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Molecular Characterization of Uropathogenic Markers in *Escherichia coli* Isolated from Pregnant Women having Urinary Tract Infection

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Abstract: Uropathogenic *Escherichia coli* (UPEC) is one of the most frequent causative organism causing urinary tract infections which is a common risk factor during pregnancy. The present study was carried out to detect the uropathogenic mackers in *E. coli* causing UTI among pregnant women. A total of 300 samples were investigated from pregnant women attending different antenatal clinics at Bhubaneswar and Puri. In the present study, *E. coli* was found to be the highest predominant uropathogen (74.3%) and thus the identified *E. coli* isolates were screened for characterization of uropathogenic factors by recommended conventional and molecular methods. A series of tests were performed for the detection of virulence related genes including pathogenecity island, hemolysin production, 'cytotoxic necrotizing factor- 1', 'S-fimbrial adhesion', afimbrial adhesion, type P frimbriae, type I frimbriae, serum resistance associated etc. among the *E coli* isolates.

Key Words: Urinary tract infection, Uropathogenic *Escherichia coli* (UPEC), Uropathogenic Markers,.

1. INTRODUCTION

UTIs have been observed as the most common bacterial infection by many physicians, especially in case of women (Winickoff *et al*., 1981). UTIs affect 10-20% of women in United States annually and account for million office visit per year. Outpatient expenditures for patients with UTI in US approach one billion dollars (Powers, 1991).

UTI is one of the most recurrent medical complications during pregnancy. Risk for development of acute pyelonephritis, preterm birth and unexplained peri-natal death may increase in the bacteriuric women due to the physiological changes, both hormonal and mechanical. The contributing factors which increase the severity of disease include dilation of the ureters and renal pelvises, increase in urinary pH and decrease in the ureteric muscle tone. The moist environment of the female peritoneum favors microbial growth and predisposes females to bladder contamination (Ebie *et al*., 2001).

Bacterial infections are mediated through certain steps like adhesion to the host epithelial cells, colonization, proliferation, secretion of enzymes or toxins, utilization of metabolites and finally destruction of host tissue resulting in pathogenesis (Ira *et al*., 2013). Each organism has its unique mechanism of invasion and pathogenesis and it is mediated by several virulence factors. The presence of *E. coli* can cause a multitude of infections, especially if it penetrates into unnatural sites. Amongst them, the serotypes of *E. coli* related to pathogenicity in urine are medically termed as Uropathogenic *E. coli* (UPEC) (Raksha *et al*., 2003).

E. coli relies on a number of pathogenic factors to cause extraintestinal infections. These factors assist *E.coli* in surviving hostile conditions and include ability of *E.coli* to adhere to uroepithelial cells, haemagglutination, serum resistance, hemolysin production, cell surface hydrophobicity, siderophore formation and gelatinase production and others (Boon *et al.*, 2006; Raksha *et al.*, 2003).

Adherence, a process that appears to promote UPEC survival within the urinary tract, stimulates UPEC entry into host epithelial cells (Bower *et al*., 2005). The primary adherence factors encoded by UPEC, and many other microbes, are supra-molecular, filamentous adhesive organelles known as pili or fimbriae. Adherance is the single most important marker that causes dramatic progression of UTI to pyelonephritis (Johnson, 1991).

"Virulence factors are encoded by genes clustered on so-called pathogenicity islands," (Hacker *et al.*, 1990). In addition, other genes may be part of particular regions on the bacterial chromosomes termed pathogenicity island (a pathogenicity island enables bacteria to induce disease), which are present in the

genome of pathogenic strains of given species but absent in the non-pathogenic variants of species (Bingenbidois *et al.,* 2002; Skyberg *et al.*,2006).

Common adhesive organelles elaborated by UPEC are type 1, P, S, and F1C pili encoded by the fim, pap,and sfa operons respectively. Individual UPEC genomes can carry >10 fimbrial gene clusters, the majority of which have been characterized in only brief detail, making the contribution of each pilus type to UPEC virulence difficult to distinguish (Miyazaki *et al*., 2002; Snyder *et al*., 2005; Snyder *et al*., 2006).

The UPEC strains harbor various virulence factors that aid the progression of the infectious process, like adhesins, toxins, serum resistance and invasion that are needed to overcome the host defense system (Lee *et al.,* 2016). Pap (pyelonephritis-associated pili) and *Afa* (afimbrialadhesin) are the most common adhesions (Santo *et al.,* 2006). "Adherence of *E. coli* to uroepithelial cells may defend the bacteria from being washed away by urine flow, increasing their ability to multiply and invade renal tissue. The binding of P-fimbrial adhesin to cell receptors of the renal tissue triggers specific signaling pathways leading to mucosal inflammation and tissue damage" (Lee *et al.,* 2016). Afimbrial adhesion *Afa* has been proved to be responsible for the existence of chronic interstitial nephritis. Medical investigation suggested that UPEC strains with *Afa* adhesins have properties that favor the occurrence of chronic UTIs (Tarchouna *et al.,* 2013). Apart from adhesins, exotoxins such as α-hemolysin and cytotoxic necrotizing factor 1 (*CNF*1) are also important uropathogenic factors. HlyA (α-haemolysin) lipoprotein is the most important secreted virulence factor of UPEC strains (Bien *et al.,* 2012). The cytolytic effect of HlyA encoded by α-*hly* plays a role in the invasion of bacteria through the epithelial barrier (Lee *et al.,* 2016). This toxin is able to lyse nucleated host cells for several reasons: better crossing of the mucosal barriers, having access to host nutrients and iron stores, damaging effectors immune cells, and inducing the apoptosis in T lymphocytes, neutrophils, and renal cells (Bien *et al.,* 2012). Cytotoxic necrotizingfactor 1 (CNF1) has been shown to have a role in dissemination and persistence of cells in the urinary tract.

2. MATERIALS AND METHODS

A research study was conducted on 300 urine samples, collected from the pregnant women with informed consent of the participant those attending different antenatal clinics at Bhubaneswar and Puri. Identification of the uropathogens was done by conventional and molecular approaches (16s rRNA gene sequencing) (Collee *et al*., 1996).

2.1. Characterization of Uropathogenic markers of the *E. coli* isolates:-

After identification of the uropathogenic isolates a total of 183 cases were found positive for UTI. It was observed that *E.coli* causes highest number of UTI in the studied population and thus 136 number of *E. coli* isolates (out of 183 positive cases) obtained, were screened for the detection of uropathogenic markers by a series of tests which are as follows-

- **Hemolysin production**- Plate hemolysin test was performed and presence of a zone of complete lysis of erythrocytes around the colony and clearing of the medium was considered as presence of hemolysin production (Sharma *et al*., 2007; Mishra *et al.* 2018).
- **Haemagglutination-** This was detected by clumping of erythrocytes by fimbriae of bacteria in the presence of D-mannose.. MRHA indicate expression of P. fimbriae and MSHA indicate expression of type 1 fimbriae virulence marker (Mishra *et al.* 2018).
- **Cell surface hydrophobicity**- Salt aggregation test, in which the bacteria was tested for their hydrophobic property by using different molar concentration of ammonium sulphate. Those which aggregate with salt particles and formed clumps were considered as hydrophobic (Mishra *et al.* 2018).
- **Gelatinase production**- Developments of opacity around colonies were considered as positive for gelatinase production (Mishra *et al.* 2018).

- **Serum resistance**- Test was done to detect serum resistance by using fresh culture of isolates. Serum resistance ability of the bacterial isolates to serum bactericidal activity was determined by the proportion of bacteria existing after 180 minutes of incubation with serum in comparison with the original count. Those bacteria were considered as serum sensitive, when viable-count drops to 1% of the original (initial) value, and resistant when more than 90% of organisms survive after 180 minutes" (Sharma *et al*., 2007; Mishra *et al.* 2018).
- **Siderophore production** - The test was performed by using chrome azurol sulfonate (CAS) agar diffusion assay, in which CAS detects colour change of CAS-Iron complex from blue to orange halo, which was taken as positive after chelation of the bound iron by siderophores (Vagarali *et al*., 2008; Mishra *et al.* 2018).
- **Detection of Biofilm Formation** - Biofilm formation was assessed by Tissue Culture Plate method (tcp) i.e.'TCP assay'. Adherent bacterial cells usually form biofilms on all sides of the wells of tissue culture plates. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader at wavelength of 620nm and were graded as per Christensen *et al*. (1985). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. (Tolle *et al*., 1998; Costerton *et al*., 1999).

Again for the genotypic characterization of uropathogenic markers by molecular methods, eight primers for uropathogenic *E.coli* was selected from the previous literature available and the selected markers are : PAI, *hyl*, *cnf*, *sfa*, *afa*, *pap*, *fimH*, *traT*, (Oliveira *et al*., 2011; Merza *et al*.,2015; Mladin *et al*., 2009; Chapman *et al*., 2006; Ahmed *et al*., 2017; Neamati *et al*., 2015). The above mentioned markers were applied for the detection of virulence related genes including pathogeneicity island, hemolysin production, 'cytotoxic necrotizing factor- 1', 'S-fimbrial adhesion', afimbrial adhesion, type P frimbriae, type I frimbriae, serum resistance associated etc. among the *E coli* isolates.

The oligonucleoside sequences of the primers used in PCR reaction for the uropathogenic marker genes are expressed in Table-2.1. Exactly following the process mentioned in a recent research article, "the reaction mixture for amplification of each gene were performed in 25µl volume Eppendrof tubes having a mixture of 2.5µl of 10XPCR buffer, 2.5µl dNTPs (0.2mM), 1µl of each primer including forward and reverse

(10pmol/ µl except for *hyl* 30pmol/µl), 0.2µl *Taq* Polymerase (1unit) and 2µl (25- 50ng) of genomic DNA. The volume was adjusted to 25µl by adding together 15.8µl of sterile de-ionized distilled water. The amplification was performed in a thermal cycler (G storm). After amplification, the amplified products were visualized using ethidium bromide staining and gel electrophoresis done on 1.5% (w/v) of agarose in Tris/Borate/EDTA (TBE) buffer"(Merza *et al*.,2015). The amplification conditions for each virulence genes are illustrated in Table-2.2**.**

Table-2.1:- Oligonucleotide sequence of the Primers used in multiplex polymerase chain reaction for the eight virulence genes.

Virulence Genes	Oligonucleotide sequence (5'-3') Forward and Reverse	Size of amplicons
PAI	**F-GGACATCCTGGTACAGCGCGCA** **R-TCGCCACCAATCACAGCCGAAC**	**930 bp**
hyl	**F-AGATTCTTGGGCATGTATCCT** **R- TTGCTTTGCAGACTGTAGTGT**	**565bp**
cnf	**F- AAGATGGAGTTTCCTATGCAGGAG** **R- CATTCAGAGTCCTGCCCTCATTATT**	**498 bp**
sfa	**F-GTGGATACGACGATTACTGTG** **R-CCGCCAGCATTCCCTGTATTC**	**240 bp**
afa	**F-GCTGGGCAGCAAACTGATAACTCTC** **R-CATCAAGCTGTTTGTTCGTCCGCCG**	**750 bp**
pap	**F-GACGGCTGTACTGCAGGGTGTGGCG** **R- ATATCCTTTCTGCAGGGATGCAATA**	**328 bp**
FimH	**F-TGCAGAACGGATAAGCCGTG** **R-GCAGTCACCTGCCCTCCGGTA**	**508 bp**
traT	**F-GGTGTGGTGCGATGAGCACAG** **R-CACGGTTCAGCCATCCCTGAG**	**290 bp**

Table-2.2:- Amplification conditions of the PCR-based analysis for the detection of different uropathogenic markers .

Genes	Initial Denaturation	Thermocycling conditions			Final extension
		Denaturation	Annealing	Extension	
PAI	94°C for 1 min 1 cycle	94°C for 60 sec 30 cycles	63°C for 30 sec	72°C for 90 sec	72°C for 5 min 1 cycle
hyl	94°C for 4 min 1 cycle	94°C for 30 sec 30 cycles	55°C for 30 sec	72°C for 60 sec	72°C for 5 min 1 cycle
cnf	95°C for 3 min 1 cycle	94°C for 30 sec 25 cycles	68°C for 30sec	68°C for 4 min	72°C for 10 min 1 cycle
sfa	95°C for 3 min 1 cycle	94°C for 30 sec 30 cycles	63°C for 30sec	68°C for 4 min	72°C for 10 min 1 cycle
afa	94°C for 5 min 1 cycle	94°C for 60 sec 30 cycles	63°C for 60 sec	68°C for 3 min	72°C for 7 min 1 cycle
pap	94°C for 5 min 1 cycle	94°C for 60 sec 35 cycles	65°C for 60 sec	72°C for 60 sec	72°C for 7 min 1 cycle
FimH	94°C for 5 min 1 cycle	94°C for 60 sec 35 cycles	55°C for 60 sec	72°C for 2 min	72°C for 10 min 1 cycle
traT	94°C for 2 min 1 cycle	94°C for 60 sec 30 cycles	63°C for 30sec	72°C for 90 sec	72°C for 5 min 1 cycle

3. RESULT-

The site of infection and any predisposing factors affect the prognosis and management of urinary tract infections. UTI may be referred to as the condition in which bacteriuria establish and then multiply within the urinary tract. In the present study, *E.coli* was the most recurrently isolated organism having 74.3% of occurrence.

Table-.3.1: Detection of various pathogenic markers in the *E.coli* isolates (N=136).

Pathogenic Markers	No. of positive cases (136)	Percentage (%)
Hemolysin	57	41.9%
Haemagglutination (MRHA & MSHA)	86	63.2%
Cell-surface hydrophobicity	38	27.9%
Gelatinase test	87	63.9%
Serum resistance	115	84.5%
Siderephore production assay	103	75.7%
Biofilm formation	19	13.9%

(Note- **MRHA**= Mannose Resistant Haemagglutination and **MSHA**=Mannose Sensitive Haemagglutination)

In the present study, *E. coli* was the most prevalent microorganism causing urinary tract infection. The incidence of different pathogenic markers of the uropathogenic *E.coli* isolates (Table -1) indicated serum resistance as the most frequent uropathogenic marker having a rate of 84.5% followed by siderophore production having 75.7% frequency, i.e. 103 isolates produce siderophore as a virulence factor. A frequency of 27.9% was observed for cell surface hydrophobicity, i.e. only 38 isolates out of 136 *E. coli* strains were positive to salt aggregation test. Biofilm formation was found to have the least frequency i.e. 13.9 % (19 out of 136). The frequencies of positive isolates for haemagglutination and gelatinase test were found to be almost equivalent at 63.2 % and 63.9 % respectively.

Fig.-3.1. Detection of Uropathogenic markers in *E. coli*

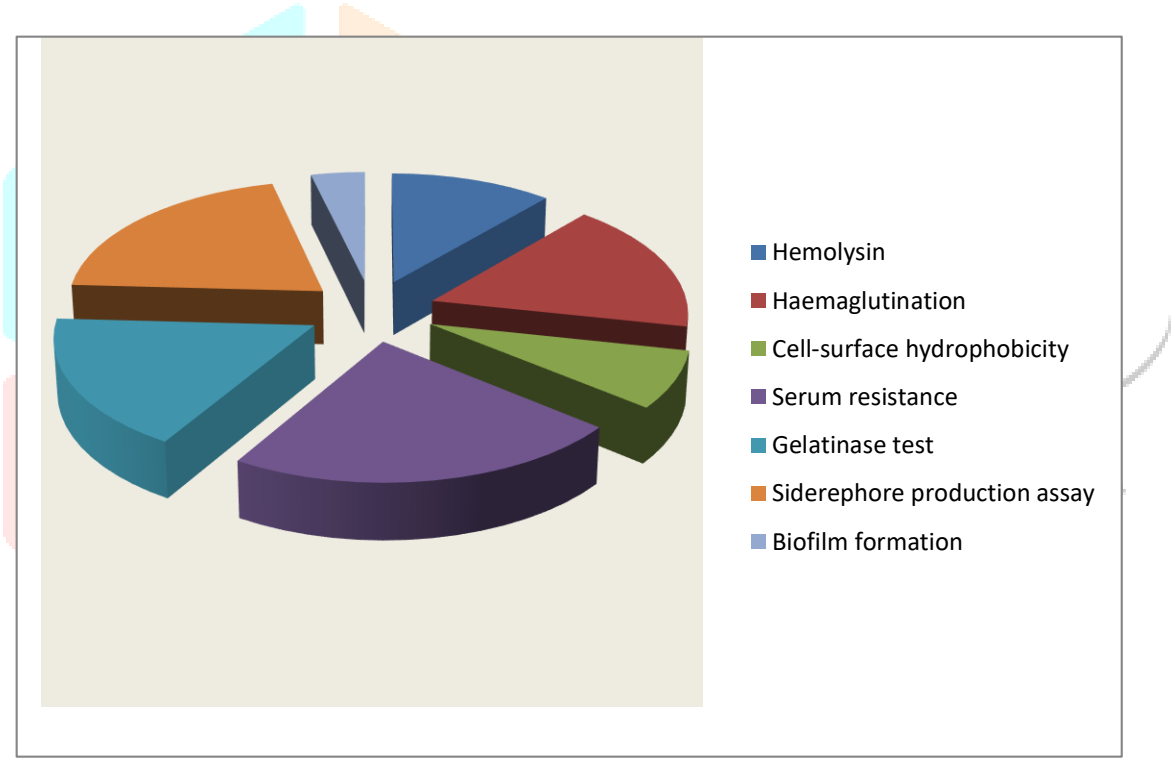
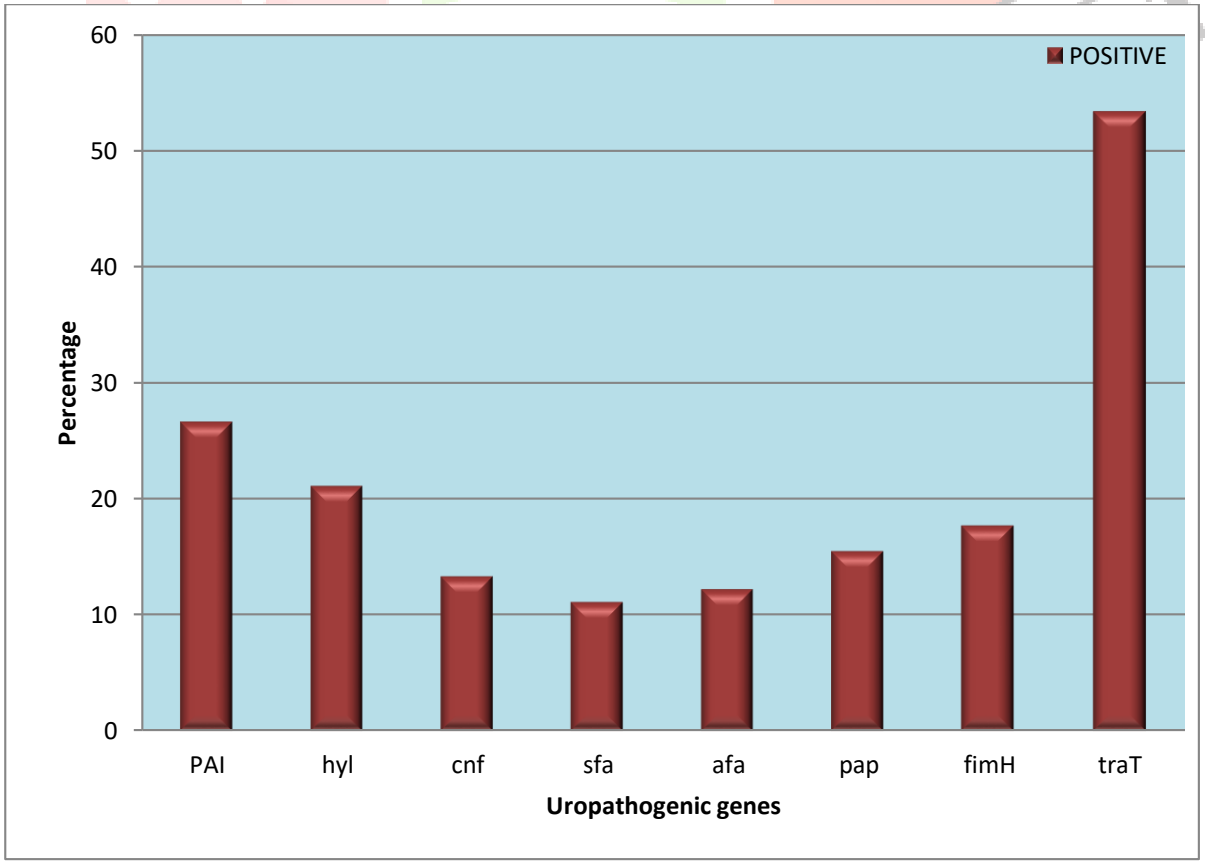


Table-3.2. Incidence of uropathogenic genes in the *E.coli* isolates

Uropathogenic *E. coli* is the most frequent bacterial isolates of urinary tract infections in the present study. Molecular identification of bacterial isolates was also done by 16s rRNA gene sequencing followed by BLAST search analysis. BLAST search analysis revealed that, query coverages of 90 bacterial isolates was matched with *E.coli*. Out of the 90 PCR confirmed *E coli* isolates, the incidence of uropathogenic factor genes are summarized in table 3.2.

Genes	No. Positive & Percentage n(%)
PAI	24(26.6)
hyl	18 (21.1)
cnf	12(13.3)
sfa	10(11.1)
afa	11(12.2)
pap	14(15.5)
fimH	16(17.7)
traT	48(53.3)

From these results, it was evident that *traT* responsible for serum resistance was the most assertive marker among all other virulence related genes accounting for 53.3% of UPEC isolates i.e. 48 out of 90 cases were positive for *traT* that responsible for serum resistance followed by PAI and *hyl* those were detected in 26.6% (24) and 21.1% (18) respectively, while less prevalence of *afa* gene was found with rate 22.7% and *sfa* with a frequency of 11.1% contributing the least prevalent gene in the isolated UPEC strains.



4. DISCUSSION

Bacteriuria, whether it may be symptomatic or asymptomatic, is common in the period of pregnancy. This prospective study aimed at finding the prevalence of urinary tract infections in pregnant women and different predisposing factors affecting growth of UTIs. A total of 300 samples from pregnant woman were included in the study.

Asymptomatic bacteriuria in pregnancy is of great concern as it leads to many maternal and fetal complications. Research of pathogenic markers of uropathogenic *E.coli* and other organisms causing UTI helps in better understanding of pathogenesis, complications thereby so that effective interventions can be made available. The common pathogenic markers include cell surface hydrophobicity, colonization factor, presence of capsule, serum resistance, resistance to phagocytosis, hemolysin production, enterotoxin and siderophore producing ability, presence of fimbriae and haemagglutination ability (Hedge *et al*., 2008).

In the present study analyzing the pathogenic markers (Table 1), among 136 *E.coli* isolates, it was observed that about 115 (84.5%) were serum resistant and among the pathogenic markers, serum resistance was the predominant pathogenic factor. This was similar to the study conducted by Sharma *et al.*, (2007). Sharma *et al*., (2007) demonstrated that among 152 isolates of urinary *E.coli*, 36 (23.7%) were hydrophobic, 132 (86.8%) were serum resistant.

From the present study it is confirmed that UTI caused mainly by uropathogenic *E. coli* (UPEC) in the studied region various virulence factors can be attributed to UPEC pathogenicity that lead to severe complications. The pathogenicity of UPEC strains happened by the presence of virulence factors was already confirmed by Tiba *et al.*, 2008. and the pyelonephritis developed due to the presence of P fimbriae (Jhonson *et al.*, 2000; Ruiz *et al.*, 2002; Oliveira *et al.*, 2011, Hagan *et al.*, 2007). The surface virulence factors of UPEC, such as adhesions, are invariably responsible for colonization of bacteria in the urinary tract. The present study results displayed that 15.5 % of UPEC isolates carried *pap* gene. The frequency of *pap* gene in UPEC isolates used for present research was found to be lesser than the ones found in Brazil, Tunisia, China,

(Tarchouna *et al.,* 2015; Abe *et al.,* 2008). However, it is in consonance with the findings of studies conducted by Santo *et al*. (2006) and their gene frequencies of 17% and 14% respectively. A review of literature suggests that the frequency of *pap* gene can fluctuate between 0% and 77% (Tarchouna *et al.,* 2015; Jhonson *et al.,* 2000; Oliveira *et al.,* 2011). These observations, lead to the conclusion that prevalence of these genes varies based on geographical placement. Also, the variation in frequency of *pap* gene in different studies can be levied on the fact that UPEC strains utilize a variety of adhesins to bind to the urinary epithelial cells and start the infection. Hence, the strains lacking the *pap* operon may use other adhesins encoding operons such as *afa*, and *sfa* for binding. The PAI marker showed a frequency of 26.6% in the current study, but this differs significantly from studies by Johnson et al. (2000) and Neamati *et al.,* (2015) who reported frequencies of 71% and 61.3% respectively for PAI markers among UPEC isolated from the patients. Usually, invasive pathogens are highly resistant to lethal activity of serum; and the role of *tra T*-protein in resistance of bacteria to serum is very important. The current study propounds that 53.3% of UPEC isolates contained *tra T* gene and it was the most prevalent virulence determinant and serum resistance-associated gene in the current study. Oliveira *et al.*, (2011) also zeroed-in on similar findings as he reported *tra T* gene to be the most prevalent virulence gene having 76% of UPEC strains among the urine samples. These results amply exhibit *tra T* to be, not only a common, but also important virulence factor. Hence, it may be deemed to be a target for therapeutic interventions. The present research identified eight distinct patterns among UPEC strains but high variations of the virulence genes compositions were reported by Oliveira *et al*. (2011) and Johnson *et al.,* (2000).

5. CONCLUSION

Controlled trials and large-scale studies act as prerequisites for ascertaining the pathogenic potential of isolates. Many studies have shown that complications during pregnancy are more common in bacteriuric women. This study has revealed that the most effective mode to find bacteriuria in culture is screening for bacteremia during pregnancy. Even though conspicuous effect of pathogenic factors cannot be directly related to clinical scenario of asymptomatic bacteriuria/UTI during pregnancy, the importance of virulence

mechanism needs to be explored for individual uropathogens in order to understand their epidemiological and pathogenic role in UTI.

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