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A Study on Antibiotic Susceptibility Pattern and Detection of Virulence Markers of Uropathogenic Escherichia coli Isolated From Patients in Hospital

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ABSTRACT

Urinary tract infection (UTI) is one of the most common infectious diseases encountered in clinical practice. Emerging resistance of the uropathogens to the antimicrobial agents due to biofilm formation is a matter of concern while treating symptomatic UTI. But studies addressing the issue of biofilm production by uropathogens in Indian scenario are scarce. Aim of the study was to study biofilm formation and antimicrobial sensitivity pattern of uropathogens. Bacteria are responsible for about 95% of UTIs. The emergence of anti-microbial resistance in uropathogens may lead to poor treatment outcomes in individuals with UTIs. The knowledge of the microorganism and antibiograms are important for the empirical treatment of UTIs. A cross-sectional study was carried out over 12 months with a focus on the identification of bacterial pathogens causing UTI and the evaluation of their antibiogram. In total, 583 urine samples were collected from individuals with suspected UTIs and inoculated on recommended media. Isolation and identification of the bacterial strains were performed using standard microbiological protocols. The frequently identified isolates were *Escherichia coli* (68.9%), followed by *Klebsiella pneumoniae* (8.9%) and *Staphylococcus aureus* (6.7%). The highest percentages of resistance have been observed against tested antibiotics. The majority of the isolates were extended spectrum β-lactamase producers (85.2%) and multidrug-resistant (98.3%). We observed that Gram-negative bacteria were the main cause of UTIs where the predominant microorganism was *E. coli.*

Keywords: Antimicrobial Agents, Urinary Tract Infection, Antibiotic Susceptibility, Uropathogens, *Escherichia coli*, Community-Acquired, Virulence Factors, Multidrug Resistance, Antibiogram.

INTRODUCTION

Urinary tract infection (UTI) is the colonization of the urinary tract by pathogenic microorganisms. Infection is caused by fungi, bacteria and viruses. The infection has prolonged admissions in hospital, morbidity in general population and high financial cost implications to the patients. Majority of UTIs are caused by bacteria that are found in the bowel and live as normal flora and often result from faecal and perineal areas. These organisms are capable invading the tissues of the urinary tract and adjacent tissues causing lower urinary tract infections and upper tract infections. In general population and hospital set up, UTI is a common infection although there are new and more powerful antibiotics in use but bacterial resistance persists. The spectrum of causative agents and their antimicrobial resistance pattern has been dynamic worldwide.

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Routine culture and sensitivity is not carried out for UTI patients in hospitals of developing countries therefore antimicrobial agents are administered before laboratory results of urine culture are available this phenomenon creates multidrug resistance. Wide use of beta-lactam antibiotics empirically, prolonged intake of antibiotics for inappropriate duration and long term hospital stay lead to persistence and spread of virulent organisms (UPEC) and end up in a major threat called resistance. Multi drug resistant (MDR) pathogens increase the morbidity and mortality of urinary tract infections in India. Therefore, regular monitoring of antibiotics resistance profile is very essential for treatment and also to prevent the spread of resistant strains in both hospital and community.

This emergence of multidrug-resistant isolates has further complicated the efficacy of antibiotics, reducing therapeutic options in the health services, and subsequently increasing medical costs, morbidity, and mortality rates. Even more demanding is the fact that the susceptibility profile of uropathogenic bacteria varies depending on the type of healthcare facility, geographic location, environment, and, the period of evaluation of susceptibility profiles of pathogens

Due to limited studies on virulence markers (VM) of UPEC causing UTI, the present study was undertaken to know the prevalence of UPEC, VMs identification and its antibiotic sensitivity. It is hoped that this information will definitely help to reduce the morbidity, hospital stay and also will provide valid information for effective hospital infection control.

MATERIALS AND METHODS

- **Ethical Considerations**
  This work was carried out with Institutional Ethical committee approval.
- **Study Design and Study Sites**
  This study was a point prevalence study, in which the number of individuals with a disease in a time interval is divided by the total number of individuals in a population.

❖ Design of Study - Cross sectional analytical study.

❖ Setting of Study - Scientific Path Lab, Department of Microbiology, Dr Turna Max Hospital, Sitarganj, Susheela Tiwari Hospital and Medical College and Research, Haldwani, Nainital, Uttarakhand.

- **Period of Study**
  The study was conducted over a period of one year (October 2018 – November 2019)
- **Sample Size**
  Total number of *E.coli* isolated from urine samples during the study period is around 583.
- **Inclusion Criteria**
  - ✓ Specimens collected from all clinically suspected cases of UTI of all age groups, of both OPDs and IPDs.
  - ✓ Conditions in which asymptomatic UTI occurs. (Diabetic Mellitus, Pregnancy)
  - ✓ Single isolate of *E.coli* per patient was included in the study.
  - ✓ Urine samples collected both via naturalis and catheters.
- **Exclusion Criteria**
  - ✓ Patients on antibiotics during last one month.
  - ✓ Samples obtained from the collection bag in catheterized patients.
  - ✓ Insignificant bacteriuria.
  - ✓ Polymicrobial growth in culture.

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- **Data Collection**
  All data were entered in Microsoft excel spread sheet.
- **Collection of Urine Samples**
  - **Clean-Catch Midstream Urine Sample:** The patients were given proper instructions to collect the sample to avoid contamination from anterior urethra. Female patients were instructed to clean the area around the urethral opening and let out first few drops of urine and to collect the mid stream urine after holding the labia apart.
    The male patients were asked to collect the midstream by retracting the foreskin into a sterile screw-capped, leak proof and transparent container.
  - **Catheterized Sample:** Sample was collected from the indwelling catheters under aseptic precautions. The catheter tube was clamped off above the port, cleaned with 70% ethanol and the urine was aspirated via a sterile needle and syringe.

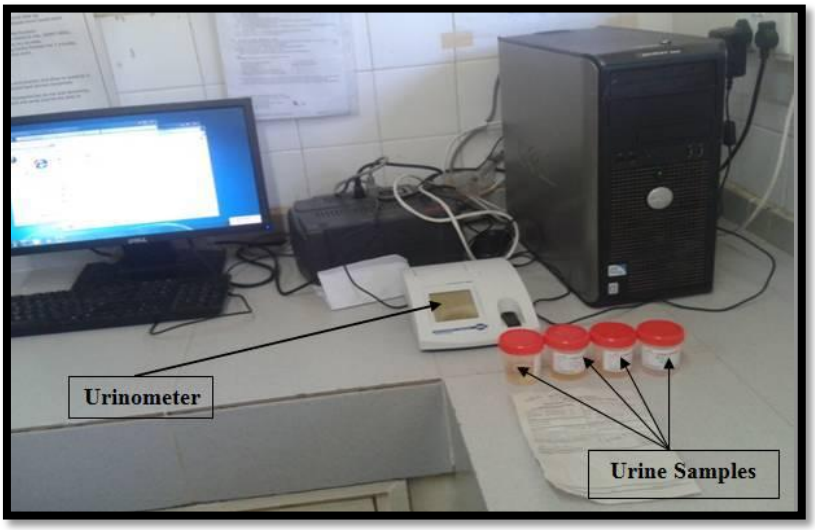

Figure 1: Processing of Urine Specimens in Sterile Containers

- **Specimen Transport:** The collected samples were labeled, transported to the laboratory and processed immediately to ensure maximum recovery of the pathogen and also to minimize the multiplication of commensals. They were refrigerated up to 24hrs in case of any delay.
- **Specimen Processing**
  - **Macroscopy:** All the urine samples were examined macroscopically and whether they were clear or turbid were noted.
  - **Microscopy:** Wet preparation of fresh uncentrifuged urine was done by transferring a drop of urine to a grease free clean glass slide and a cover slip was placed over it carefully without letting air bubbles. The slide was examined first under the low power 10x and then high power 40x for the presence of pus cells (significant if 10 WBC/mm3 of urine), microorganisms, RBCs, casts and crystals.

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- **Isolation:**
  - ❖ A semi-quantitative culture method was performed by using sterile 4.0mm calibrated loop delivering 0.01mL of urine. A loopful of urine sample was plated on Nutrient agar and Cysteine-Lactose-Electrolyte Deficient (CLED) agar and incubated overnight at 37 ^{0}C.
  - ❖ The number of isolated colonies was multiplied by 100 for the estimation of Colony Forming Units (CFU)/mL of the urine sample. As per Kass criteria, the growth was considered as significant if colony count is $\geq 10^5$ CFU/mL and in case of catheterized sample it is considered as significant when the count is $\geq 10^3$ CFU/mL and insignificant if $<10^3$ CFU/mL.
- **Identification:** *E.coli* was identified with the following preliminary and biochemical tests done by standard recommended laboratory methods.

Table 1: Identification of *E.coli*

Biochemical Reactions of *E.coli*	
Grams Staining	**Short Gram Negative *Bacilli***
Mobility of Hanging Drop Method	Motile
Catalase Test	Positive
Oxidase Test	Negative
Nitrate Reduction Test	Nitrate Reduced
Indole Test	Produced
Methyl Red	Positive
Voges Proskeur	Negative
Simmons Citrate	Not Utilized
Urease Test	Not Produced
Triple Sugar Iron	Acid Slant/ H_2S not produced
Mannitol Motility Medium	Fermented and Motile

- **DETECTION OF VIRULENCE MARKERS OF UPEC**

  ❖ **Hemolysin Production**

  Hemolysin production was detected by the method adopted by Cavalier. The *E.coli* isolates were inoculated on 5% sheep blood agar and incubated overnight at 37°C. Presence of hemolysin was detected by the formation of clear zone around the colonies.
  Positive: Zone of hemolysis around the colony.
  Negative: No zone of lysis.

  ❖ **Hemagglutination**

  Direct bacterial haemagglutination test was performed by slide method. All the *E.coli* isolates were inoculated into 1% nutrient broth and incubated at 37 °C for 48 hours for full fimbriation. The red blood cells (human 'O' blood group) were taken and washed

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three times in normal saline. Then it was made up to 3% suspension in fresh saline. One drop of suspension was added to a drop of 48 hours broth culture of *E.coli* on a concave slide. Then the slide was rotated for 5 minutes at room temperature. Appearance of clumping was taken as positive for haemagglutination.

- ✓ **MRHA:** Presence of haemagglutination in 2% W/V D-mannose.
- ✓ **MSHA:** Inhibition of haemagglutination in 2% W/V D-mannose.

Gelatinase Test

Gelatinase production was tested by stab method as done by Hass D etal 99. A heavy inoculum of the test isolates was inoculated by stabbing 4-5 times on the tube containing nutrient gelatin medium and incubated at 37°C for 24 hours. Then the tubes were removed from the incubator and placed in the refrigerator (4°C) for 15-30 minutes. Production of enzyme gelatinase was observed by tilting the tubes. If the medium flows when the tube is tilted, it is considered as positive. If the medium does not fall by tilting the tube, it gets solidified and considered as negative.

Biofilm Production

Quantitative assessment of biofilm production was performed by the method adopted by Christensen GD et al. 4 – 5 colonies from overnight culture plates were inoculated in a test tube holding 10ml of trypticase soy broth added with 1% glucose. After overnight incubation at 37°C, all tubes were decanted and washed with phosphate buffered saline. After that, all the tubes were dried and stained with 0.1% safranine. Excess stain was removed and washed with deionized water.
Biofilm was observed once the tubes were dried. Biofilm formation was considered as positive by appreciating visible film lines along the sides and bottom of each tube and considered as negative when there was ring formation at the interface and clearing of tubes.

Negative – 0; Weak positive – 1+; Moderate positive – 2+ ; Strong positive – 3+

Siderophore Production Assay

This test was performed by universal chrome azurol sulfonate (CAS) agar diffusion method. The CAS assay detects the color change of CAS-Iron complex form blue to orange after chelation of the bound iron by siderophores. A strong ligand L (e.g., a siderophore) is added to a highly colored iron dye complex; when the iron ligand complex is formed, the release of the free dye is accompanied by a color change.
This was done by inoculation of all the test isolates in nutrient broth and centrifugation. Then the sediment was taken and inoculated on CAS agar plates and incubated at 37°C for 48h and observed for colour change.

- ✓ **Positive :** Colour change from blue to orange.
- ✓ **Negative :** No colour change.

Serum Resistance

All the test isolates were inoculated into 1% Nutrient Broth and incubated overnight at 37°C. Then they were diluted in 5ml nutrient broth. The diluted suspensions were incubated at 37°C for 2hours to make it 105 CFU/ml. The cultures were centrifuged (1500 g for ***5*** min) and the deposit was resuspended in ***5*** ml of phosphate buffered saline.

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Antibiotic Susceptibility Testing

This test was performed using Kirby Bauer disc diffusion method. In this technique organisms isolated were inoculated in normal saline with the help of sterile wire loop. Briefly, colonies were taken from 24 hours culture plates into nutrient broth. The turbidity formed was adjusted to an equivalent of 0.5 McFarland. (1x 10^8 cells/ml) resulting in semi-confluent growth. The test organism was streaked over the surface of Muller Hinton agar plates using sterile cotton swabs. Discs impregnated antibiotics which were commercially available were placed on plates firmly by means of sterile forceps aseptically and the inoculated plates were incubated for 24 hours at 37°C. The antibiotic discs were then placed on the inoculums surface at 20-25 mm distance between the two discs. Afterwards diameters of zone of inhibition were measured in mm.

Antibiotic discs used were Ampicillin (AMP), Amoxicillin-clavunalic acid (AMC), Trimethoprim-Sulfamethoxazole (COT), Ciprofloxacin (CIP), Nitrofurantoin (NIT), Gentamycin (GEN), Amikacin (AK), Cefotaxime (CTX), Ceftriaxone (CTR), Ceftazidime (CAZ), Cefipime (CPM), Pipercillin-Tazobactum (PIT) and Imipenem (IPM) - Purchased from Himedia lab.

Quality control test was performed with *E.coli* ATCC 25922 strain. The isolates which showed resistance to at least three or more than three groups of antibiotics were considered as multi drug resistant *E.coli*. MDR *E.coli*. Zone size of all the isolates against the antibiotics were recorded and noted.

RESULTS AND DISCUSSIONS

Table 2: Screening and Rate of Detection of Virulence Markers in UPEC

COMPONENTS	POSITIVE	NEGATIVE
Biofilm Formation	120	160
Serum Resistance	164	116
Hemolysis	166	114
Haemaggltination	167	113
Gelatinase	104	176
Siderophore Production	112	168

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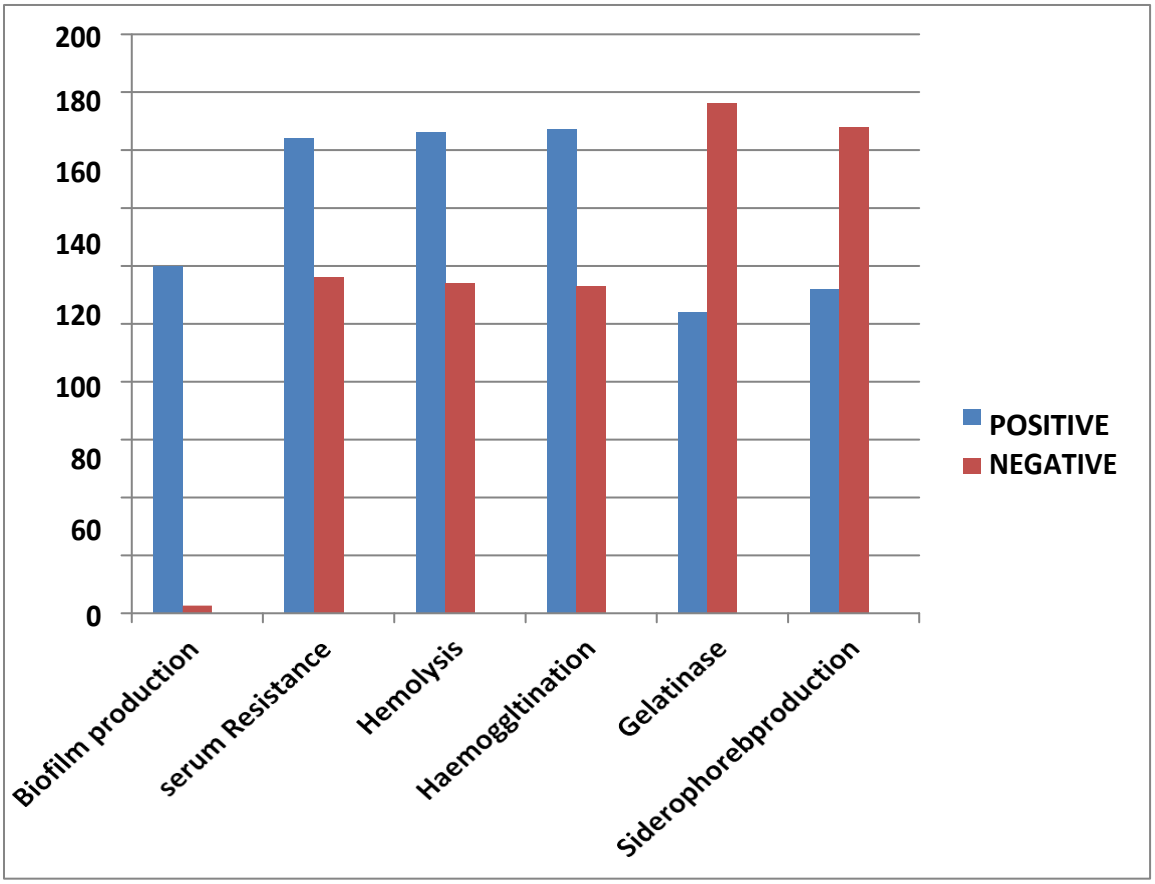

Figure 2: Screening and Rate of Detection of Virulence Markers In UPE

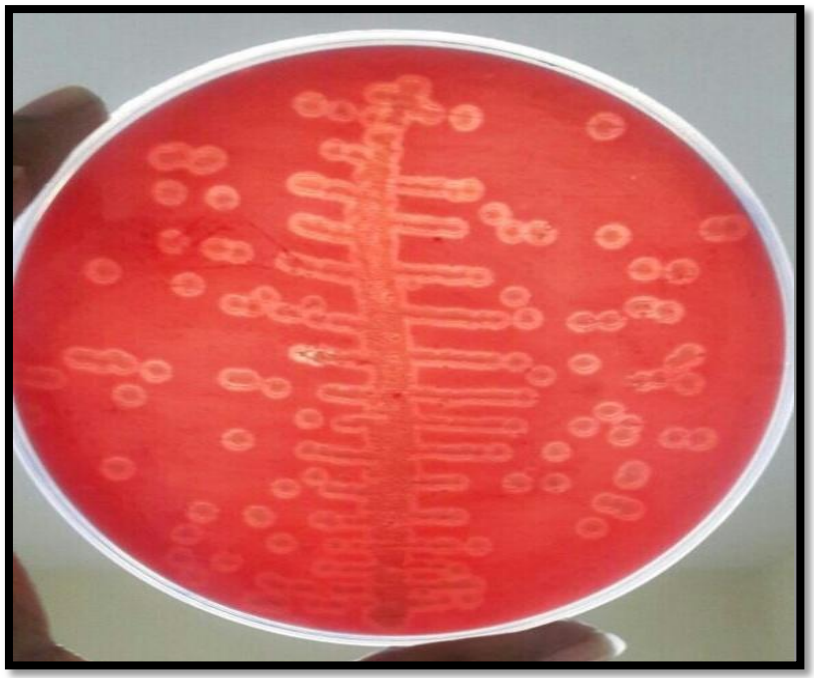

Figure 3: Sheep Blood Agar Plate - Hemolysis Around Each Colony

In the present study, it was observed that 59.3% of isolates produced hemolysin. Hemolysin production is associated with severe form of UTI by colonization of virulent UPEC since it is toxic to urothelial cells, causes inflammation and enters into the blood stream.

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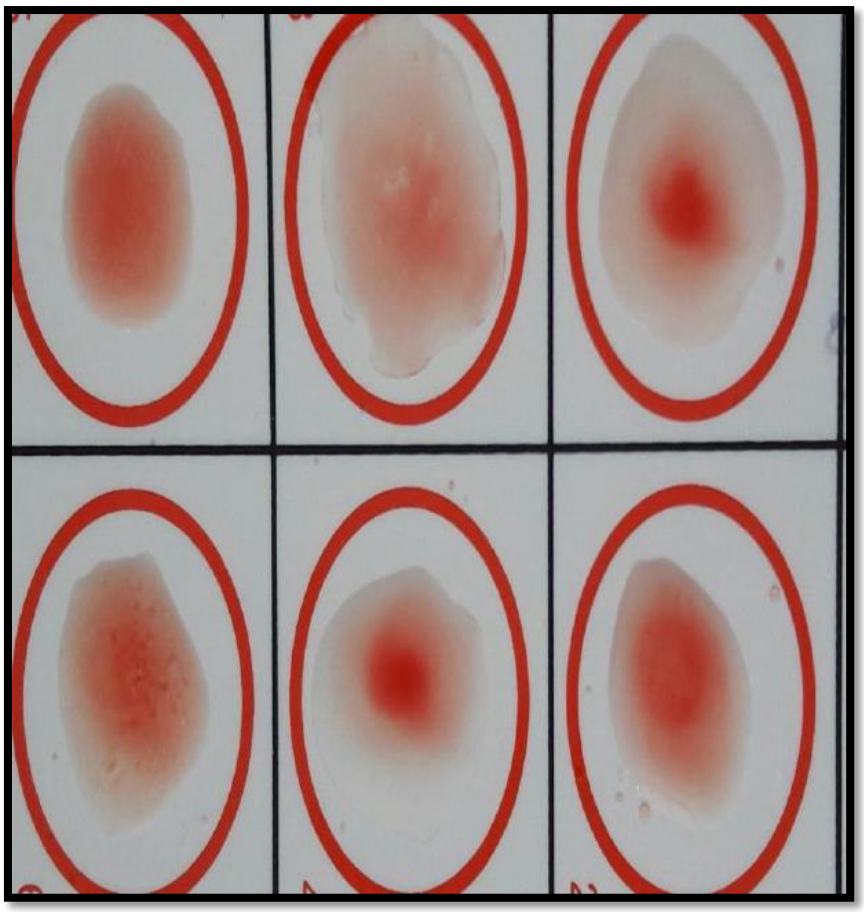

Figure 4: Haemeagglutination - Slide Method MRHA and MSHA

Fimbriae are responsible for adherence by pathogenic organisms to the host epithelial cells. Thus, MRHA property of UPEC is considered as an important virulence factor in UTI.

Figure 5: Nutrient Gelatin Agar Tube-Gelatinase Positive and Negative

Gelatinase Creation

Gelatinase creation by UPEC was least studied. The present investigation showed that lone UPEC isolates produced gelatinase. It is rather than the studies where just 6.6% isolates produced gelatinase. Based on the observations of the present investigation, prevalence of Hemeagglutination was high followed by Hemolysin creation and serum resistance property. The least virulence factor discovered was gelatinase creation.

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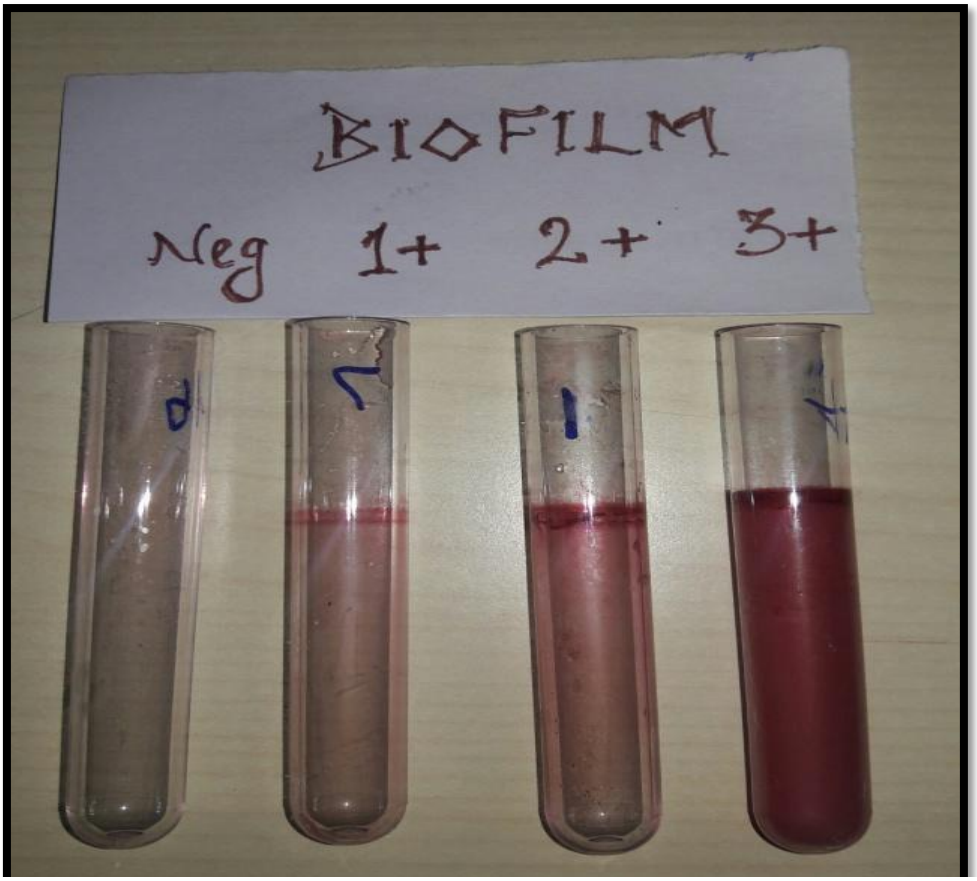

Figure 5: Biofilm By Tube Method Negative 1+, 2+, 3+

Biofilm Creation

It was observed that 52.95 % of UPEC produced solid biofilm whereas in the examination done produced solid biofilm. It is in opposition to the investigation performed where just 17.2% were solid biofilm producers. Biofilm creation is constantly considered as serious problem in case of patients with indwelling catheter as it accelerates trouble in management and anti-infection resistance. In this examination additionally, it has been discovered that UPEC isolates among catheterized samples showed biofilm arrangement. Of these, were discovered to be solid biofilm producers. It is harsh with the investigation in which like results in which of isolates of UPEC from catheterized samples produced solid biofilm.

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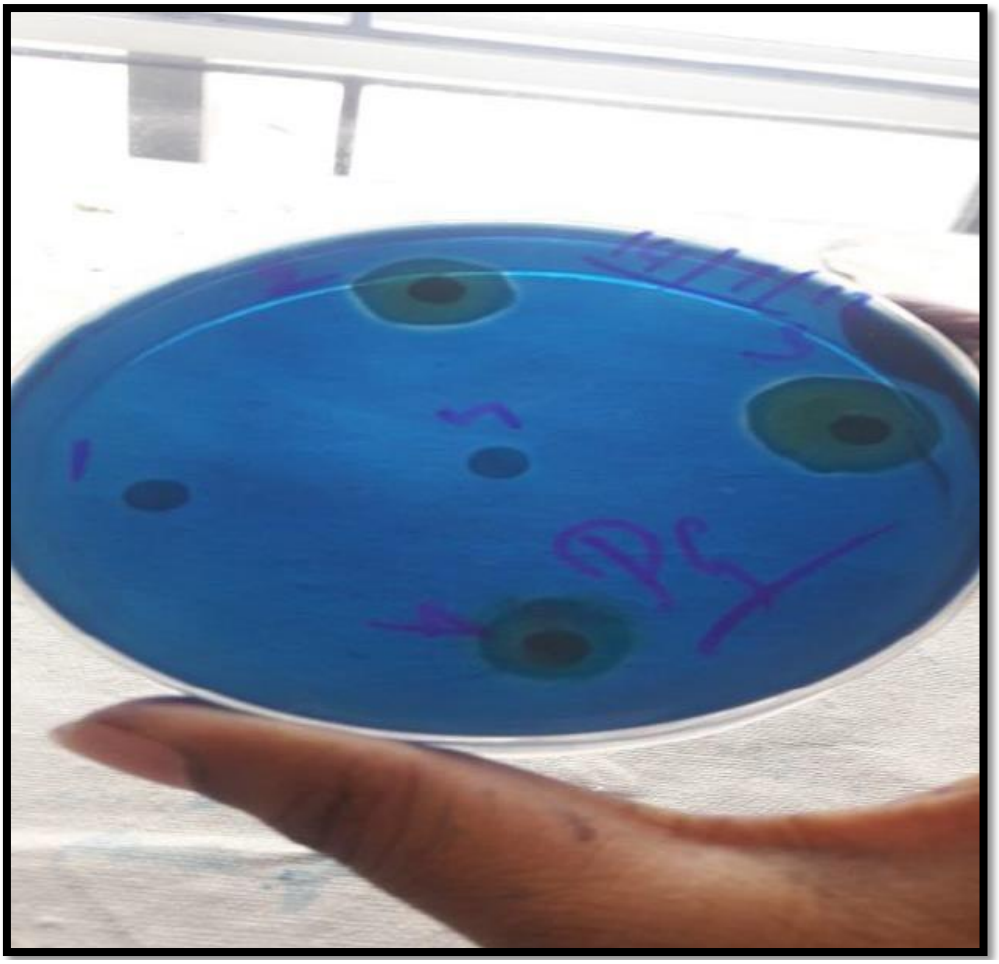

Figure 6: Chrome Azurol Sulfonate Agar Plate-Siderophore Production (Orange)

Siderophore Creation

Siderophore creation was seen in (86%) isolates in this investigation. It is like the results of other studies performed who showed that 86%, 75% and 93% isolates produced siderophore respectively. The majority of the researchers suggested that siderophore creation consistently helps in the development of pathogen and keeps up its endurance inside the host in the pathogenesis of UTI.

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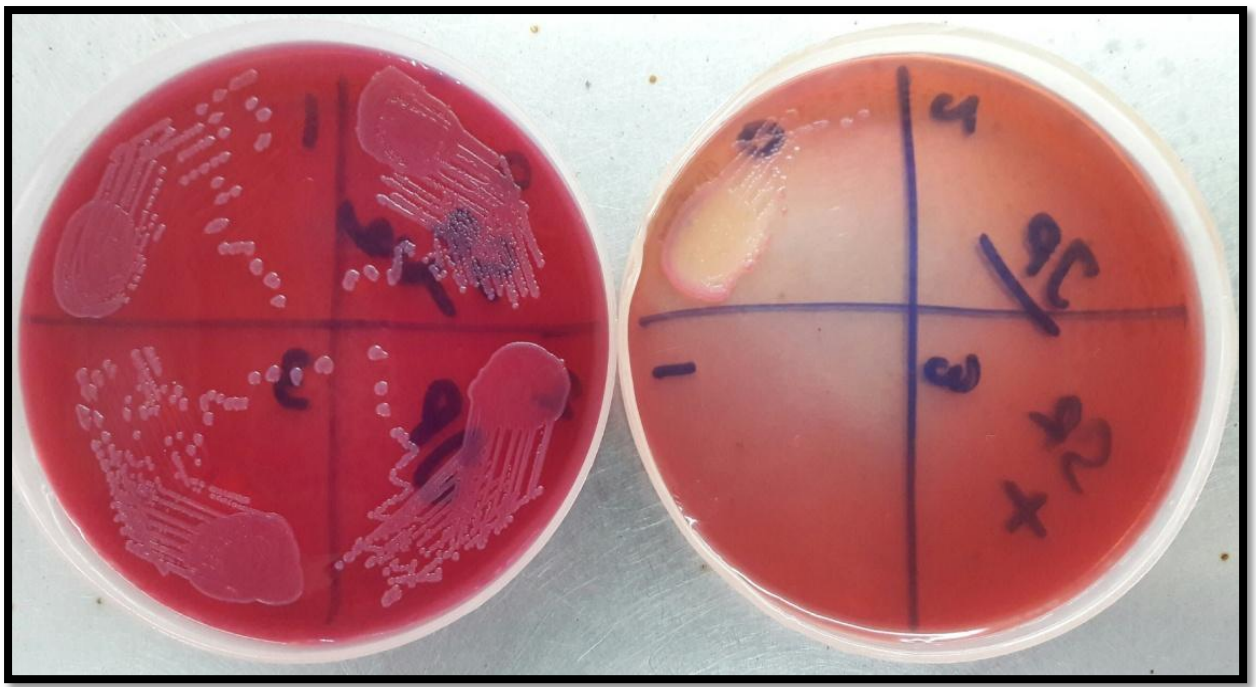

Figure 7: Serum Sensitive-Growth Seen At 0hr, 1hr, 2hrs and 3hrs of Incubation. Serum Resistant-Growth Seen Only At 0 hrs of Incubation

In the current study, 58.63% of isolates showed resistance to serum bactericidal action. High degree of serum resistance is correlated with increased virulent property of bacteria.

Culture Characteristics

Figure 8: Nutrient Agar Plate
Large, Translucent Colonies with Significant Growth

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Figure 9: Muller Hinton Agar Plate Showing Zone of Inhibition of *E.coli*

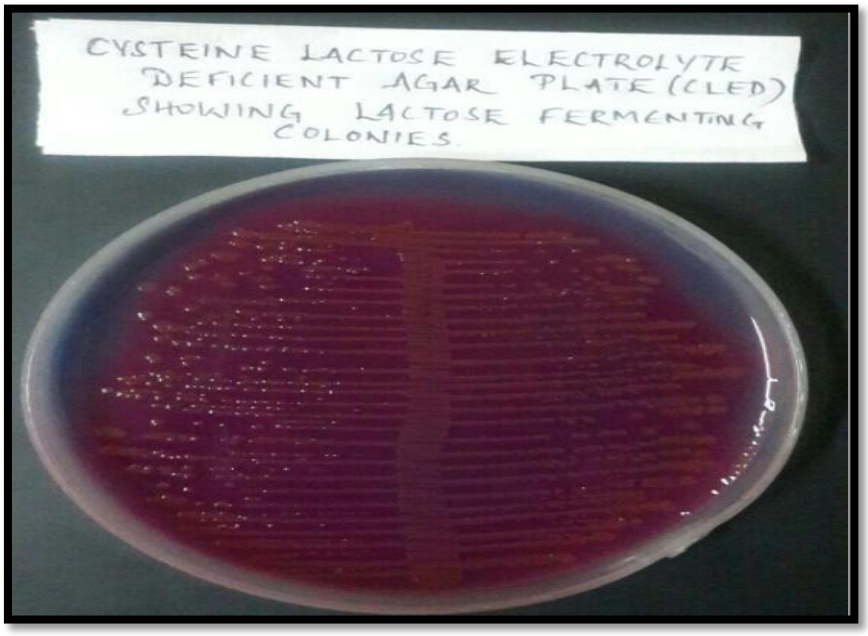

Figure 10: CLED Plate - Lactose Fermenting Colonies

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DISCUSSION

UPEC is the most important group of pathogens causing UTIs. Many molecular studies proved that urovirulent *E.coli* expresses many cell surface factors and secretes toxins and enzymes, among them few are peculiar in causing UTIs. Considering high morbidity and mortality rate of UTIs due to the spread of resistant and virulent strains among the community and in the hospital, UPEC should be focused and paid more attention. These virulence factors are expressed in different frequencies in different states. Phenotypic characteristics of UPEC isolates and their correlation with antibiotic resistance patterns in patients with UTI are not well known.

Susceptibility pattern of Gram-negative bacteria showed that all of the isolates were sensitive to nitrofurantoin (100%). The rest of isolates were sensitive to Ciprofloxacin (79.8%), Cefotaxime (75.3%), Amoxicilin-clavulinic acid (72.8%), Gentamicin (67.6%), Nalidixic acid (65.6%), Cotrimoxazole (46.6%), and Ampicillin (44%) which indicated that their susceptibility pattern of Gram-negative bacteria were Gentamicin (93.3%), Chloramphenical (83.3%), Contrimoxazole (73.3%) and Amoxicilin-clavulinic acid (70%) were highly resistant. Availability and indiscriminate use of commonly used antibiotics without health care workers prescription lead to an increased multidrug resistance. Due to the increasing multidrug resistance among uropathogens, the health care workers are left with a limited choice of routinely used antibiotics to choose from for the treatment of urinary tract infections. This can be attributed the fact that bacteria undergo mutation which makes their susceptibility vary from one geographical to the other.

All the six virulence factors were positive in only 7 isolates (2.51%), five were detected in 32 isolates (12.58%), four in 68 (24.46%), three in 70 (25.17%), two in 58 (20.86%), only one virulence factor was found to be positive in 29 isolates (10.43%) and 11 isolates (3.95%) did not show positivity for any virulence factor. It was also recorded that Multiple VFs (≥ 4) were seen in 106 isolates (38.12%).

CONCLUSION

Urinary tract infections (UTIs) are the most common bacterial infections. Of the 1989 urine samples received in Microbiology Laboratory, 676 showed significant growth. Of these 278 was belonged to *E.coli* (UPEC).

The prevalence of UTI was 59.35% in females and 40.64% in males. In both patients with complicated and uncomplicated UTIs *E.coli* was the most commonly encountered organism. When these 278 isolates of *E.coli* were subjected to detect these various virulence factors like hemolysin production, siderophore production, biofilm formation, gelatinase production, MRHA property and serum resistance property. Of these, 166 (59.7%) showed Hemeagglutination, 165 (59.35%) produced hemolysin, 163 (58.63%) showed serum resistance property, 119 (42.8%) were biofilm positive, 111 (39.9%) produced siderophore and 103 (37%) produced gelatinase in the above order. 106 isolates (38.12%) expressed Multiple VFs (≥ 4). Hemolysin production (59.8%) was the most frequently identified virulence factor in complicated UTI and serum resistance (61.53%) was found to be more common in uncomplicated UTIs.

Hemolysis (95.1%) was more frequent in Upper UTI whereas serum resistance 57.44% was common in Lower UTI. Highest resistance of UPEC was seen with Ampicillin (98.9%) followed by Amoxyclav (75.53%), Ceftriaxone (73.74%) and Gentamycin (73%), maximum sensitivity was found with Imipenem (94.6%) followed by Piperacillin-Tazobactam (89.92%). Prevalence of ESBL and MDR *E.coli* isolates were 68% and 21% respectively.

Most of MDR *E.coli* isolates (50) were from in-patients (82.6%). Isolates carried MVFs showed MDR pattern.

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I pay tribute to My Parents for lifting me up till this phase of life. I thank them for their love, trust, patience, support and bearing all kind of stress to make me what I am.

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