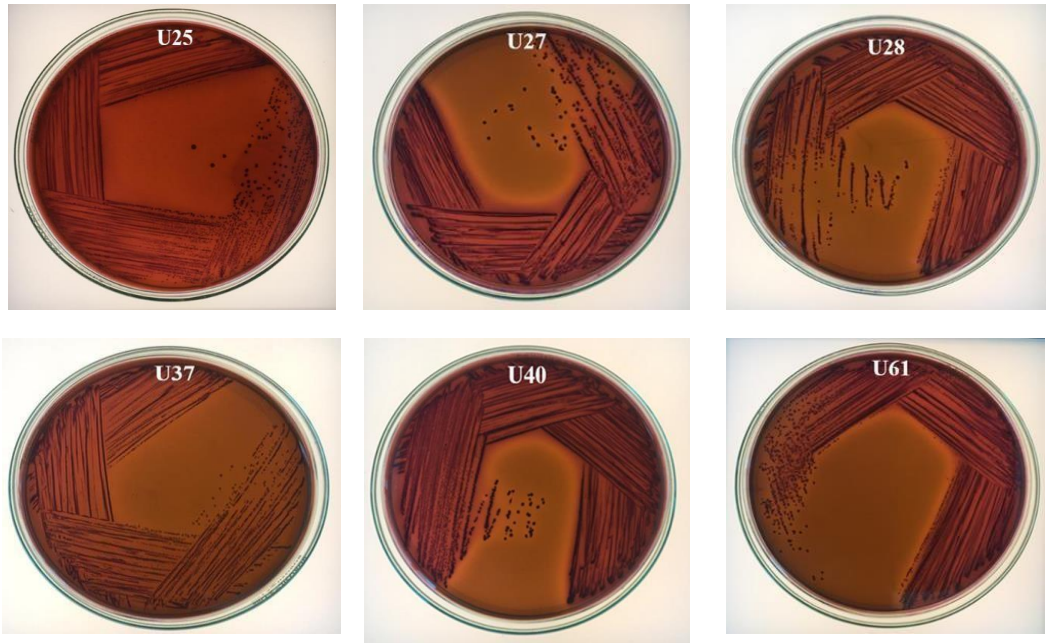


Fig.2. Pure cultures of *E. coli* on Nutrient Agar.

B. Pure cultures of *E. coli* on Nutrient Agar and biochemical investigation

Various biochemical assays were performed for the selective isolation of E. coli. The isolates positive for five different test were considered as E. coli [U1, U5, U15, U25, U58, U65]. Out the total 123 isolates, 63 positive isolates were screened as E. coli on EMB agar plates, biochemical test was conducted [Fig 3, Table I].

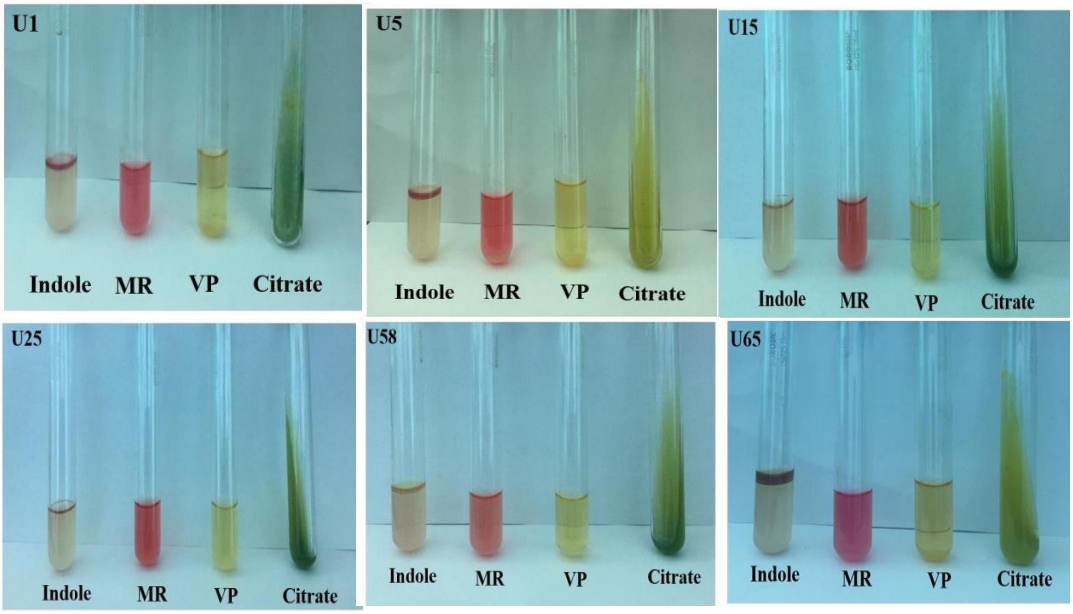


Fig. 3. Biochemical Assay of *E. coli* isolates.

TABLE I: Biochemical test for *E. coli* isolates

SL. no	Isolates *E.coli*	Biochemical tests			
		Indole	Methyl Red	Voges Proskauer	Citrate
1	U1,U3,U5,U6,U8,U10-U16,U18-U29,U32-U42,U44-U72,U88	+ve	+ve	-ve	-ve
2	U2,U7,U30,U31,U47,U73,U79,U93,U96,U98,U100,U101,U123	-ve	-ve	+ve	+ve
3	U4,U17	-ve	+ve	+ve	+ve
4	U7,U61,U77,U81,U85,U86,U89,U97,U112	+ve	-ve	-ve	-ve
5	U9,U43,U78,U99	-ve	-ve	-ve	+ve
6	U74,U82,U109,U116	+ve	+ve	+ve	-ve
7	U75	+ve	+ve	-ve	+ve
8	U80,U87,U90,U102-U108,U113	-ve	+ve	-ve	-ve
9	U83,U91	+ve	+ve	+ve	+ve
10	U84,U110,U111,U114,U115,U117-U122	-ve	-ve	-ve	-ve
11	U92	-ve	-ve	+ve	-ve

C. Isolation of bacterial strains

Gram-positive bacteria retain the primary stain (crystal violet), violet/purple, under a microscope while Gram-negative bacteria lose the primary stain and take the secondary stain which imparts red or pink color to the bacteria. The gram staining study confirmed that the isolates were gram-negative [Fig. 4].

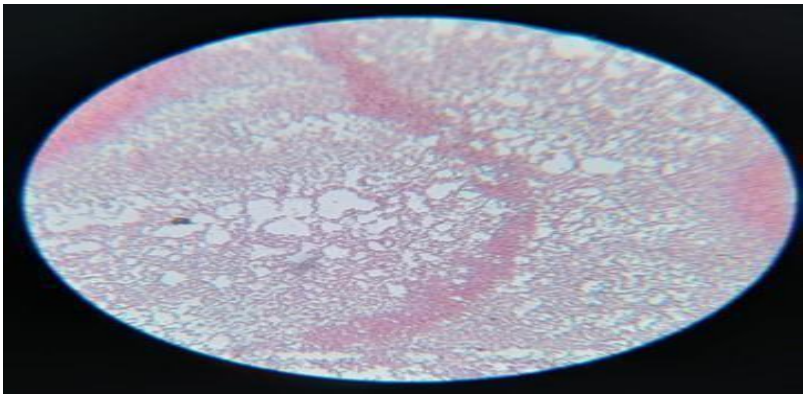
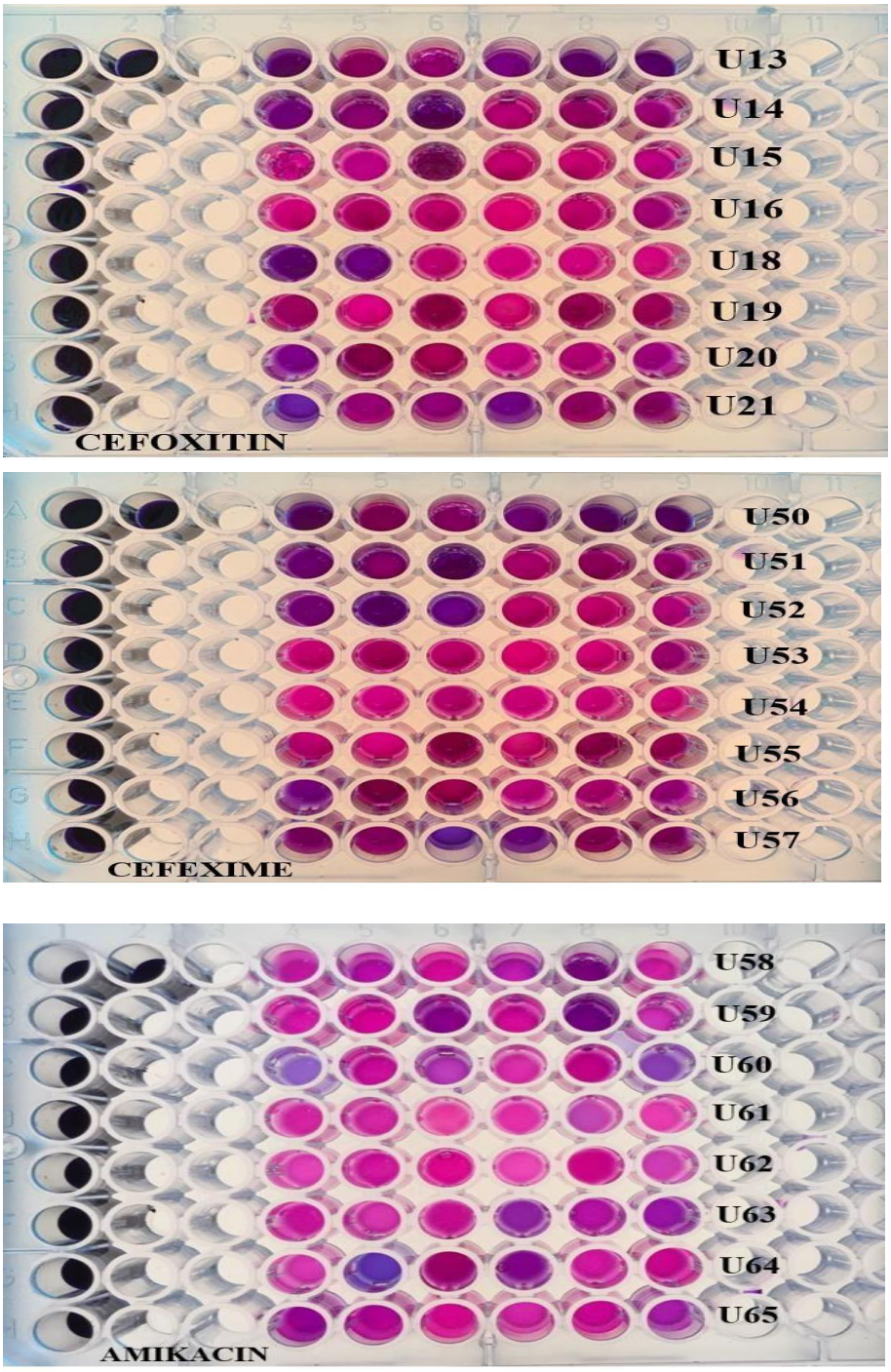


Fig. 4. Gram Negative Pink Rod-Shaped Bacilli of *E. coli.*

D. Multiple Antibiotic Resistance of Resazurin Assay

MAR was carried out to assess the resistance patterns offered by the Uropathogenic

E. coli (UPEC) strains to the commonly prescribed antibiotics. MAR was determined using 15 different antibiotics. Most of the isolates showed bacterial UTIs urine samples purple to pink color at different concentrations and were observed as antibiotic resistant. The concentration at which no color changes after the addition of resazurin was considered to be susceptible. All positive results were observed between 15 min and less than 1 hour after the addition of resazurin. Consequently, interpretation of the results was obtained in a maximum of 4 hours for the E. coli isolates [Fig 5].



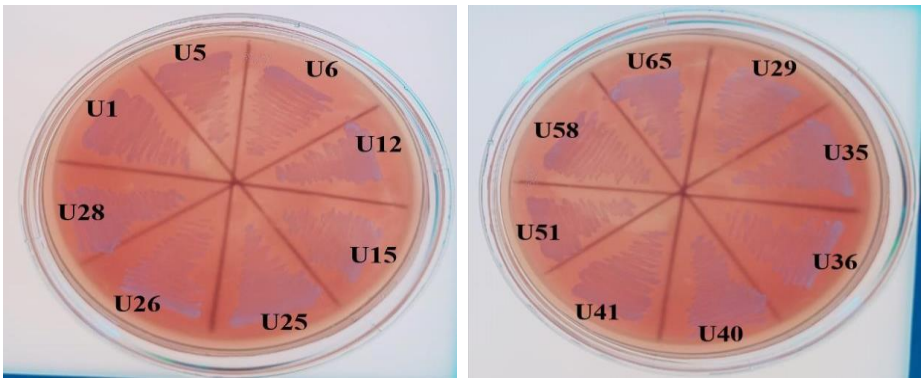
A4-A9, B4-B9, C4-C9, D4-D9, E4-E9, F4-F9, G4-G9, H4-H9: (Nutrient broth, Sample, Antibiotics)

Fig. 5. Resazurin Assay for the determination of Multiple Antibiotic Resistance.

A1- H1: Control (Nutrient broth, Resazurin) A2: Control (Nutrient broth, Antibiotic) B2: Control (Nutrient broth, NaCl)

E. Haemolysis on Blood Agar, biofilm formation by *E. coli* isolates

The selected E.coli strains were examined for Haemolysis on Blood Agar



[U1,U5,U6,U12,U15,U25,U26,U28,U29,U35,U36,U40,U41,U51,U58,U65]. E. coli isolates producing a clear zone of hemolysis on blood agar were recorded as positive (Fig 6). All sixteen isolates were selected for testing the biofilm. Out of which nine isolates exhibited strong biofilm formation, three isolates were weak biofilm producers and four isolates showed moderate biofilm production [Fig 7, 8 and Table II].

Fig. 6. Haemolysis on Blood Agar.

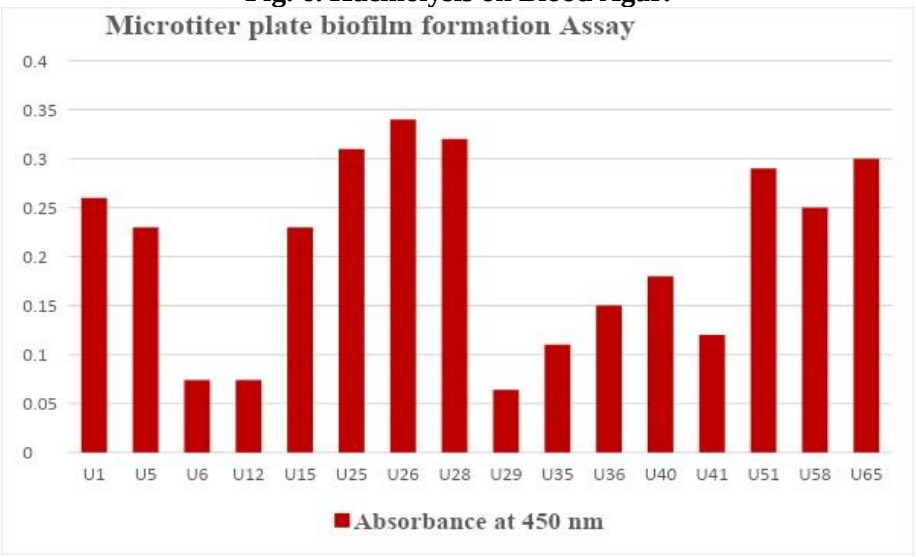


Fig. 7. Graph representing biofilm formation by *E. coli* isolates.

Ac = 0.052, 2Ac = 0.104, 4Ac = 0.208, values < 0.052 do not indicate biofilm formation, values > 0.052 indicates weak biofilm formation, values between 0.1-0.2 indicates moderate biofilm formation, values > 0.2 indicates strong biofilm.

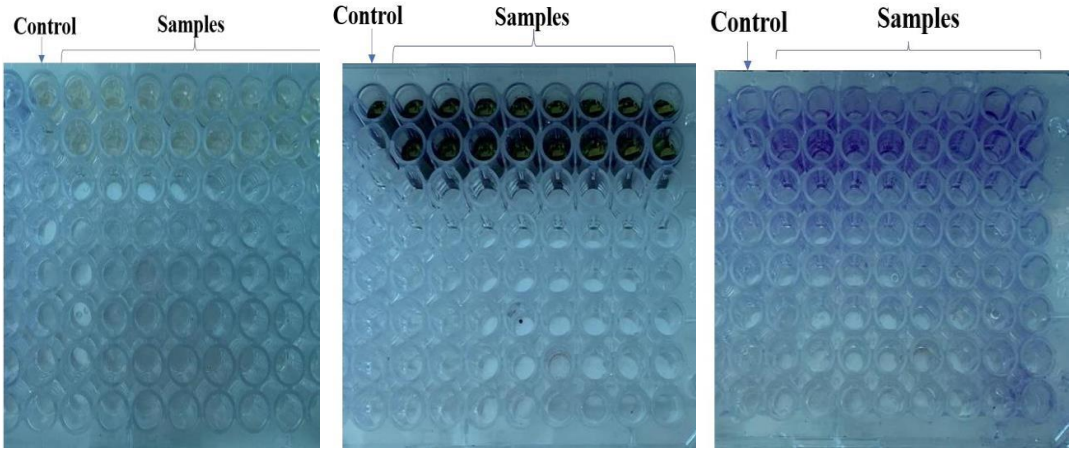


Fig. 8. Biofilm Formation Test.

TABLE II. Rate of Biofilm formation by *E. coli* isolates

S.No	SAMPLES	BIOFILM PRODUCTION
1	U1,U5,U15,U25,U26,U28,U51,U58,U65	Strong
2	U6,U12,U29	Weak
3	U35,U36,U40,U41	Moderate

F. Initial screening of ESBL and Modified Double Disc Synergy Test

The E. coli isolates showing resistance to any two of the cephalosporin antibiotics tested were considered positive [U58] for ESBL production in an initial screening. The isolates obtained were subjected to screening for ESBL production using initial screening [Fig 9]. Of these isolates tested, zone of inhibition ceftazidime, cefotaxime, and ceftriaxone, were observed for isolates respectively. Initially screened E. coli isolates that showed resistance to third-generation cephalosporins were subjected to a phenotypic confirmatory test by modified double disc synergy test [U58] to detect the ESBL production. Based on the confirmatory test, 8 of the isolates were confirmed as ESBL producers. ESBL production was found to be negative in the rest of the isolates [Fig 10].

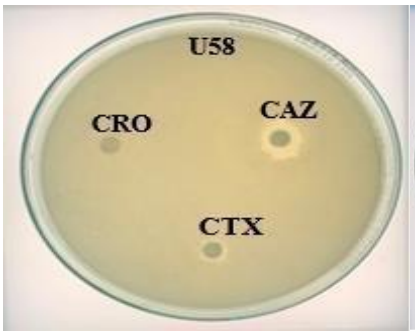


Fig. 9. Initial screening of ESBL.

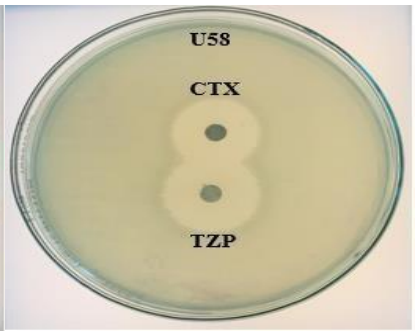


Fig. 10. Modified Double Disc Synergy Test.

G. DNA Isolation and Amplified DNA

The genomic DNA of selected samples was isolated using the Phenol chloroform isoamyl method (PCI). The quality of the DNA isolated was checked using agarose gel electrophoresis (U1, U5, U15, U25 and U26, U51, U58, U65). A positive result was obtained in all of the isolates 1500 bps [Fig 11,12].

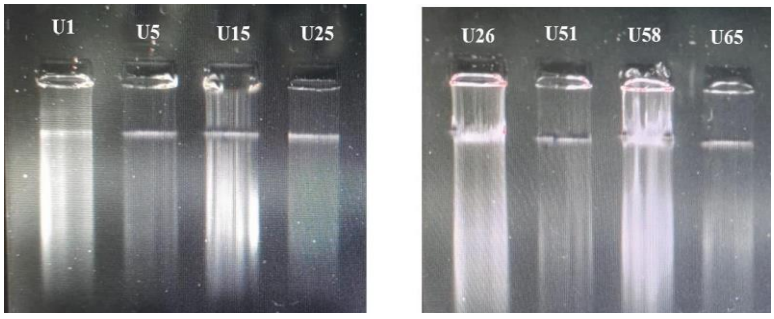


Fig. 11. Gel image for DNA Isolation.

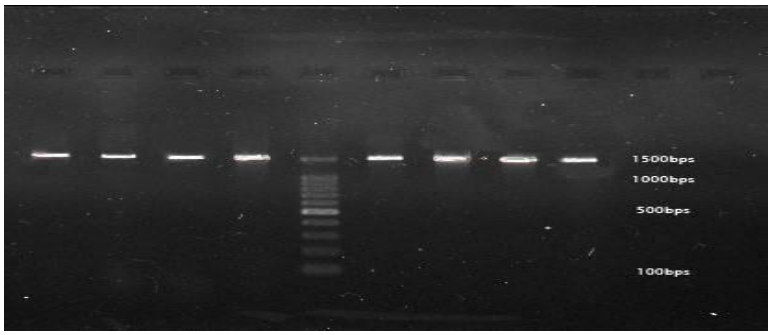
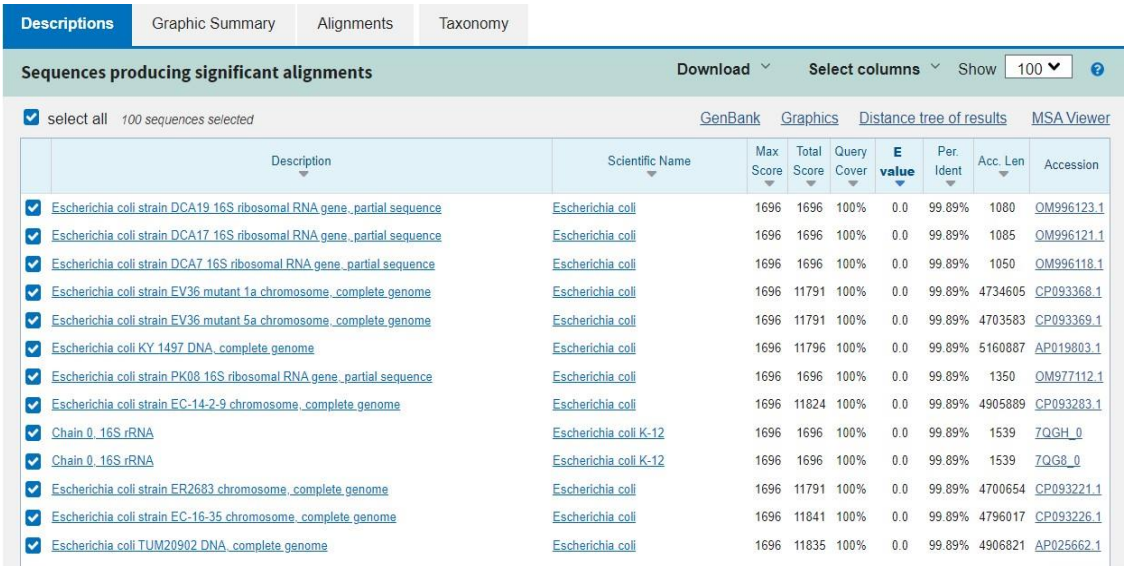


Fig. 12. Gel Image of Amplified DNA.
Lane 1: U1, Lane 2: U5, Lane 3: U15, Lane 4: U25, Lane 5: 100 bp DNA Ladder

Lane 6: U26, Lane 7: U51, Lane 8: U58, Lane 9: U65

H. BLAST result of 16S Ribosomal DNA Sequence of U65 Isolate and Phylogenetic grouping After PCR reaction completion, analyse a part of the reaction mixture by agarose gel electrophoresis. The sample DNA solution yields amplification products of approximately 1499bp [Fig 13, 14].



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Escherichia coli strain DCA19 16S ribosomal RNA gene, partial sequence	Escherichia coli	1696	1696	100%	0.0	99.89%	1080	OM996123.1
Escherichia coli strain DCA17 16S ribosomal RNA gene, partial sequence	Escherichia coli	1696	1696	100%	0.0	99.89%	1085	OM996121.1
Escherichia coli strain DCA7 16S ribosomal RNA gene, partial sequence	Escherichia coli	1696	1696	100%	0.0	99.89%	1050	OM996118.1
Escherichia coli strain EV36 mutant 1a chromosome, complete genome	Escherichia coli	1696	11791	100%	0.0	99.89%	4734605	CP093368.1
Escherichia coli strain EV36 mutant 5a chromosome, complete genome	Escherichia coli	1696	11791	100%	0.0	99.89%	4703583	CP093369.1
Escherichia coli KY 1497 DNA, complete genome	Escherichia coli	1696	11796	100%	0.0	99.89%	5160887	AP019803.1
Escherichia coli strain PK08 16S ribosomal RNA gene, partial sequence	Escherichia coli	1696	1696	100%	0.0	99.89%	1350	OM977112.1
Escherichia coli strain EC-14-2-9 chromosome, complete genome	Escherichia coli	1696	11824	100%	0.0	99.89%	4905889	CP093283.1
Chain 0, 16S rRNA	Escherichia coli K-12	1696	1696	100%	0.0	99.89%	1539	7QGH_0
Chain 0, 16S rRNA	Escherichia coli K-12	1696	1696	100%	0.0	99.89%	1539	7QG8_0
Escherichia coli strain ER2683 chromosome, complete genome	Escherichia coli	1696	11791	100%	0.0	99.89%	4700654	CP093221.1
Escherichia coli strain EC-16-35 chromosome, complete genome	Escherichia coli	1696	11841	100%	0.0	99.89%	4796017	CP093226.1
Escherichia coli TUM20902 DNA, complete genome	Escherichia coli	1696	11835	100%	0.0	99.89%	4906821	AP025662.1

Fig. 13. BLAST result of 16S Ribosomal DNA Sequence of U65 Isolate.

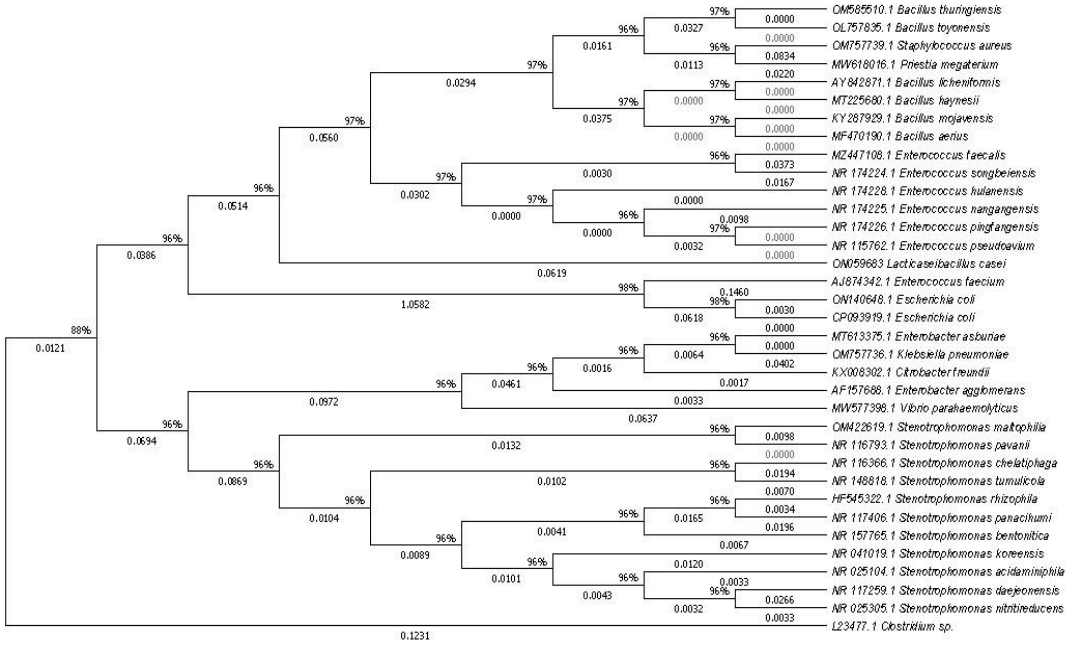


Fig. 14. Phylogenetic grouping of 16S Ribosomal DNA.

IV. DISCUSSION

In case of study, a sum total of 123 urine samples from UTI patients were collected from varied hospitals, located from Kerala, India of which the most of patients were woman and adult age groups. This monitoring was similar to other information in India respectively [34], [35]. The present exploration value the antibacterial heterogeneity profiles of bacteria from UTI-positive urine samples collected from the several hospitals in Kerala, India, predicated on culture and self-reliant techniques in microbiology, and Gram-positive & negative bacteria maintain the primary stain (crystal violet) bacilli, results for the particular species have been reported similarly earlier [36], [37], [38] especially E. coli and take the secondary stain which imparts red or pink color to the bacteria someone ESBL enzyme are deliberate as serious organism causing disease to it hospital in UTIs patients [39]. The raised MDR

patterns of identified bacteria against different groups of antibiotics. Resistance to the BL group of antibiotics was related to be associated with the capacity to produce ESBL, suitable of degrading the BL ring. The present study is an undertake on the isolation and characterization of ESBL-producing Gram-positive bacteria, exceptionally E coli from urine samples of UTI patients in a specialized area of different hospitals in Kerala.

The detection assay was performed to check for the presence of ESBL and one strain, was found positive for ESBL production. The ESBL-producing capability of might have contributed to its antibiotic resistance pattern to some extent [40], [41]. However, resistance to antibiotics was found in the entire 12 isolates microtiter plate assay. The vast majority of E. coli strains isolated in our study are sensitive to imipenem, therefore, Most strains of ESBL-producing E coli were resistant to gentamicin, and ciprofloxacin. The mechanisms of this resistance are not yet clearly started, but some authors suggest the co-transmission of ESBL and other antibiotic-resistance genes by the same conjugative plasmid. This may suggest that the producing of ESBLs by *Ecoli* the family limit the treatment option available for the management of the infections caused by this group of anti-bacteria. Thus the members of the *Ecoli* family that were proven to produce ESBL pose a serious problem in the clinical management of UTI which activate to put powerful effort in the avoidance of ESBL eventuality.

Biofilm-yielding bacteria are of chief medical consequence as they antibiotic resistantance to antimicrobial agents and hence, complicated to treat the various infections [36]. Biofilms facilitate the assign of plasmid from only one bacterium in its vicinity, let the spread of antibiotic resistance [42], [43]. However, the biofilm-creating capability of the 16 strains was tested by the resazurin microtiter plate method. Out of the nine isolates were found suitable of bearing strong biofilms. The capacity of biofilm development week of the three isolates might have convinced the antimicrobial sensitivity patterns of the four isolates exhibited moderate biofilm production [44], [45], [46]. Therefore, Ecoli (sp) was found positive for both ESBL-yielding and biofilm-producing assays, but still resistant to most antibiotics compared to the other 16 isolates. Ac = 0.052, 2Ac = 0.104, 4Ac = 0.208, values < 0.052 do not indicate values > 0.052 indicate weak (U6, U12, U29) between 0.1-0.2 indicates moderate (U35, U36, U40, U41) values > 0.2 indicates strong (U1, U5, U15, U25, U26, U51, U58, U65) biofilm formation for Ecoli sp.

This study expose other the bacteria in UTIs had produce a various mechanism of resistance to many antibiotics. The results sure state that the bacteria in urine samples acquired diverse ways of escaping the effects of antibiotics and emerging multi-drug resistance mechanisms. UTI are obligated for suggestive morbidity, and incline factors thus infections. Proper knowledge of stem-isolated bacteria by a recovery of infections in a hospital unit, further on the species identification and antibiotic sensitivity testing routine, has set off a necessity to find the mode of communication of the bacteria to the host. The phenotypic generally has restriction. If many phenotypic characteristic permit for analogous bacterial species, the reflection of phenotypic traits may vary in live conditions for the bacteria. The bacterium that appears continually feels its environment, actuates or inhibits some of its genes to be consistent site. This elevates a problem the reproducibility of the results can be uncomfortable for a string of comparisons strains if one is based on phenotypic traits. The E. coli isolates displaying resistance to any two of the cephalosporin antibiotics tested were deliberate positive for ESBL production in the earliest screening. The isolates earned were exposing to screening for ESBL production using initial screening. Of these isolates tested, zone of inhibition ceftazidime, cefotaxime, and ceftriaxone, were observed for isolates respectively. Initially screened E. coli isolates that showed resistance to thirdgeneration cephalosporins were subjected to a phenotypic confirmatory test by modified double disc synergy test to detect the ESBL production. Based on the confirmatory test, 8 of the isolates were confirmed as ESBL producers. ESBL production was found to be negative in the rest of the isolates.

V. CONCLUSION

The present study aimed to decide the antibiotic resistance patterns between the isolated *E.coli* bacterial strains from infected UTI urine samples. It was found positive for both ESBL production and biofilm formation. The possible to produce ESBL enzymes and biofilms may be related with antibiotic resistance mechanisms but is not the only way of remove the effect of antibiotics. The physicians must be mindful of the antibiotic susceptibility profiles of the bacteria before suggestion an individual antibiotic treatment. The exercise of antibiotic resistance profiles in the treatment of UTI and the selection of suitable

antibiotics may be of large future possible. The surfacing antibiotic resistance mechanisms in the bacteria from urine samples indicate that UTIs may suit more dangerous soon. These subjects can stimulate the gravity of more immersion to therapeutic tyranny, especially for high-risk patients. Furthermore, the limitation in cluth broad-spectrum and third-generation cephalosporins appear to be critical for infection control programs.

ACKNOWLEDGEMENT

We thank **Dr. D. Kaplana.** Principal, Sree Narayana Guru College, for the facilities provided is gratefully acknowledged.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

REFERENCE

1. Mihankhah N, Khoshbakht R, Raeisi M, V. Raeisi V. Prevalence and antibiotic resistance pattern of bacteria isolated from urinary tract infections in Northern. Iran J Res Med Sci. 2017; 22: 1–5.
2. Delcaru C, Alexandru I, Podgoreanu P, et al., Microbial biofifilms in urinary tract infections and prostatitis: Etiology, pathogenicity, and combating strategies. Pathogens. 2016; 5:1–12.
3. Fair RJ, TorY. Antibiotics and bacterial resistance in the 21st century, Perspect Medicin Chem. 2014; 6: 25–64.
4. Farajnia S, Alikhani MY, Ghotaslou R, Naghili B, Nakhlband A. Causative agents and antimicrobial susceptibilities of urinary tract infections in the Northwest of Iran. Int J Infect Dis. 2009; 13:140–4.
5. Nickel JC. Management of urinary tract infections: Historical perspective and current strategies: Part 2 – modern management. J Urol. 2005; 173: 27–32.
6. Spoorenberg V, Hulscher ME, Akkermans RP, Prins JM, Geerlings SE. Appropriate antibiotic use for patients with urinary tract infections reduces length of hospital stay. Clin Infect Dis. 2014; 58: 164–9.
7. Sharma N, Gupta A, Walia G, Bakhshi R. Pattern of antimicrobial resistance of *Escherichia coli* isolates from urinary tract infection patients: A threeyear retrospective study. J Appl Pharm Sci. 2016; 6: 62–5.
8. Naber KG, Schito G, Botto H, Palou J, Mazzei T. Surveillance study in Europe and Brazil on clinical aspects and antimicrobial resistance epidemiology in females with cystitis (ARESC): Implications for empiric therapy. Eur Urol. 2008; 54: 1164–75.
9. Kim YM, Yang EM, Kim CJ. Urinary tract infection caused by community acquired extended-spectrum -lactamase-producing bacteria in infants. J Pediatr. 2017; 93: 260–6. [10] Das AP, Kumar PS, Swain S. Recent advances in biosensor based endotoxin detection. Biosens Bioelectron. 2014; 51: 62-75.
10. Bal B, Armstrong PB, Das AP. Development of indigenous bio-sensing methodology for rapid and low cost endotoxin detection system. Sens Netw Data Commun. 2016; S1 005.
11. Zee AVD, Roorda L, Bosman G, Ossewaarde JM. Molecular diagnosis of urinary tract infections by semi-quantitative detection of uropathogens in a routine clinical hospital setting. PLoS One. 2016; 11:0150755.
12. Das AP, Bal B, Mahapatra PS, Chromogenic Biosensors for Pathogen Detection, Biological and Pharmaceutical Applications of Nanomaterials, CRC press, Taylor & Francis. 2015.
13. Edlin RS, Copp HL. Antibiotic resistance in pediatric urology. Ther Adv Urol. 2014; 6:54-61.
14. Pobiega M, MaciagJ, Wesolowska MP, Chmielarczyk A, Romaniszyn D, Ziolkowski G, Heczko PB, Mach JW, Bulanda M. Urinary tract infections caused by *Pseudomonasaeruginosa* among children in Southern Poland: virulence factors and antibiotic resistance. J Pediatr Urol. 2016; 12: 361- 366.
15. Beyene G, Segaye WT. Bacterial uropathogens in urinary tract infection and antibiotic susceptibility pattern in Jimma university specialized hospital, southwest Ethiopia. Ethiop J Health Sci. 2011; 21: 141-146.
16. Cappuccino JG, Sherman N. Microbiology A Laboratory Manual, fififth ed., The Benjamin/Cummings Publishing Company Inc. 1999; 129; 8053-7646- 1, p. 129. [18] Holt JG, Krieg NR, Sneath PHA, Staley JT, Bergey's Manual of Determinative Bacteriology, ninth ed., Williams & Wilkins, Baltimore (MD), 1994.
17. Bartholomew JW, Mittwer T. The Gram stain, Bacteriol Rev. 1952; 16: 1-29.
18. Patel JB, Cockerill FR, Alder J, Bradford PA, Eliopoulos GM, Hardy DJ, Hindler JA, Jenkins SG, Lewis JS, Miller LA, Powell M, Swenson JM, Traczewski MM, Turnidge JD, Weinstein MP, Zimmer BL. Performance

standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. Clin Lab Stand Inst. 2014; 34. [21] Fekete T, Tumah H, Woodwell J, Truant A, Satischandran V, Axelrod P, Kreter B. Acomparison of serial plate agar dilution, bauer-kirby disk diffusion, and the vitek automicrobic system for the determination of susceptibilities of Klebsiella spp., Enterobacter spp., and *Pseudomonas aeruginosa* to ten antimicrobial agents. Diagn Microbiol Infect Dis. 1994; 18: 251-258.

19. Eleje GU, Adinma JI, Ghasi S, Ikechebelu JI, Igwegbe AO, Okonkwo JE, Okafor CI, Ezeama CO, Ezebialu IU, Ogbuagu CN. Antibiotic susceptibility pattern of genital tract bacteria in pregnant women with preterm premature rupture of membranes in a resource limited setting. Int J Gynaecol Obstet. 2014; 127: 10-14
20. Cavalieri SJ, Bohach GA, Snyder IS. Escherichia coli a-hemolysin: characteristics and probable role in pathogenicity. Microbiol Rev. 1984; 48: 326-343.
21. Freeman DJ, Falkiner FR, Keane CT. A new method for the detection of the slime production by the coagulase negative Staphylococci. J Clin Pathol. 1989; 42: 872-874. [25] Niveditha S, Pramodhini S, Umadevi S, Kumar S, Stephen S. The isolation and the biofilm formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs). J Clin Diagn Res. 2012; 6:1478-1482.
22. Christensen GD, Simpson WA, Bismo AL, Beachery EH. The adherence of the slime-producing strains of *Staphylococcus epidermis* to smooth surfaces. Infect Immune. 1982; 37: 318-326.
23. Rout S, Dubey D, Panigrahy R, Padhy RN. Surveillance of extended spectrum b lactamase producing bacteria in an Indian teaching hospital. J Taibah Univ Med Sci. 2014; 9: 274-281.
24. Jean SS, Lee WS, Bai KJ, Lam C, Hsu CW, Yu KW, Liao CH, Chang FY, Ko WC, Wu JJ, Chen YH, Chen YS, Liu JW, Lu MC, Liu CY, Chen RJ, Hsueh PR. Relationship between the distribution of cefepime minimum inhibitory concentrations and detection of extended-spectrum b-lactamase production among clinically important Enterobacteriaceae isolates obtained from patients in intensive care units in Taiwan: results from the Surveillance of Multicenter Antimicrobial Resistance in Taiwan (SMART) in 2007. J Microbiol Immunol Infect. 2015; 48: 85-91.
25. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: a Laboratory Manual, second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
26. Mohanty S, Ghosh S, Nayak S, Das AP. Isolation, identification and screening of manganese solubilizing fungi from low grade manganese ore deposits. Geomicrobiol J. 2017; 34: 309-316.
27. Dowd SE, Zaragoza J, Rodriguez JR, Oliver MJ, Payton PR. Windows. NET network distributed basic local alignment search toolkit (W.ND-BLAST). BMC Bioinforma. 2005; 6: 93.
28. Sanket AS, Ghosh S, Sahoo R, Nayak S, Das AP. Molecular identification of acidophilic Manganese (Mn) solubilizing bacteria from mining effluents and their application in mineral beneficiation. Geomicrobiol J. 2016; 1-10.
29. Ghosh S, Mohanty S, Nayak S, Sukla LB, Das AP. Molecular identification of indigenous manganese solubilizing bacterial biodiversity from manganese mining deposits. J Basic Microbiol. 2015; 55: 1-9.
30. Hasan AS, Nair D, Kaur J, Baweja G, Deb M, Aggarwal P. Resistance patterns of urinary isolates in a tertiary Indian hospital. J Ayub Med Coll Abbottabad. 2007; 19: 39-41.
31. Ullah F, Malik S, Ahmed J. Antibiotic susceptibility pattern and ESBL prevalence in nosocomial *Escherichia coli* from urinary tract infections in Pakistan. African Journal of Biotechnology. 2009; 8.
32. Iliyasu M, Uba A, Agbo E. Phenotypic detection of multidrug resistant extended spectrum beta-lactamase ESBL producing *Escherichia coli* from clinical samples. African Journal of Cellular Pathology. 2018; 9: 25–32.
33. Khosravi AD, Hoveizavi H, Mehdinejad M. Prevalence of *Klebsiella Pneumoniae* encoding genes for CTX-M-1, TEM-1 and SHV-1 extended-spectrum beta lactamases ESBL enzymes in clinical specimens. Jundishapur Journal of Microbiology. 2013; 6–15. [38] AL-agamy MH, Shibl AM, Tawfik AF. Prevalence and molecular characterization of extended spectrum β-lactamase-producing *Klebsiella Pneumoniae* in Riyadh, Saudi Arabia. Annals of Saudi medicine. 2009; 29: 253–257.
34. Rout S, Dubey D, Panigrahy R, Padhy RN. Surveillance of extended spectrum b lactamase

producing bacteria in an Indian teaching hospital. J Taibah Univ Med Sci. 2014; 9: 274-281.
35. Luzzaro F, Perilli M, Amicosante G, Lombardi G, Belloni R, Zollo A, Bianchi C, Toniolo A. Properties of multidrug-resistant, ESBL-producing *Proteus mirabilis* isolates and possible role of beta-lactam/beta-lactamase inhibitor combinations. Int J Antimicrob Agents. 2001; 17: 131-135.
36. Pagani L, Migliavacca R, Pallecchi L, Matti C, Giacobone E, Amicosante G, Romero E, Rossolini GM. Emerging extended-spectrum b-lactamases in *Proteus mirabilis*. J Clin Microbiol. 2002; 40: 1549-1552.
37. Molin S, Nielsen TT. Gene transfer occurs with enhanced efficiency in bio- biofilms and induces enhanced stabilization of the biofilm structure. Curr Opin Biotechnol. 2003; 14: 255-261.
38. Normark BH, Normark S. Evolution and spread of antibiotic resistance. J Int Med. 2002; 252: 91-106.
39. Pirog JK, Bogiel T, Skowron K, Wieckowska E, Gospodarek E. *Proteus mirabilis* biofilm qualitative and quantitative colorimetric methods-based evaluation. Braz J Microbiol. 2014; 45:1423-1431.
40. Jacobsen SM, Shirtliff ME. Proteus mirabilis biofilms and catheter-associated urinary tract infections. Virulence. 2011; 2: 460-465.
41. Stickler DJ, Morgan SD. Modulation of crystalline *Proteus mirabilis* biofilm development on urinary catheters. J Med Microbiol. 2006; 55: 489-494.