International Journal of Clinical Obstetrics and Gynaecology

ISSN (P): 2522-6614
ISSN (E): 2522-6622

www.gynaecologyjournal.com
2018; 2(6): 31-42
Received: 21-09-2018
Accepted: 24-10-2018

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Characterization, prevalance and antimicrobial susceptibility pattern of bacterial uropathogens isolated from pregnant women at Lahore general hospital, Lahore, Pakistan

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Abstract
Urinary tract infection (UTI) is common in ladies living in developing countries which may progress to complications such as pyelonephritis and preterm delivery during pregnancy. The present study provides an insight for causative agent of UTI, their prevalence in pregnant ladies and its association with age, metabolic disorder and gestational period. Total of 375 midstream samples were collected from pregnant women, pure culture were segregated on selective media and identified through analytical profile index (API) to evaluate prevalence of uropathogens in UTI and ASB patients. Isolated uropathogenic *E. coli* were further characterized by polymerase chain reaction (PCR) using specific primers for genotype *cjrA, cjrB,* and *cjrC.* Among 375 midstream urine samples of pregnant women, 160 cases of UTI and ASB ($\geq 10^5$ CFU) were recorded. API analysis of such samples showed 65(40.6%), 55(34.35%) and 40(25%) of *E. coli*, *Enterococci* and *Staphylococci* respectively. Furthermore, PCR based characterization of *E. coli* revealed 42(64.61%) prevalence of both *cjrA* and *cjrB* genotype in asymptomatic and urinary tract infected patients. Prevalence of uropathogen in UTI suspected patients was found significantly higher in 20-40 age group 77(74.75%), diabetic patients 90(87.37%) and women reported in first trimester of gestation period 52(50.48%). Antibiotic susceptibility test results revealed that PCR confirmed uropathogenic *E. coli* was highly sensitive to Ceftriaxone, Amikacin, Nitofurantoin, Gentamicin and Ciprofloxacin with percentage of 79.2%, 76.4%, 75%, 61% and 52% respectively. Whereas, these isolates were resistant to Ampicillin 89%, Cefuroxime 70%, Amoxicillin 65%, Tobramycin 43% and Ceftazidime 22%.

Keywords: urinary tract infection, asymptimatic bacteriuria, uropathogenic *E. coli*

1. Introduction
Urinary tract infections are common problem in females and need serious attention particularly during pregnancy. It is the inflammatory response of epithelium of urinary tract to bacterial invasion, which is typically associated with bacteriuria and pyuria (1). Lower UTI (cystitis**)** was characterized with the symptoms of suprapubic pain or tenderness, urgency, frequency of urination or dysuria. Upper UTI (pyelonephritis) was diagnosed (without another obvious etiology) with or without urinary symptoms (2). In pregnant women Urinary tract infection (UTI) is a common health problem whose diagnostic process may get complicated as it can be either symptomatic or asymptomatic. Symptomatic bacteriuria is one of the fatal cause of premature birth, high fatality rate and postpartum complications. Urinary tract infections are common in female due to short urethra, its dilatation in various stages of pregnancy make it easy access to faecal flora [1]. Mostly 80-90% of infections caused by *Escherichia coli.* Other organism are *Klebsiella spp, Staphylococcus aureus. Pseudomonas spp* [3, 4]. and less common cause of UTI are *enterococci, Staphylococcus saprophyticus, Chlamydia spp, Candida albicans, Gardenerella vaginalis*. Colonization of group B streptococcus associated with preterm rupture of membrane and labour [5]**.** Urine in bladder is normally sterile [6] the presence of bacteria in urine is known as bacteriuria [7]. Significant bacteriuria is defined as presence of 10^5 colony forming units CFU per millilitre of urine [8]. Asymptomatic bacteriuria (ASB) is the presence of bacteria in urine without symptoms of acute urinary tract infection [9, 10]. Asymptomatic bacteriuria (ASB) commonly occurs in both pregnant and non-pregnant women [11]. The antibacterial location and position of urinary and reproductive genital in females make them prone to infection specially after conceiving. Uropathogen could have the ability to pose severe

threats in terms of creating micro changes in epithelium, inflammatory responses and invasiveness. Such conditions may lead to adverse gynaecological outcomes which includes low-birth weight, pre-mature birth and even high mortality rate. Furthermore, hormonal and anatomical changes during pregnancy and its effect on the dilatation of the ureters and renal pelvis may create ample space and time for uropathogen to get entered and cause infection. This combined with short nature of female urethra and perineal colonization by enteric organism, predispose to UTI and pyelonephritis [12-15].

It is widely accepted that uropathogenic *E. coli* (UPEC) originates from the distal gut microbiota [16-18]. To cause ascending UTI, it needs to overcome and adapt to different distinct host environments, such as the bladder, the kidneys and even the bloodstream. It is known that UPEC requires a combination of multiple virulence genes to cause infection[19]. The virulence gene combination of a UPEC strain may determine the pathogenesis process employed by this strain to cause infection.

Ascending infection of uropathogen has another prospect in development of cystitis and pyelonephritis. In either of the cases, involvement of UPEC make them complicated for the medical professionals to treat because of their versatility and high resistance of antibiotics. The severity of such complications can be controlled by screening symptomatic bacteriuria and giving prompt treatment before conceiving and during different stages of pregnancy [20]. Therefore, it is important to visit medical professionals for interpretation of urinalysis at different stages of pregnancy to prevent imperial treatment. Screening recommended is at first visit or between12-16 week of gestation [21].

2. Materials and Method

2.1 Study Design and Protocol

2.1.1 Sample Size

The sample size was calculated by using the formula of Kish & Lisle (1965) states that [22]

n = z2 p (1-p) / d2

Where, z = Score for 95% confidence interval = 1.96

p = Prevalence (To estimate the proportion of pregnant women with UTI)

d = Sampling error that could be tolerate= 5%.

1-p= Probability

Present study was conducted in co-ordination with Gynaecology and obstetrics department of Lahore General Hospital (LGH) located on Ferozpur road Lahore, Pakistan. The study population comprised of all pregnant women attending the antenatal outdoor patient department in hospital during study period. Five ml mid-stream urine samples were collected from each of the 375 candidate in sterile plastic container and stored at 4 °C (Fig. 9).

2.2 Isolation and Growth of Bacteria

Specified grams of CLED agar, nutrient agar, mannitol salt agar, eosine methylene blue agar were weighed using digital balance (Shimadzu Scientific Instruments-Japan) and suspended in 1000ml glass flask containing distilled water according to manufacturer instructions (Oxoid Ltd- England). Media was sterilized by autoclaving at 121°C for 15 minutes and mixed well before pouring. Urine samples were streaked with sterile inoculating platinum loop on surface of media and incubated at 37 °C for 24 hours (Fig. 10, 11).

2.3 Gram Staining

Every pure culture grown on the surface of selective medium showing standard characteristics colony were selected for Gram Staining. It was performed according to standard procedure. Smear was established by placing a loopful of culture on microscope glass slide re-suspended with drop of distilled water in circular pattern. The smear was fixed by passing 3-4 times on flame followed by heat air drying. Straining was performed according to the method described by Bergey [23] (Fig. 12).

2.4 Biochemical Assay

The analytical profile index of Gram negative rods and Gram positive cocci isolated from urine samples was determined through RapID One Pannel (Remel-Thermo Fisher Scientific, USA) and API 20 (Biomerieux-USA), following instructions of the manufacturer (Fig. 13, 14).

2.5 Polymerase Chain Reaction (Pcr)

All the samples including positive known bacteria were subjected for extraction of DNA by using QAIamp DNA Mini kit (QIAGEN-Germany). The extracted DNA of the samples were subjected to PCR for amplification of the desired genes *cjr*A, (223bp) *cjr*B (397bp) and *cjr*C (513bp) of uropathogenic *E. coli*. For the PCR reaction, 2 µl of 10X PCR buffer, 1 µl of forward and reverse primer, 2 µl of 80mM $MgCl_2$, 2µl of DNTPs mixture and 1 µl of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were added to the reaction mixture. Reaction volume was obtained by adding 9 µl of Nuclease-free Water and 2 µl of extracted DNA sample. The PCR tubes were incubated in thermal cycler (Applied Biosystems) with an initial denaturation of 94 °C for 2 mints. Followed by 30 cycles of denaturation at 94 °C for 30 secs, annealing at 56 °C for 30 secs. and extension at 72 °C for 45 secs. Final extension was conducted at 72 °C for 5 minutes (Fig. 15, 16).

Table I: Sequences of Oligonucleotide primers *E. coli.* Gene Primers Amplicon size Sequences

***cjr*A**	Forward Primer Reverse Primer	223bp AAAGGGTGGTCCTGGGAGAT ACGTCAGTTGCTGGCTTTCA
***cjr*B**	Forward Primer Reverse Primer	397bp CGAAGTTCAGCCCGCTATGT GCRRRCCCAAGATGCCTCAG
***cjr*C**	Forward Primer Reverse Primer	518bp AAACCTCAGCGCAAAATCGT AGGCTTCAGGAATGGGTTCA

2.6. Culture Sensitivity Test

Antimicrobial susceptibility test (AST) was performed for all the isolates using Kirby-Bauer's disk diffusion method described by Bauer *et al*., 1966. All procedures were done as recommended by Clinical and Laboratory Standard Institute (CLSI, 2012). Individual colonies were suspended in normal saline to 0.5 McFarland and using sterile swabs the suspensions were inoculated on Muller Hinton agar for 18-24hr. Antibiotics tested for susceptibility were Ampicillin (10 µg), Amikacin (30 µg), Cephradine (10 µg), Ceftriazone (30 µg), Nitrofuration (5 µg), Amoxicillin (25 µg), Ciprofloxacin (30µg), Tobramycin (30 µg), Gentamicin (10 µg), Ceftazidime (30 µg), Cefuroxime (30 µg) and Erythromycin (5 µg) (Fig. 17, 18).

3. Result

The samples collected from pregnant women attending outpatient departments (OPD) of LGH were categorized into significant ($\geq 10^5$/ml CFU) and non-significant ($\leq 10^5$/ml CFU) groups. It was recorded that out of 375 samples, 160 (42.66%) were declared as significant and 215 (57.33%) were identified as non-significant (Fig. 1). However, among 160(42.66%) confirmed bacteriuria samples, 103 (64.37%) were graded as the

cause of UTI as compare to the rest of 57 (35.62%) samples declared as asymptomatic bacteriuria (Fig. 2)

Overall prevalence of UTI patients in significant samples based on colony characteristics, Gram staining, microscopy and analytical profile index (API) analysis of each isolate revealed that *E. coli*, *Enterococci* and *Staphylococci* were 42 (40.77%), 35 (33.98%) and 26 (25.24%) respectively (Fig. 3). PCR amplification of the extracted DNA of each UPEC showed percentage presence of *cjr*A 35(83.33%) and *cjr*B 35(83.33%) as compare to *cjr*C (0%) which did not show amplification for either of the samples.

Analytical profile index (API) analysis of 103 UTI suspected patients in age group 20-40 showed highest prevalence of *E. coli* 31 (40.25%) followed by *Enterococci* 26 (33.77%) and *Staphylococci* 20 (25.97%). Whereas PCR based molecular analysis confirmed highest prevalence of *cjr*A and *cjr*B 28 (90.32%) of uropathogenic *E. coli* in same group of patients (Fig. 4).

Significant bacterial count 32 (56.14%) was recorded in urine sample of 20-40 years asymptomatic age group. Out of these positive samples 13 (40.62%), 11 (34.37%) and 8 (25%) were declared as API confirmed *E. coli*, *Enterococci* and *Staphylococci* respectively. However, 5 (38.46%) Uropathogenic *E. coli* genotype *cjr*A and *cjr*B were detected positive through PCR (Fig. 4).

On the basis of metabolic disorder, Out of 103 samples 90 (87.37%) diabetic and UTI suspected patients were showed significant prevalence of *E. coli* 37 (41.11%), *Enterococci* 31 (34.44%) and *Staphylococci* 22 (24.44%) in analytical profile index analysis. Whereas the prevalence of virulence genes *cjr*A and *cjr*B of UPEC in diabetic patients were 19 (51.35%) (Fig. 4).

Moreover, the urine samples of diabetic pregnant women suspected for ASB showed significant bacterial count 53 (92.98%) for API analysis. The percentage prevalence is 22 (41.50%), 18 (33.96%) and 13 (24.52%) for *E. coli*, *Enterococci* and *Staphylococci* respectively. PCR based identification of Uropathogenic *E. coli* gene *cjr*A and *cjr*B were positive for 5 (22.72%) samples in ASB diabetic patients (Fig. 5).

In UTI suspected patients, analytical profile index based analysis revealed that significant bacteriuria 52 (50.48%) was observed in pregnant women of first trimester. The positive samples showed high prevalence of *E. coli* 21 (40.38%) followed by *Enterococci* 18 (34.61%) and *Staphylococci* 13 (25%). However, 19 (90.47%) were positive for UPEC virulence gene through PCR (Fig. 6).

Furthermore, 38 (66.66%) urine samples of ASB pregnant women first trimester were recorded for significant bacterial count having percentage positivity of *E. coli* 16 (42.10%), *Enterococci* 13 (34.21%) and *Staphylococci* 9(23.68%) in analytical profile index analysis. ASB pregnant women with first trimester showed highest prevalence, the frequency of UPEC gene *cjr*A and *cjr*B was 5 (31.25%) (Fig. 6).

The PCR confirmed uropathogenic *E. coli* showed varying susceptibility patterns to 12 antimicrobial drugs as shown in Table-2. The results revealed that the isolate was highly sensitive to Ceftriaxone 79.2%, Amikacin 76.4%, Nitofurantoin 75%, followed by Gentamicin and Ciprofloxacin with percentage of 61% and 52% respectively. It showed low susceptibility to Erythromycin 18.2% and Cephradine 8.7% Whereas, the isolated *E. coli* was highly resistant to Ampicillin 89%, Cefuroxime 70%, Amoxicillin (65%), Tobramycin (43%) followed by Ceftazidime with percentage of 22.

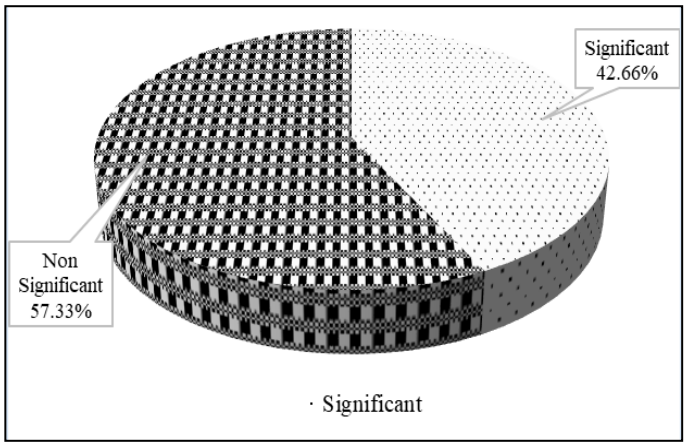


Fig 1: Overall prevalence of significant and non-significant cases

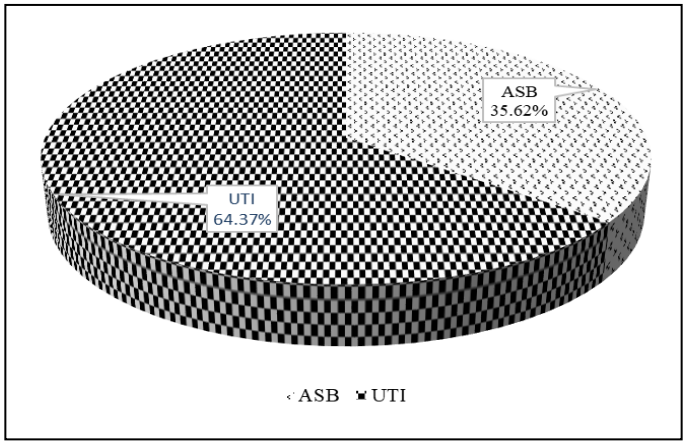


Fig 2: Overall percentage prevalence of UTI and ASB

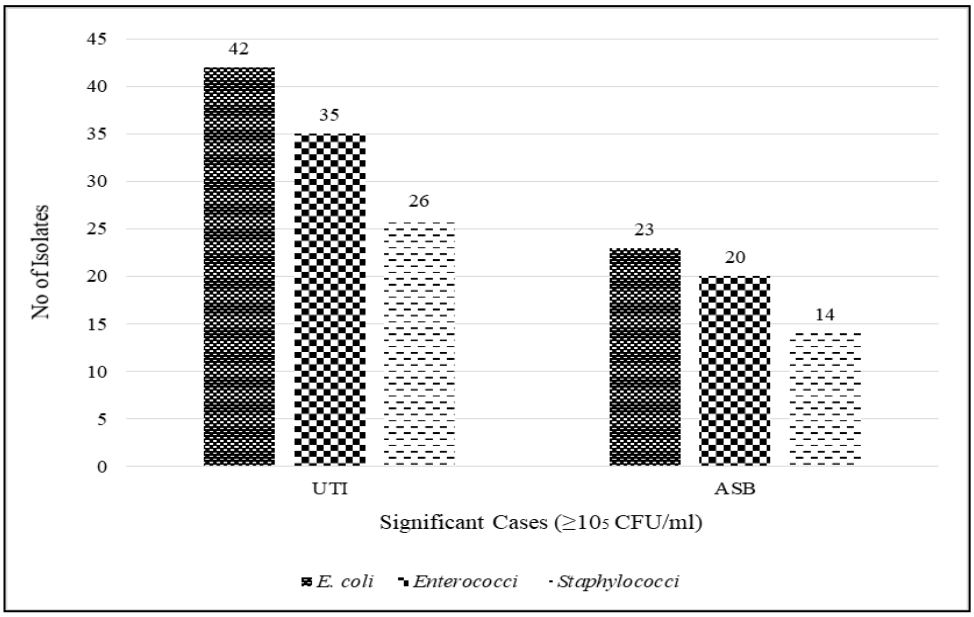


Fig 3: Prevalence of Uropathogens *Enterococci, Staphylococci* and *E. coli* in UTI and ASB patients

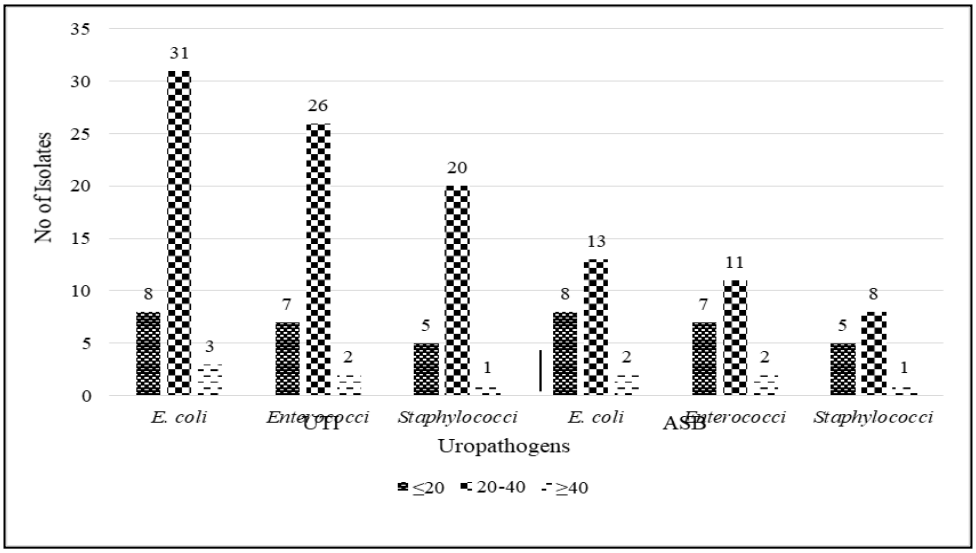


Fig 4: Age based prevalence of Uropathogens in Significant cases

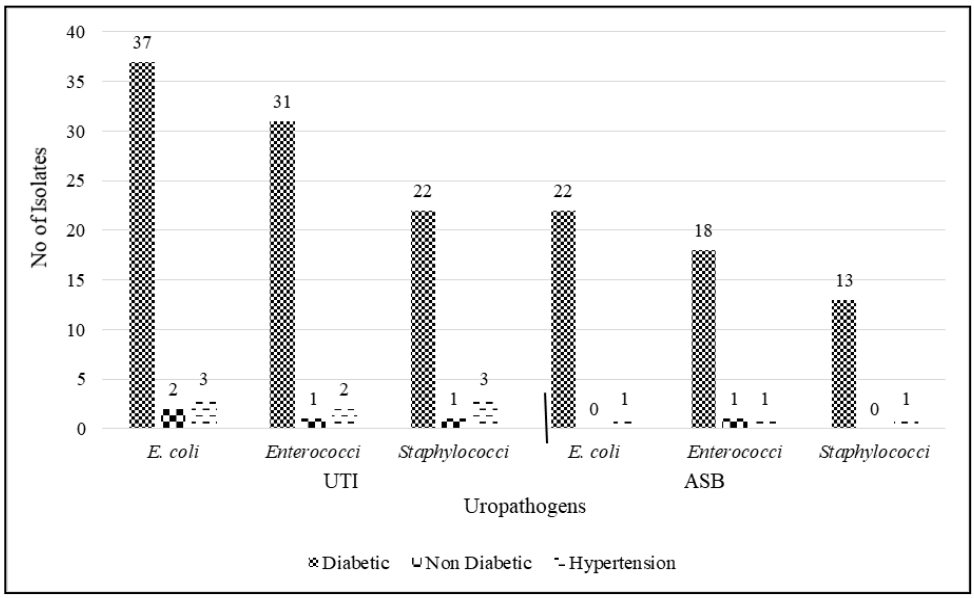


Fig 5: Metabolic disorder based prevalence in UTI and ASB

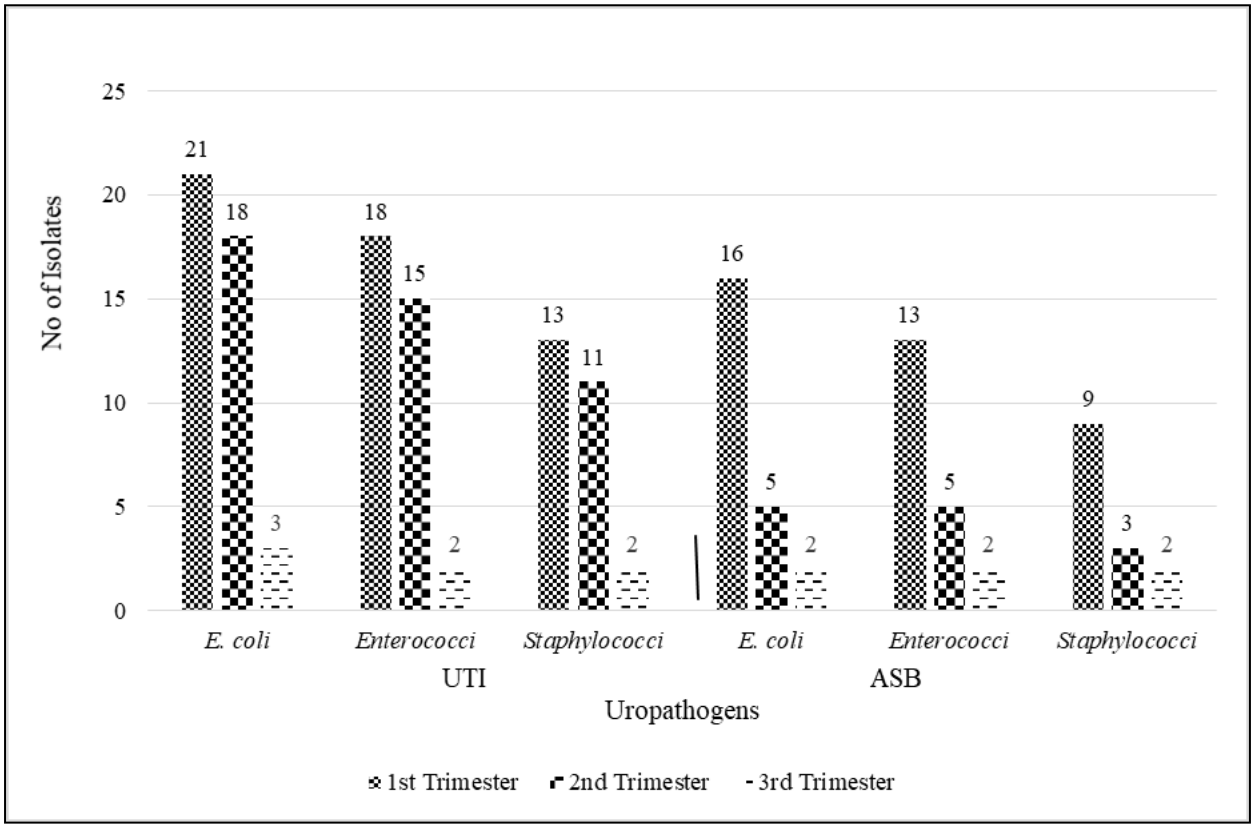


Fig 6: Gestational period associated with prevalence of Uropathogens

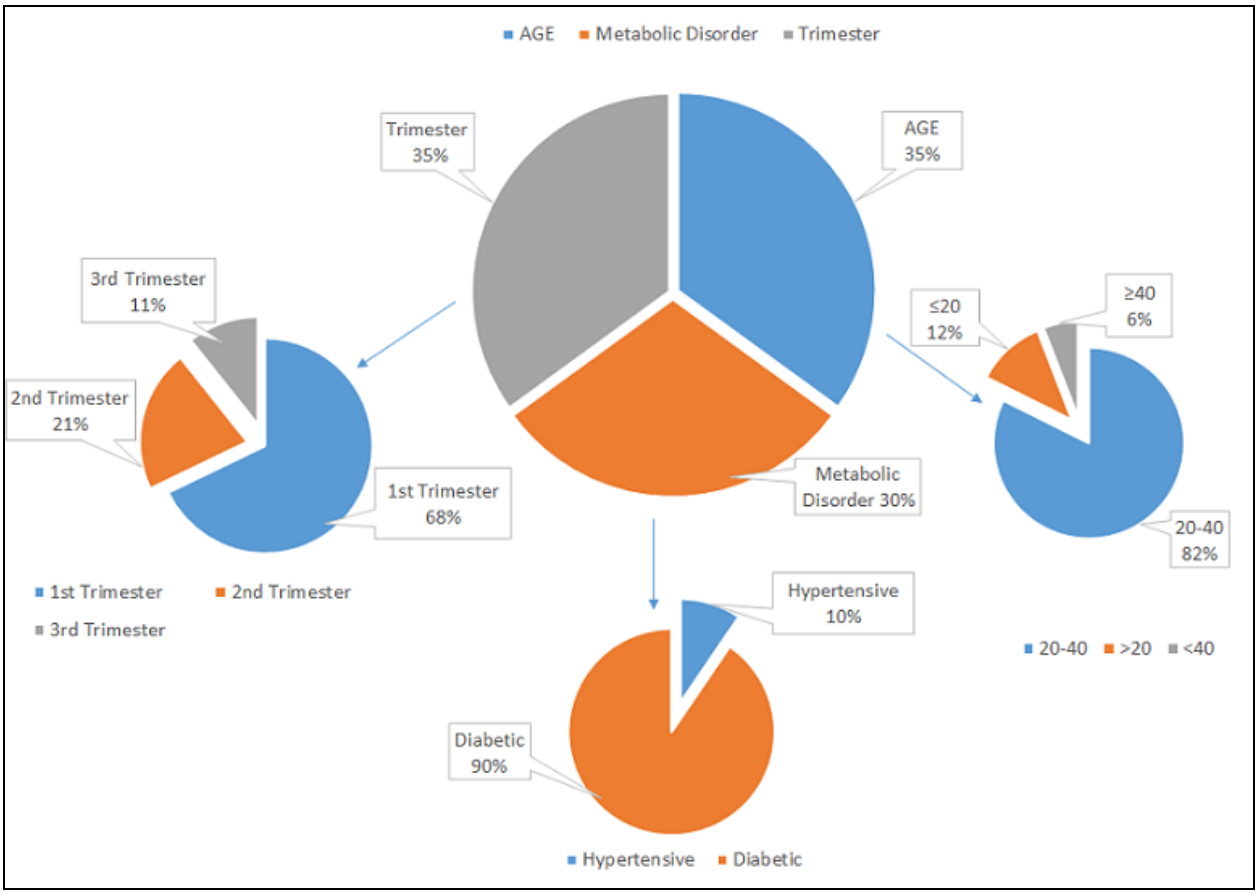


Fig 7: Genotype prevalence of UPEC associated with age, metabolic disorder and gestational period in UTI patients

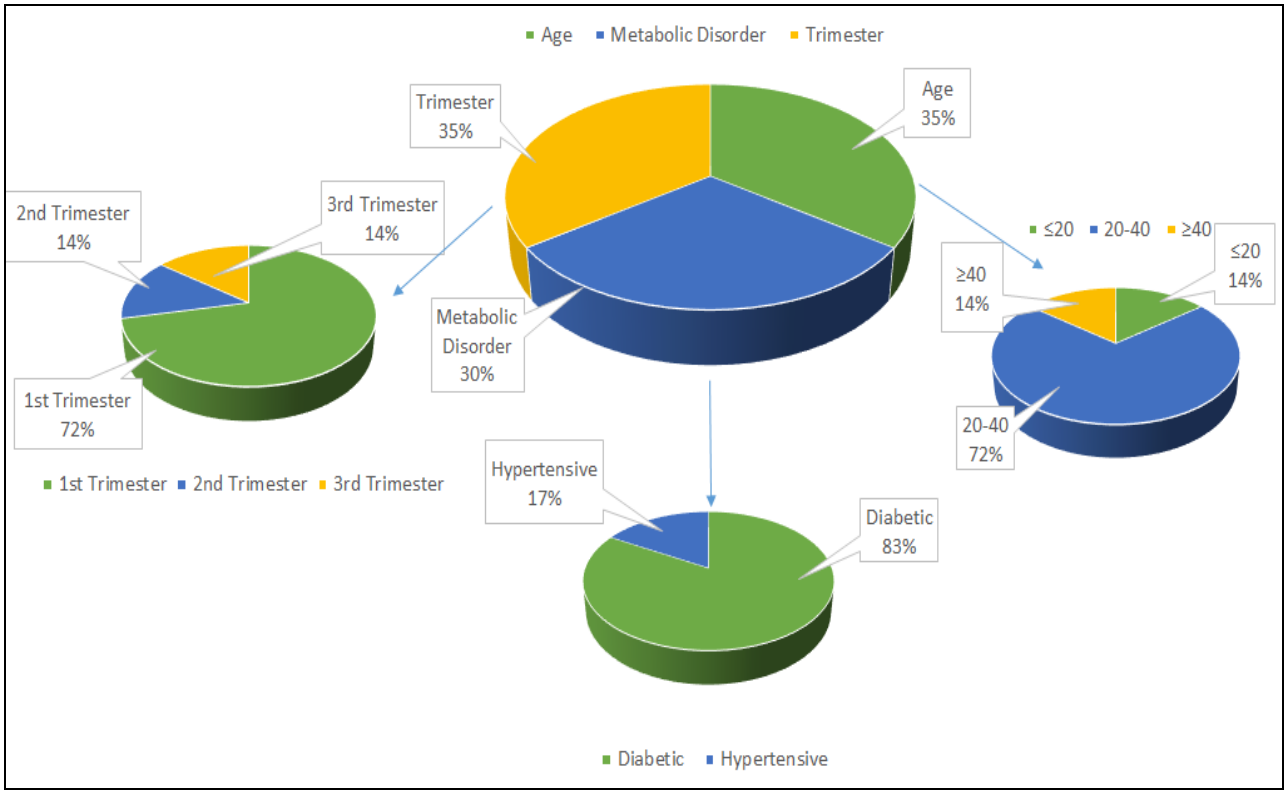


Fig 8: Prevalence of *E. coli* virulence gene *cjrA* and *cjrB* associated with age, metabolic disorder and gestational period in ASB patients.

Table 2: Anti-biogram of Uropathogenic *E.coli* isolated from UTI patients

Antibiotics (µg)	Antibiotic susceptibility pattern of isolated Uropathogenic *E. coli*	
	Sensitive	Resistant
Ceftriaxone (30µg)	79.2%	20.8%
Amikacin (30µg)	76.4%	23.6%
Gentamicin (10µg)	61%	39%
Nitrofurantion (5µg)	75%	25%
Ciprofloxacin (30µg)	52%	48%
Erythromycin (5µg)	18.2%	81.8%
Ampicillin (10µg)	11%	89%
Ceftazidime (30µg)	88%	22%
Cephardine (10µg)	8.7%	9I.3%
Amoxicillin (25µg)	35%	65%
Tobramycin (30µg)	57%	43%
Cefuroxime (30µg)	30%	70%

Fig 9: Collection of Urine Samples from UTI and ASB patients

Fig 10: Media Preparation, Pouring and isolation of Uropathogens on Nutrient and Selective Medium

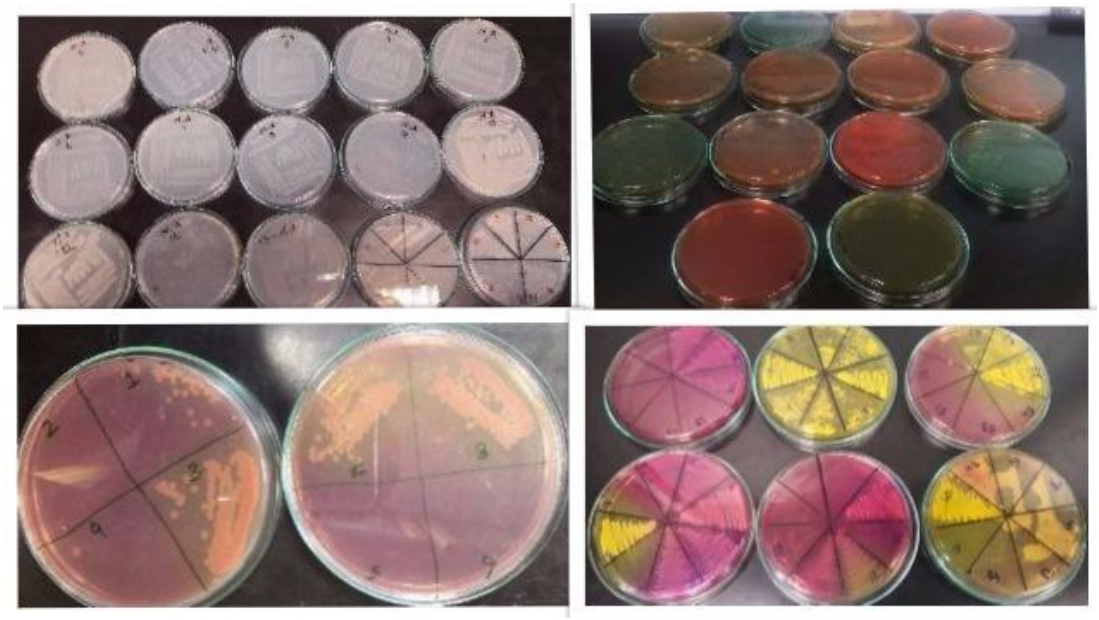


Fig 11: Isolation and growth of different bacteria on Selective Medium

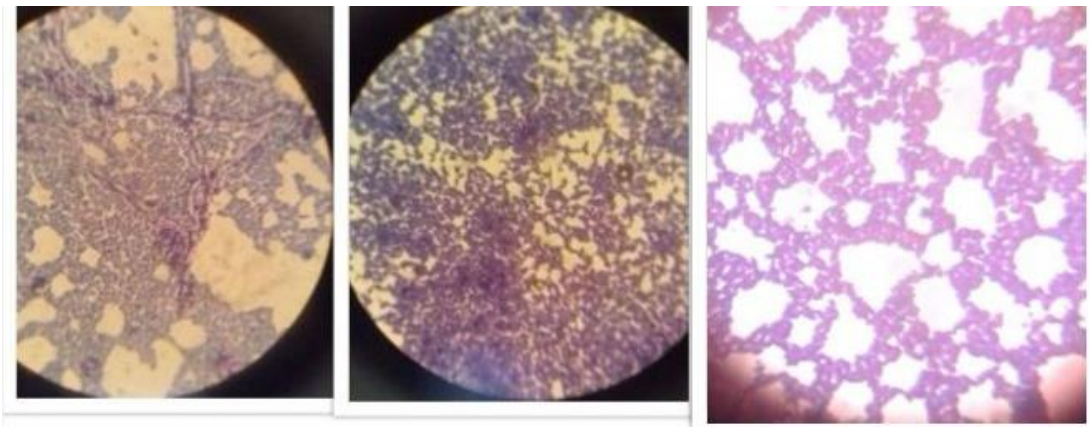


Fig 12: Gram staining and Microscopy

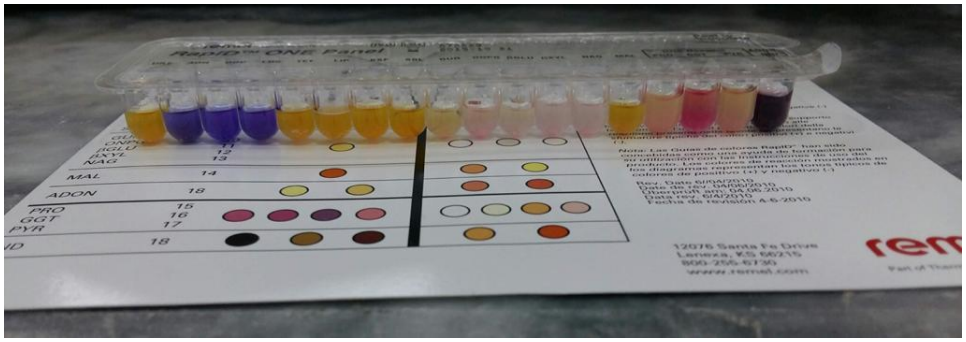
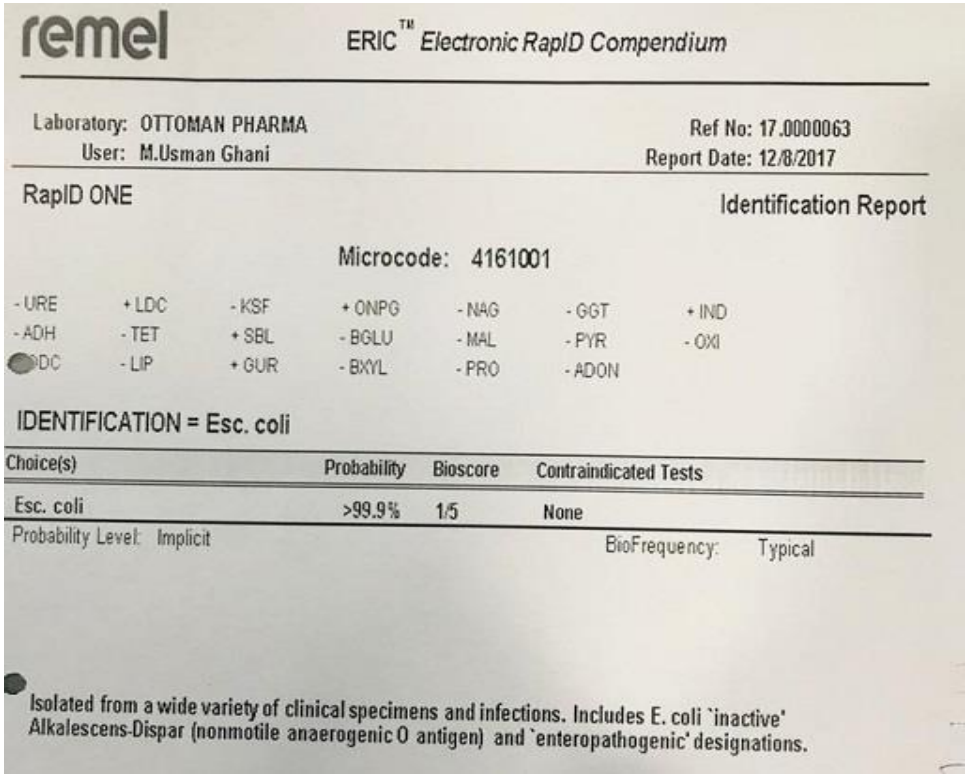


Fig 13: Analytical Profile Index Kit



remel ERIC™ *Electronic RapID Compendium*

Laboratory: OTTOMAN PHARMA
User: M.Usman Ghani

Ref No: 17.0000063
Report Date: 12/8/2017

RapID ONE — Identification Report

Microcode: 4161001

- URE	+ LDC	- KSF	+ ONPG	- NAG	- GGT	+ IND
- ADH	- TET	+ SBL	- BGLU	- MAL	- PYR	- OXI
- DC	- LIP	+ GUR	- BXYL	- PRO	- ADON	

IDENTIFICATION = Esc. coli

Choice(s)	Probability	Bioscore	Contraindicated Tests
Esc. coli	>99.9%	1/5	None

Probability Level: Implicit — BioFrequency: Typical

Isolated from a wide variety of clinical specimens and infections. Includes E. coli `inactive' Alkalescens-Dispar (nonmotile anaerogenic O antigen) and `enteropathogenic' designations.

Fig 14: Analytical Profile Index Report

Fig 15: Polymerase chain reaction of Extracted DNA and Electrophoresis analysis of PCR product of isolated Bacterial Uropathogens

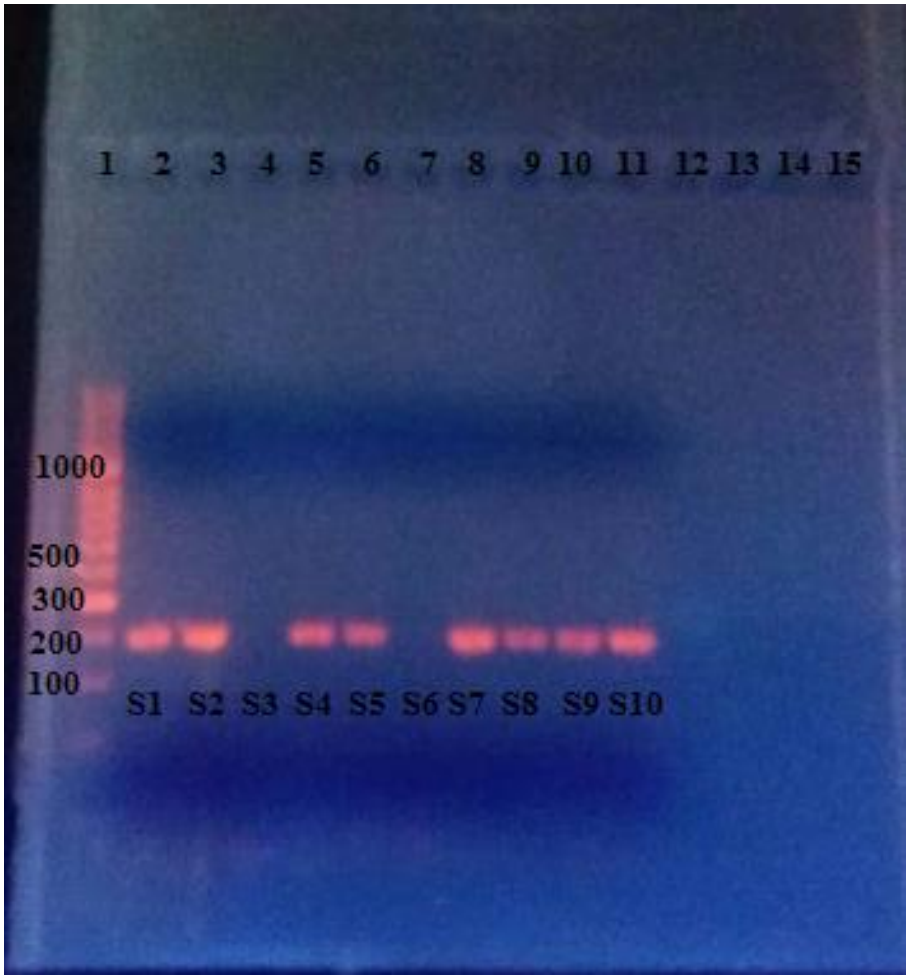


Fig 16: Electrophoresis analysis of PCR products

Fig 17: Kirby-Bauer's disk diffusion method for Antimicrobial susceptibility test

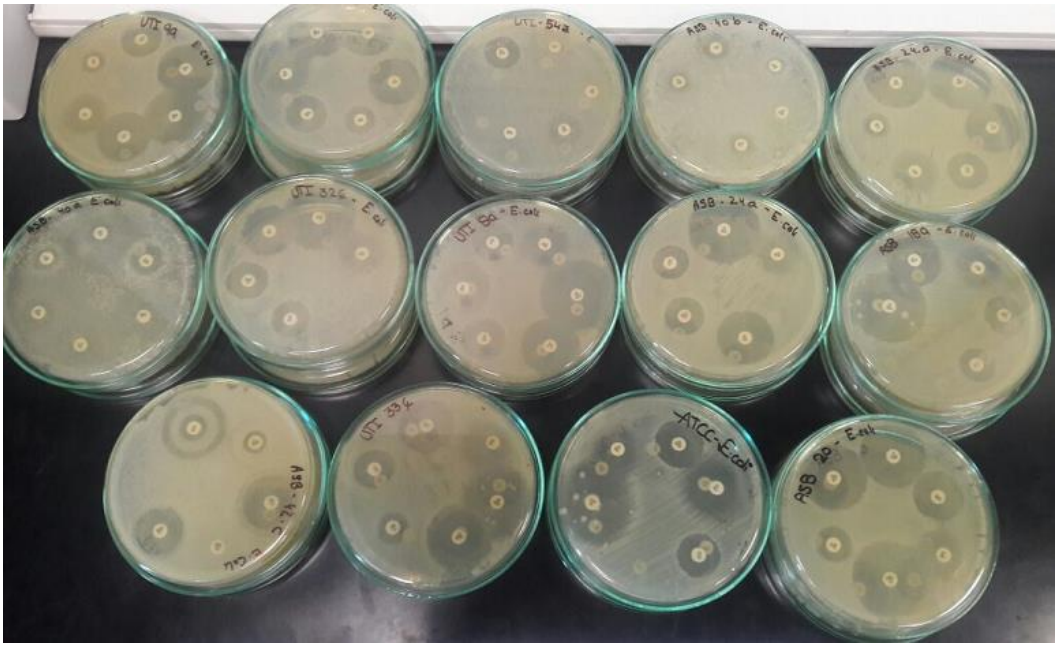


Fig 18: Anti-biogram of Uropathogenic *E. coli* isolated from patients

4. Discussion

In the current study symptomatic and asymptomatic pregnant women attended antenatal outdoor patient department of LGH, Pakistan were screened for the prevalence of uropathogens. The pure cultures grown on the selective medium were identified through commercially available API kit showed percentage prevalence of *E. coli* 40.62% followed by *Enterococcus* 34.37% and Staphylococcus 25%. The *E. coli* isolates were further analyzed for their genotypes using specific primers for UPEC *cjrA*, *cjrB* and *cjrC*. Furthermore, prevalence of bacteriuria in pregnancy is co-related with their associated risk factors such as age, metabolic disorders and gestational period were documented which contribute UTI among pregnant women.

The overall prevalence of significant bacterial cases ($\geq 10^5$CFU/ml) among 375 samples documented in the current study was 42.66%. Whereas, the prevalence of symptomatic and asymptomatic bacteriuria was found to be 64.37% and 35.62% respectively. The results collaborate with Nasir Uddin and Khan who observed that prevalence of symptomatic bacteriuria is significantly higher as compare to asymptomatic bacteriuria [24]. Whereas, percentage of symptomatic bacteriuria is significantly lower in developed countries. This variation may be due to the differences in the environments, social habits of the community, socio-economic statuses and the standards of personal hygiene[25].

In this study, gram-negative bacteria such as *E. coli* in UTI suspected pregnant women was more prevalent (40.77%) than gram-positive bacteria including *Enterococci* (33.98%) and *Staphylococci* (25.24%). Similar findings have been reported in Tanzania and Tikur Anbessa Specialized Hospital Addis Ababa, Ethiopia [26, 27]. *E. coli* was the most predominant bacteria isolated from all UTI cases, similar results were reported in Nigeria, Sudan, and Yemen [28-30]. *E. coli* is considered uropathogenic due to a number of virulence factors specific for colonisation and invasion of the urinary epithelium, such as the P-fimbria and S-fimbria adhesions[31]. *Enterococci* and *Staphylococci* were the second and third most predominant pathogen isolated.

Genotypic prevalence of *cjrA* and *cjrB* of uropathogenic *E. coli* was compartively higher (64.61%) than *cjrC* (0%) in all UTI and ASB patients. Moreover, the high prevalence of *cjrA* and *cjrB* (83.33%) in UTI patients revealed its virulence nature in the form of clinical presentation in terms of typical signs and symptoms. It is reported that the *cjrA, cjrB* and *cjrC* gene is predicted to be involved in iron acquisition, which may contribute to urovirulence.

Prevalence of *E. coli* in UTI suspected pregnant women based on age groups, individuals of the 20-40 years age group had comparatively high incidence of infection (40.25%) than age group ≥40 years and ≤20 years. Akobi *et al*., (2014), recorded similar observation. Whereas, our findings contradictory to the observations of Kawser *et al*., (2011) in which the age group 21-25 showed highest ranked of infection. This divergence could be due to high sexual activity and multiparous pregnancy.

The prevalence of symptomatic urinary tract infections in female diabetic patients has been reported as increased [32, 33] compare to non-diabetic individuals. The mechanisms for the greater susceptibility of the diabetic urinary tract to infection include decreased antibacterial activity of the urine as a result of dilution of inhibitory substances such as urea, defects in polymorphonuclear leukocyte function or cellular immunity as a result of hyperglycaemia and increased adhesive capacity of bladder epithelium [34]. On the basis of metabolic disorder in the current study, incidence of *E.coli* in diabetic pregnant women having UTI infection was significantly higher (41.11%) than Non-diabetic and hypertension individuals. Gestational diabetes mellitus (GDM) complicates up to 5% of pregnancies and has been associated with an increased risk of both fetal and maternal morbidity[35]. In another recent study, the prevalence of UTI was significantly increased in women with GDM as compared to non-diabetic pregnant women [36] which support the finding of our study. Similarly, the study conducted by Hani Faidah reported that diabetes represent the most influencing factor for UTI in pregnant women followed by hypertension [37].

In current study, symptomatic pregnant women in first trimester showed the high frequency of *E. coli* (40.38%), complemented by second and third trimester. This is in line with the observations Yahodara and Turpin who reported 40.08% of women are infected by uropathogens in first trimester of gestation period [38, 36]. The higher incidence of symptomatic bacteriuria in first trimester might be caused by hormonal changes occurring prior to occurrence of anatomical changes. Unexpectedly, Roy and Nath declared the second and third

trimesters respectively as having the greatest rate of infection, which do not counteract the current situation [39, 40].

Selection of appropriate antibiotic and effective dosage after early detection is vital to limit the infection. The frequent bacterial isolate recovered from the urine samples of symptomatic pregnant women is the *Escherichia coli*. The results are similar to the observations reported by Ahmed showed that the PCR confirmed UPEC were sensitive to Ceftriaxone Amikacin, Nitofurantoin, Gentamicin and Ciprofloxacin whereas, the isolates were highly resistant to Ampicillin, Cefuroxime, Amoxicillin, Tobramycin and Ceftazidime [41].

The contradiction might be due to antibiotic resistant pattern could be generated due to self and discriminated use of antibiotic. The resistance to these drugs is an indication of earlier exposure of the isolates to these drugs, which may have enhanced resistant development. These drugs are very common due to low cost and often purchased without prescription in different areas.

An irrational and unnecessary use of antibacterial agents can result in the emergence of bacterial strains that exhibit multidrug resistance[42]. In the present study most of the isolated pathogens showed multiple drug resistance of two and more antibacterial agents tested. Similarly, multidrug resistances in bacterial uropathogens were reported [43-45]. It suggests a need for continuous monitoring of uropathogens in pregnant women and antibiotic susceptibility testing before antibiotic prescription in order to ensure adequate treatment of urinary tract infection.

5. Conclusion

Symptomatic bacteriuria is common in pregnant women that progress to gynecological complications and posing great risk to the mother and child. Analytical profile index is valuable technique to identify the causative agent however, identification of UPEC could be more precisely confirmed through using PCR for better understanding of urinary tract infections. Irrespective of age, association of gestation period and influence of metabolic diseases, UTI cases were recorded in all categories. Therefore, periodic physical examination and urinalysis should be done of symptomatic and asymptomatic antenatal women. Whereas, culture shall be tested for antibiotic sensitivity before antibiotic prescription or empirical treatment in order to minimize the adverse effect on mother and child. Regardless of the category, all the E. coli are sensitive to Ceftriaxone, Amikacin, Nitofurantoin, Gentamicin and Ciprofloxacin while showed resistant to Ampicillin, Cefuroxime, Amoxicillin, Tobramycin and Ceftazidime. It is suggested that pregnant women shall be screened and treated with sensitive antibiotics in first trimester. Huge number of antibiotics showed resistance during antibiotic susceptibility test therefore, it is dire responsibility on the shoulders of drug regulatory bodies to take control over manufacturing and distributing channels in the best interest of community.

6. Acknowledgement

I wish to thank various people for their contribution to this research work Director of Ottoman Pharma, Pakistan for providing financial and technical support; Dr. Shumaila and Dr. Fadia, Medical officers of LGH, for their help in collecting the samples and all the technicians who helped me in procedural execution.

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