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Virulence Markers, Extended Spectrum Beta Lactamase detection and Antibiotic Susceptibility pattern of Uropathogenic E Coli in Clinically Suspected Urinary Tract Infections.

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ABSTRACT

To detect prevalence of Uropathogenic *Escherichia coli* (UPEC) and their Virulence Markers (VMs) along with its ESBL detection in addition to antiobiotic sensitivity pattern (ASP) in clinically suspected cases of urinary tract infection. A Total of 100 E.coli isolates from clinically suspected UTI cases were studied and screened for VMs namely haemolysin, mannose-resistant and mannose-sensitive haemagglutination (MRHA and MSHA), cell surface hydrophobicity and siderophore production and also Antibiotic susceptibility pattern was done using Kirby bauer disc diffusion method. Further, isolates were subjected for extended spectrum beta lactamase (ESBL) detection using predictor disc approximation method. The prevalence of UPEC is 75%. The VMs pattern exhibited by UPEC isolates were siderophore production in 76% (57/75) of isolates; 72 %(54/75) of them showed haemolysin production; 60 %(45/75) haemagglutination (HA) and 56 %(42/75) are positive for cell surface hydrophobicity. Antibiotic sensitivity of UPEC showed maximum sensitivity to amikacin (90.7%) and exhibited least sensitivity to tetracycline (21.3%). In ESBL detection, 57% showed ESBL production (43 UPEC and 14 non UPEC). The association between production of ESBL by UPEC and non UPEC isolates were statistically significant (P< 0.001).UTI are treated empirically without culture and sensitivity resulting in the occurrence of higher prevalence of UPEC, emphasize the need for cost effective routine screening for the Virulence markers. This can be used as good tool in early diagnosis and rational use of antibiotics for UTI patients.

Keywords: Uropathogenic Escherichia coli, Virulence markers, Antibiotic susceptibility pattern, ESBL

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INTRODUCTION

Urinary tract infection (UTI) is the most common site of bacterial infection both in community and hospitalized patients [1]. Escherichia coli is most commonly isolated from nosocomial infections [2] and is most common cause of UTI accounting for 85% of community acquired infections [3].

It has been traditionally described that certain serotypes of *E. coli* are consistently associated with uropathogenicity and are designated as uropathogenic *E.coli* (UPEC). The cell morphology and molecular biology studies have revealed that UPEC express several surface structures and secrete protein molecules some of them cytotoxic, peculiar to the strains of E.coli causing UTI. Hence it is important to identify UPEC from non UPEC isolates in the urinary samples [4]. These UPEC isolates express chromosomally encoded virulence markers(VMs) namely Haemolysin(H), haemagglutination(HA), Cell surface hydrophobicity(CSH) and siderophore(SPA) production etc.

UTI is usually treated empirically without culture and it contributes for about 10-15% prolongation of hospital stay due to its ability to exhibit resistant bacteria in the hospital [5,6]. Among the wide array of antibiotics, ß-lactams are the most widely used agents. But ß -lactamases continue to be the leading cause of resistance to ß-lactam antibiotics in gram negative bacteria [7, 8]. However new ß-lactamases emerged against each of the new classes of ß-lactams, that were introduced and caused resistance. The latest of these enzymes has been the evolution of Extended Spectrum ß-lactamases (ESBL) [8]. These enzymes were coded by plasmids and their ability to spread to other bacteria through the plasmids has led to dramatic increase in their prevalence worldwide. Therefore, regular monitoring of antibiotics resistance profile is crucial in rational drug prescription, complete cure and also to prevent the spread resistant strain in the hospital as well as community [5].

The information on VMs of UPEC causing UTI is limited and less studied. So the present study was undertaken to know the prevalence of UPEC, VMs identification, ESBL Detection and Antibiotic sensitivity pattern (ASP) pattern in different patient population of a tertiary care teaching hospital.

MATERIALS AND METHODS

This cross sectional study was conducted in department of Microbiology, Mysore Medical College and Research Institute for a period of 12 months .The study was initiated after institutional ethics committee approval and informed consent. The patients from the outpatient and inpatient departments were enrolled.

One hundred E. coli strains isolated from urine samples from clinically suspected UTI patients were studied for the detection of VMs. A Wet mount of uncentrifuged urine was done and considered significant if more than 5-6 pus cell/HPF, red blood cells (RBCs) and organisms were seen. The isolates were maintained by inoculating nutrient agar butts and stored at room temperature. Semiquantitative Culture was done on MacConkey agar and blood agar by standard loop method [9]. The growth of single morphotype of colony was considered and colony counts were done and termed 'significant' based on clinical history. Identification of E.coli was done using standard microbiological techniques and the E.coli thus isolated was screened for virulence markers namely; Haemolysin, HA, CSP and siderophore production.

Haemolysin

The cytolytic protein toxin secreted by most haemolytic Esch.coli is α-haemolysin. The method used for detection of α-haemolysin was as described by Silveira WDD et al [10]. The different strains of E.coli were grown in Lysogeny broth medium overnight at 37^0C.50 µL of this were cultured on the Petri dish containing 5% sheep blood agar and incubated at 37^0C for 24 hrs. Haemolysin production was detected by the presence of zone of complete lysis of the erythrocytes around the colony and clearing of the medium [11].

Cell Surface Hydrophobicity (CSH) [1]

The CSH of *E. coli* was determined by the salt aggregation test (SAT). *E.coli* grown on MacConkey agar plates were inoculated into 1ml of phosphate buffer saline pH 6.8 and turbidity was matched with McFarland tubes 6 and 7 to get colony count of 5 × 10 colonies /ml. 40 µL of 0.2 M Phosphate buffer saline pH 6.8 was

taken in first column of VDRL slide.40 µl of 1M, 1.4 M & 2M concentration of Ammonium sulphate was taken in each well of other columns of VDRL slides.40 µl of E.coli suspension was added to each of these wells. The clumps formed in different molar concentration of Ammonium sulfate was observed under the microscope and were considered hydrophobic if they aggregate in concentrations of 1.4 M [12].

Siderophore Production

This test was carried out using a method named "chrome azurol sulphonate agar diffusion assay.'' [13]. The chrome azurol sulphonate (CAS) assay detects colour change of CAS-Iron complex from blue to orange after chelation of the bound iron by siderophores. A strong ligand 'L' (e.g., a siderophore) is added to a highly coloured iron dye complex; when the iron ligand complex is formed, the release of the free dye is accompanied by a colour change. The result was taken as positive if there was a colour change from blue to orange halo.

Procedure [13]

60.5 mg CAS was dissolved in 50ml deionized water and mixed with 10 ml iron (111) solution (1mM FeCl3 – 6H20, 10mM HCl); by stirring, this solution was slowly mixed with 72.9 mg Hexadecyltrimethylammonium bromide dissolved in 40 ml water. The resultant dark blue solution was autoclaved and mixed with an autoclaved mixture of 900 ml water, 15 gm agar.30.24 gm 1.4 PIPES (piperazine diethane sulphonic acid) and 12 gm of solution of 50% (w/v) NaOH to raise the pH to the pKa of PIPES (6.8).In CAS agar plate, each hole(2.5–5 mm diameter) was filled with 25-35 ml of the broth & desferal, which was two fold serially diluted from 2.5mM. After incubation of plate at 37^0C or room temperature for 4-9 hrs, orange halo was formed around each hole.

Haemagglutination

The HA was detected by clumping of RBCs by fimbriae of bacteria in the presence of D-mannose. This test was carried out as per the direct bacterial HA test - slide method and mannose-sensitive haemagglutination test(MSHA) and mannose-resistant haemagglutination tests (MRHA).The strains of *E. coli* were inoculated into 1% nutrient broth and incubated at 37 °C for 48 hours for full fimbriation. A panel of RBCs was selected by obtaining blood from human blood group 'O'. The RBCs were then washed thrice in normal saline and made up to a 3% suspension in fresh saline. They were used immediately or within a week when stored at 3-5 °C. The slide haemagglutination test was carried out on a multiple-concavity slide. One drop of the RBC suspension was added to a drop of the broth culture and slide was rocked to and fro at room temperature for 5 minutes. Presence of clumping was taken as positive for haemagglutination [14].

MSHA was detected by the absence of haemagglutination in a parallel set of test in which a drop of 2% w/v D-mannose was added to the RBCs and a drop of broth culture. MRHA was detected by the presence of haemagglutination of 3% 'O' blood group human RBC in the presence of 2% mannose [14].

The ASP was carried out on Mueller Hinton agar using Kirby-Bauer disc diffusion method. The panels of antibiotics used were Ampicillin 10 µg, Amikacin 30 µg, Ciprofloxacin 5 µg, Norfloxacin10 µg, 10 µg, Tetracycline 30 µg, Co-Trimoxazole-1.25/23.75 µg. All these were further subjected for detection of probable ESBL producers using predictor disc approximation method.

Procedure of predictor disc approximation method [15]

Ceftazidime & Ceftazidime + Clavulanic acid discs were kept 15-20 mm apart from each other (center to center). Imipenem, an inducer, was placed in the center and on either side of it, at a 15mm distance, were placed Ceftazidime & Cefotaxime (indicators of induction). In addition another inducer Cefoxitin was placed at 15mm from Cefotaxime .This was placed opposite to that of Ceftazidime + Clavulanic acid to avoid any effect of inducible beta–lactamase on the zone of inhibition of the later.

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Interpretation

- An isolate was suspected to be an ESBL producer by screening method if it had the zone sizes for Cephalosprines-Aztreonam (30 µg) ≤27mm, Cefotaxime (30 µg) ≤27mm, cefpodoxime (10 µg) ≤21 mm, Ceftazidime (30 µg) ≤22mm & Ceftriaxone (30 µg) ≤25mm.
- Susceptible to Cefoxitin.
- Increase in zone size with addition of an inhibitor by ≥5mm.

Statistical Analysis

The parametric data is presented as frequency and percentages. The categorical data was analyzed using chi square tests with Yates corrections at p= 0.05 significance level. The statistical analysis was done using graphpad software.

RESULTS

The present study was carried out on 100 *E.coli* isolates from cases of clinically suspected UTI patients and screened for the VMs of UPEC.

Table 1 shows Age-wise distribution of UPEC and non UPEC isolates. UPEC isolates were more between 41-50 years and 51-60 years accounting 14% and 15% respectively.

Table 1: Age-wise distribution of UPEC and non UPEC isolates

Age (years)	E coli Isolates					
	Non UPEC (%)		UPEC (%)		Total (%)	
0-10	4	(4)	9	(9)	13	(13)
11-20	1	(1)	4	(4)	5	(5)
21-30	3	(3)	9	(9)	12	(12)
31-40	3	(3)	11	(11)	14	(14)
41-50	7	(7)	14	(14)	21	(21)
51-60	4	(4)	15	(15)	19	(19)
61-70	3	(3)	6	(6)	9	(9)
>70	0	(0)	7	(7)	7	(7)
Total	25	(25)	75	(75)	100	(100)

$\chi 2=3.995$; df=7.00; P=0.78

Table 2 shows Gender wise distribution, Out of 100 *E.coli* isolates female preponderance was seen in both UPEC and Non UPEC isolates.

Table 2: Gender wise distribution among UPEC and non UPEC isolates

E. coli/Gender	F (%)	M (%)	Total
Non UPEC	13(52)	12(48)	25
UPEC	40(53.333)	35(46.66)	75
Total	53(53)	47(47)	100

$\chi 2=0.013$; df=1.00; P = 0.908

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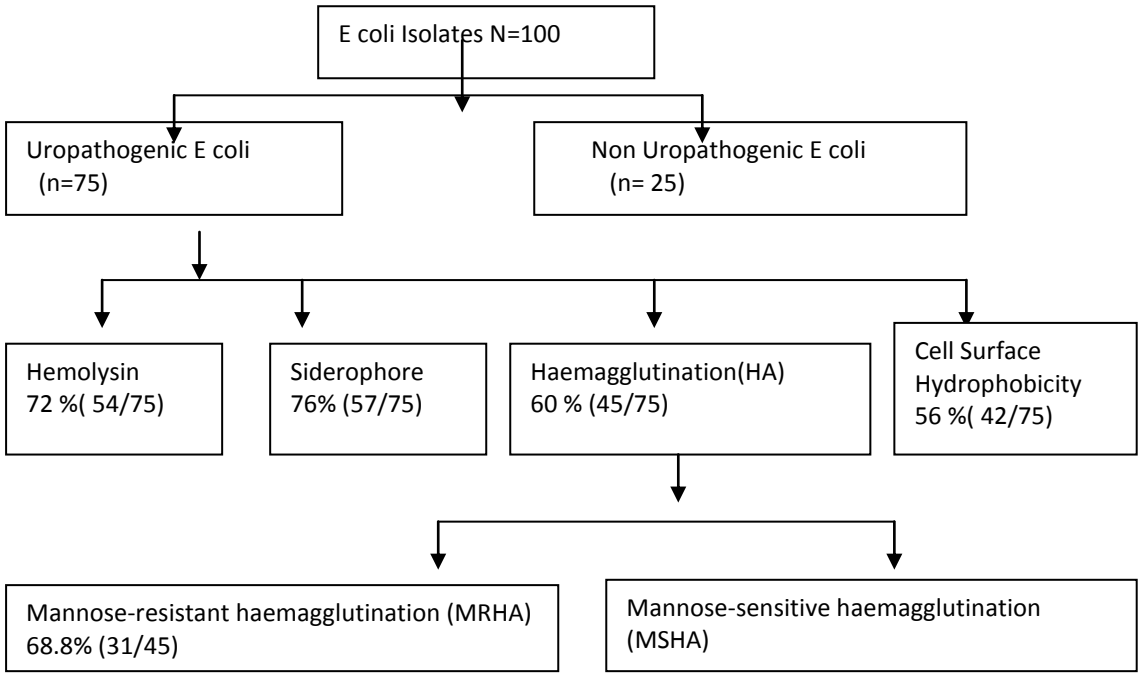
Flow Chart 1: Study of Virulence Factors



Flowchart 1 shows the study of total 100 E.coli isolates, among this 75% were UPEC and 25% were non-UPEC. The various VMs exhibited by UPEC isolates were shown. The Siderophore production was shown in 76 %(57/75) of isolates; followed by haemolysin (H) production in 72 %(54/75), haemagglutination (HA) in 60 %(45/75) and positive for cell surface hydrophobicity in 56 %(42/75) of the urinary isolates. Within HA producing UPEC, 68.8% (31/45) were Mannose-resistant haemagglutination(MRHA) and 31.2%(14/45) were Mannose-sensitive haemagglutination (MSHA).

Table 3: Antibiotic sensitivity pattern of E.coli and UPEC strains with its virulence markers

Drug(µg)	Sensitivity isolates of E.coli %	UPEC (%)	Virulence markers					
			H (%) N=54	CSH (%) N=42	SPA (%) N=57	HA (%) N=45	MRHA (%) N=31	MSHA (%) N=14
Ampicillin (10)	39	30 (40)	26(48.1)	12(28.6)	23(40.4)	17(37.8)	11(35.5)	6(42.9)
Amikacin (30)	88	68 (90.7)	50(92.6)	38(90.5)	51(89.5)	39(86.7)	27(87.1)	12(85.7)
Ciprofloxacin (5)	46	36 (48)	27(50)	22(52.4)	25(43.9)	24(53.3)	15(48.4)	9(64.3)
Norfloxacin S(10)	37	29 (38.7)	22(40.7)	16(38.1)	20(35.1)	19(42.2)	13(41.9)	6(42.9)
Gentamicin (10)	54	43 (57.3)	34(63)	21(50)	33(57.9)	25(55.6)	18(58.1)	7(50)
Co-Trimoxazole-(1.25/23.75)	38	29 (38.7)	22(40.7)	17(40.5)	22(38.6)	17(378)	12(38.7)	5(35.7)
Tetracycline (30)	21	16 (21.3)	14(25.9)	8(19)	12(21.1)	9(20)	6(19.4)	3(21.4)
	N=100	n=75						

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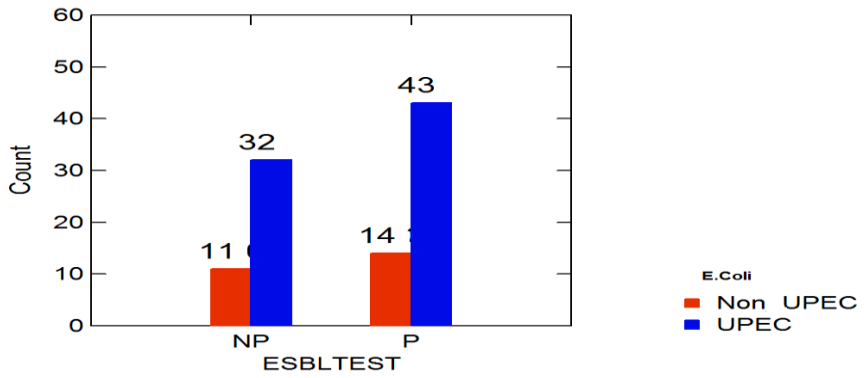
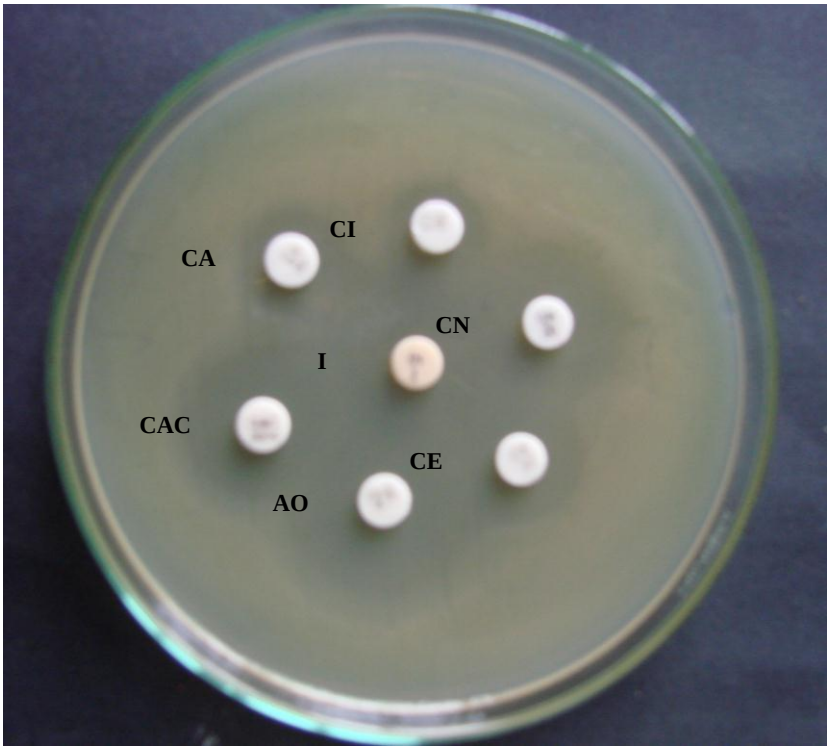
UPEC-Uropathogenic E coli
H-Hemolysin
HA- haemagglutination
CSH- cell surface hydrophobicity
MSHA -Mannose-sensitive haemagglutination
MRHA- Mannose-resistant haemagglutination
SPA –siderophore assay

Figure 1 shows ESBL analysis E.coli urinary isolates. Of which 57% showed ESBL production of which 75.44 % were UPEC and 24.66 % were non UPEC.

Figure 1: ESBL analysis



Photograph 1 shows ESBL detection using predictor Disc approximation method.



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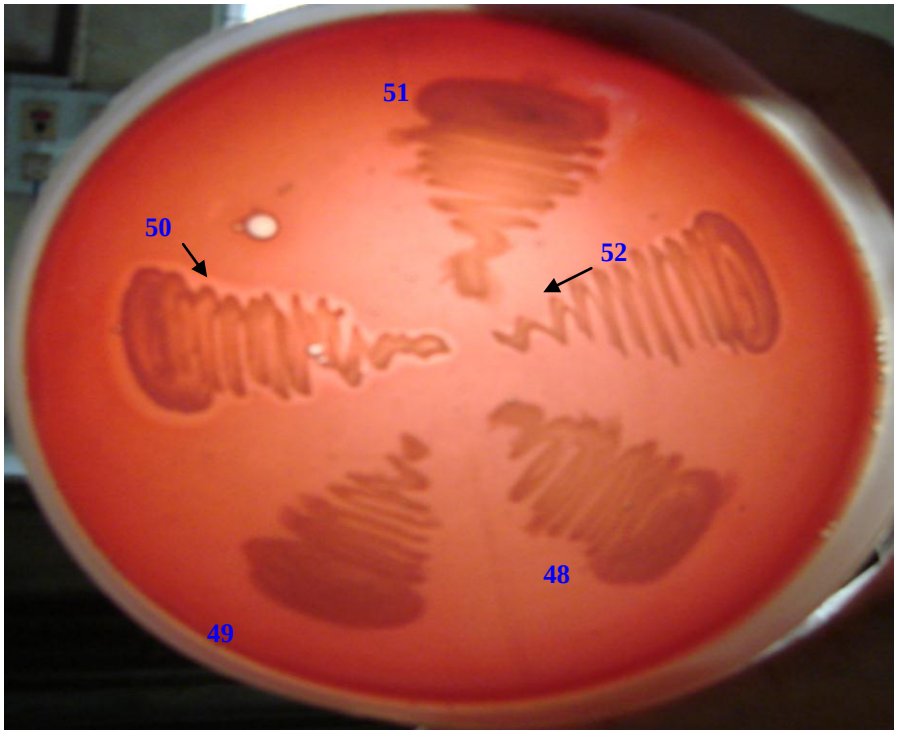
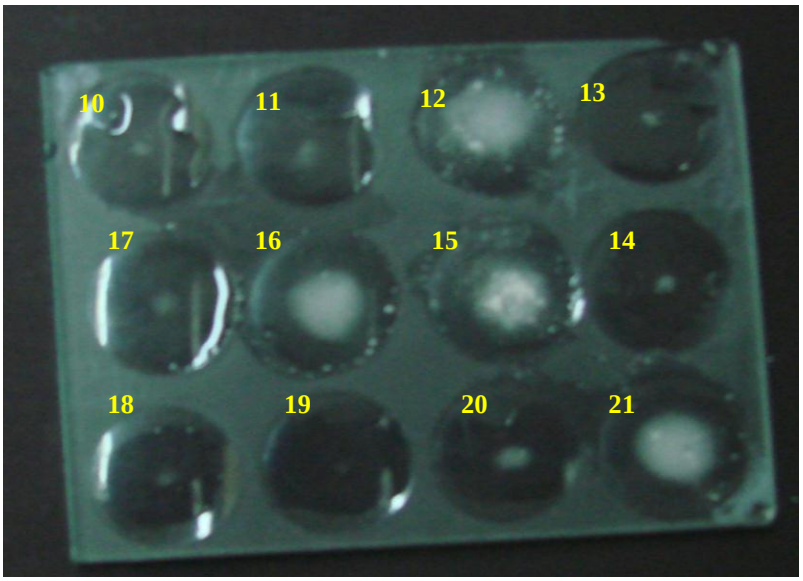
Photograph 2 shows H Test - haemolysin production in No. 50 and 52 E.coli isolates.



Photograph 3 shows CSH Test showing hydrophobicity in 12, 16, 15 and 21 E.coli isolates.



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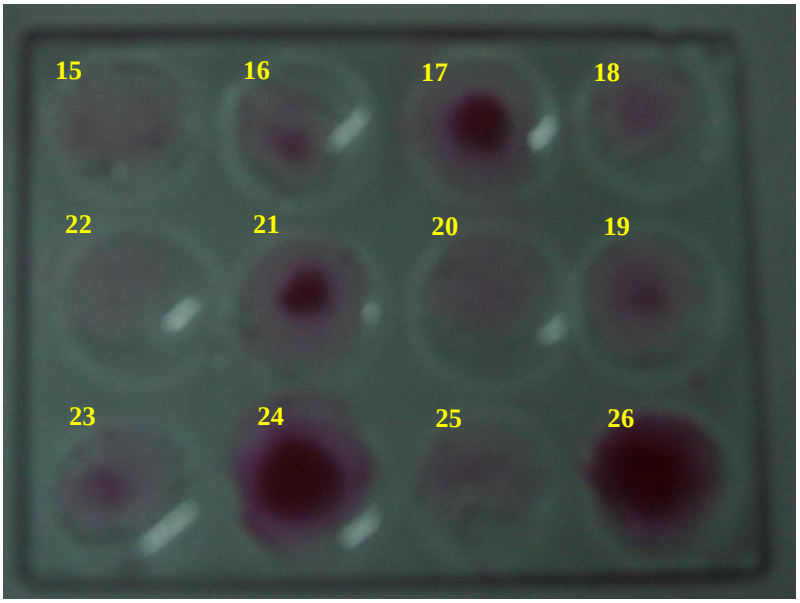
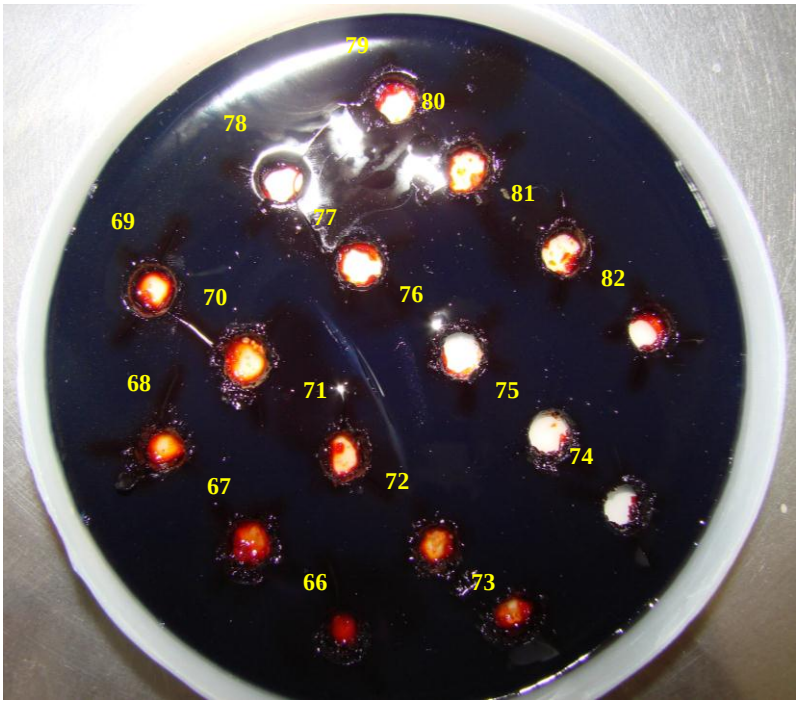
Photograph 4shows HA Test showing Haemagglutination in 16, 17, 21, 24 and 26 E.coli isolates.



Photograph 5 shows SPA Test - Siderophore production in 66,67,68,69,70,71,72,73, 76,77,78,79,80,81 & 82 E.coli isolates.



DISCUSSION

Majority of E.coli isolates were between the age group of 41 to 60 years, which falls in range of reproductive age, who are more prone for acquiring UTI. This study shows higher occurance of UTI in females. This finding correlates with Das NK et al [16] , Gales AC et al [17] and Modarres S et al [18]. This is because female have relatively short urethra and also it lies in close proximity to the warm, moist, perirectal region, which is teeming with microorganisms.

Among 100 E.coli urinary isolates, the prevalence of UPEC is 75 (75%) and showed one or more VMs, were designated as UPEC. The occurrence of multiple VMs in UPEC strains further strengthens the concept of association of UPEC with urinary pathogenicity. Many strains of *E.coli* associated with UTI produce siderophores (SPA). Bacterial siderophores compete for iron with host iron binding proteins. When bound by siderophore, the iron is taken up by special bacterial surface receptors and can be utilized by the pathogen, [19] so SPA showed in 76% of UPEC isolates. This correlates with Mandal P et al [20] 100%; Vagarali MA et al [14] 97.5% and Manjula A Vagarali [13] with 97.5%.

The haemolysin (cytotoxic necrotizing factor) detection (72%) is correlated with Silveria et al [10] (61.53%). And VMs like CSH (56%) are comparable with that of (56.36%) Raksha et al [1]. In hemeagglutination, MSHA (31.2%) are comparable with (32%) Yaseem Kausar et al [21], whereas MRHA detection (68.8%) 2 times higher in comparison with (30.9%) Raksha et al [1] and (30%)Yaseem Kausar et al [21].

In ASP study, *E coli* (88%) and UPEC (90.7%) showed maximum sensitivity to amikacin and exhibited least sensitivity to tetracycline accounting 21% and 21.3% respectively. Further study for ESBL detection, 57% were ESBL producers and among these UPEC (75.5%) and non UPEC (24.5%) isolates respectively.

CONCLUSION

UTI are treated empirically without culture and sensitivity resulting in the occurrence of higher prevalence of UPEC. This study emphasize on the need for routine screening for cost effective Virulence markers before starting a course of antibiotics and it facilitates rational use of antibiotics for UTI patients. So that further development of bacterial drug resistance is avoided.

Also further studies are needed for better understanding of interaction of different virulence markers at molecular level as most urovirulent strain express multiple virulence factors simultaneously.

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