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RESEARCH ARTICLE

MOLECLAR CHARACTERIZATION OF SURFACE ADHESINS OF UROPATHOGENIC *ESCHERICHIA COLI* FROM PATIENTS IN A TERTIARY CARE HOSPITAL.

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Abstract

Uropathogenic E.coli (UPEC) classified under Extraintestinal pathogenic *E.coli* (Expec) with multiple virulence markers encodes for surface adhesive organelles. The study was carried out to isolate and to identify *E.coli* from urine samples and to characterize the adhesive genes by molecular technique - *pap* (pyelonephritis associated pili), *fimH* (type 1 fimbriae) and *sfa* (S fimbrial adhesin).A total of 96% out of 208 *E.coli* were positive for *pap*, *fimH* and *sfa* genes with varied frequencies - *pap* 46% (96/208), *fimH 92% (192/208)* and 58% (28/208) *sfa* genes. 12.5% of isolates possess all three adhesive genes. The antibiotic pattern showed resistance to nalidixic acid (80.8%), Ceftazidime (49.5%), Cefepime (47.6%), Gentamicin (20.2%), Norfloxacin (30.8%). Fimbrial adhesion may lead to resistance towards host defence, biofilm formation resulting in relapse and recurrences of infection. Molecular studies help us to determine the multiple virulence genes and to understand the complexity and severity of UTI.

Introduction:-

Uropathogenic E.coli (UPEC) classified under Extraintestinal pathogenic *E.coli* (Expec) has multiple virulence markers which encodes for surface adhesive organelles and secretory toxins which are located on 'pathogenicity islands' determines the pathogenicity of UTI (Farzaneh Firoozeh et al.,2014). Fimbrial adhesion may be responsible for resistance towards host defence, biofilm formation resulting in antibiotic resistance, relapse and recurrences of infection.

UPEC with its multitude virulence factors breaks the inertia of mucosal barrier, persist within the urinary tract and serves as reservoir of recurrence infection (Azam Karimian etal, 2012). Type 1 pili being a common adhesion binds to uroplakin glycoproteins coded by *fim H* expressed in both pathogenic and commensal strains enhances the bacterial survival, stimulate the mucosal inflammation, invasion and responsible for inter bacterial binding called as biofilm in the central tubule. P fimbriae, the second common virulence factor enhances early colonization in tubular epithelium leading to cytokine production, pathogenesis of ascending UTI and pyelonephritis. S fimbriae binds to the epithelial and endothelial cells of lower urinary tract and kidney facilitating the dissemination within host tissues and are associated with *E.coli* causing sepsis, meningitis, ascending UTI's (Malagolini et al., 2000).

Some virulence factors can be demonstrated phenotypically by using conventional method ex., Haemagglutination for detecting P fimbriae (MRHA) and Type 1 pili (MSHA) based on agglutination using 3% RBC's cell suspension. Molecular studies help us to determine the multiple virulence factors which cannot be demonstrated conventionally

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and not expressed phenotypically due to mutation or changes in conditions of in-vivo and in-vitro. The genotypic study is the sensitive, accurate determination of virulence genes which helps us to understand the complex pathogenicity and severity of UTI.

Materials and Methods:-

Bacterial isolates:

1100 urine samples collected from outpatients of a tertiary care hospital after obtaining approval from Institutional Ethical Committee (IEC) and were processed by standard culture methods. The isolated 208 strains of *E.coli* were confirmed by biochemical tests followed by antibiotic susceptibility pattern.

DNA extraction:

DNA extraction was performed using the boiling method. Before DNA extraction, the E. coli isolates were cultured in LB broth at 37^{o} C for 18 hours. Bacteria were pelleted from 1.5 ml LB broth then suspended in 200 ml of sterile deionized water and incubated at 100^{o}C for 10 min. After centrifuging, the supernatant was used as template DNA and stored at -20^{o}C (Farzaneh Firoozeh et al.,2014)

PCR amplification:

PCR amplification of virulence genes were used to reveal the prevalence of three virulence genes including fimH, pap, sfa using specific primers. The amplification of virulence genes was carried out in a Thermal Cycler (Eppendorf Master Cycler) after standardizing the PCR conditions - initial denaturation, denaturation, annealing and extension steps.

Table 1: Virulence gene primer sequences

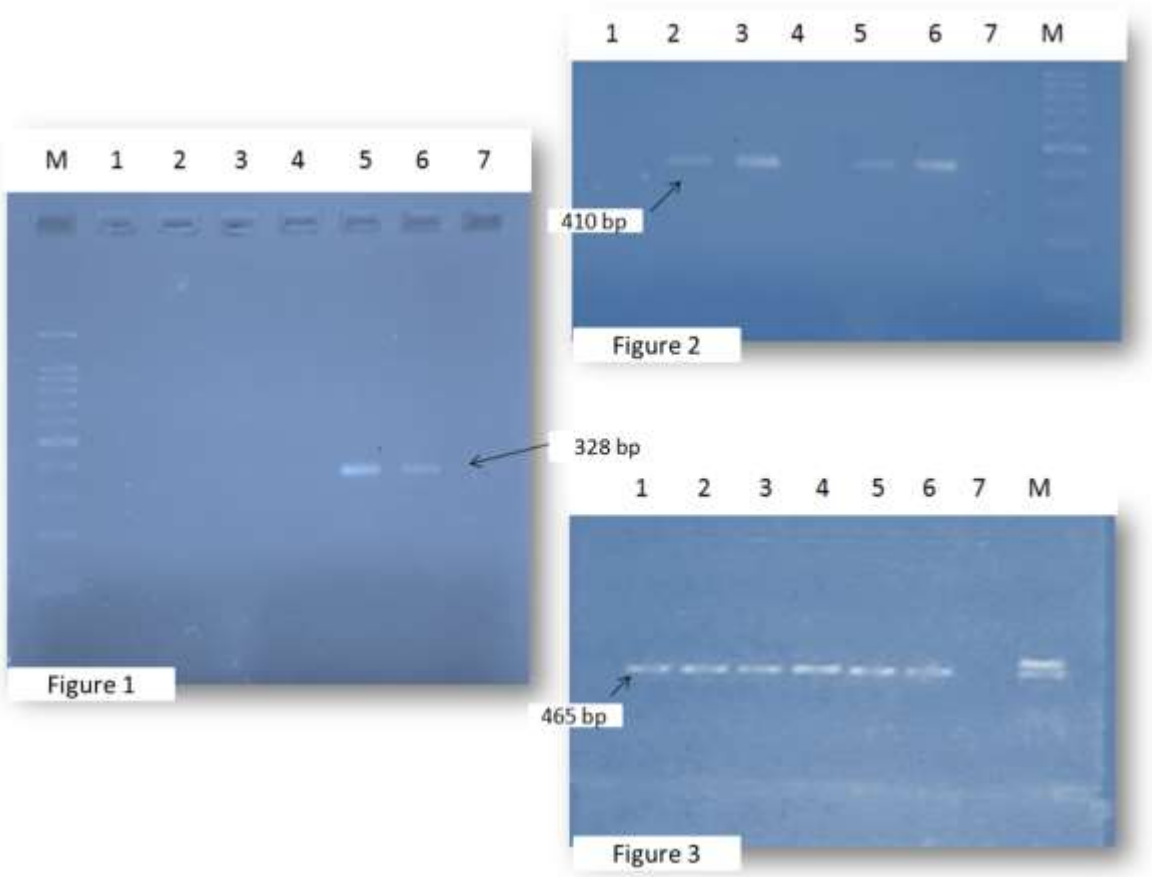
Gene	Primer sequence (5'-3')		Product size (bp)	References
pap	F	GACGGCTGTACTGCAGGGTGTGGCG	328	Soto *et al.*,2011
	R	ATATCCTTTCTGCAGGGATGCAATA		
fimH	F	AACAGCGATGATTTCCAGTTTGTGTG	465	Farmer et al.,1999
	R	ATTGCGTACCAGCATTAGCAATGTCC		
sfa	F	CTCCGGAGAACTGGGTGCATCTTAC	410	Soto *et al.*,2011
	R	CGGAGGAGTAATTACAAACCTGGCA		

The electrophoresis of PCR products was performed on 1% agarose gel with a 100-bp DNA ladder as molecular size. The gels were stained with ethidium bromide and visualized in a UV transilluminator/ Gel documentation system.

Results:-

Out of 1100 urine samples, 208 (50.9%) were *E.coli* among other culture positive isolates. 96% of the isolates possess the surface adhesive genes. The prevalence of *fimH 92.3% (192/208)* which was the highest followed by *pap* 46.2% (96/208) and 27.9% (28/208) *sfa* genes.

These adhesive genes were expressed in single or in combinational forms. The prevalence of gene coding individually were 75 (36%) isolates with only *fimH*, 4 (1.9%) with *pap* and 1 (0.5%) with *sfa* alone. Combinations were seen among these three genes - 44% possess pap and *fim H*, 31.8% pap and *sfa, 61.4% fim H and sfa.* All three adhesive genes were present in 25 (12.5%) of the isolates. Seven isolates (3.4%) possess none of the surface adhesin genes.



Agar gel electrophoresis of UPEC virulence factors – pap, sfa, fim H (328bp, 410bp, 465bp) –Lane (M) – DNA Molecular size marker (100 bp ladder). **Figure 1:** Lane 5, 6 showing positive for pap and lane 1, 2, 3, 4, and 7 with negative results. **Figure 2:** Lane 2, 3, 5, 6 positive for sfa and 1, 4, 7 negative for sfa gene. **Figure 3:** Lane 1, 2, 3, 4, 5 and 6 has the band denoting the presence of fim H gene and lane 7 is negative for fim H.

Table 2: Prevalence of different virulence genes

Virulence gene	No. of isolates	%	Virulence gene	No. of isolates	%
Positive	201	96.6	**pap**	96	46.2
			fimH	192	92.3
Negative	7	3.4	**sfa**	58	27.9

Table 3: Prevalence of virulence genes in relationship to gender

	Gender			
Virulence genes	**Male**	**%**	**Female**	**%**
pap positive	36	46.2	60	46.2
fimH positive	70	89.7	122	93.8
sfa positive	23	29.5	25	19.2

Table 4: Virulence genes among different age groups

Age group (year)	Virulence genes - Types and No. (%)		
	Pap (96)	**fimH (192)**	**Sfa (58)**
< 1 – 10	4 (4.2)	11 (5.7)	2 (3.4)
11 – 20	11 (11.5)	25 (13.0)	4 (6.9)
21 – 30	15 (15.6)	28 (14.6)	9 (15.5)
31 – 40	11 (11.5)	20 (10.4)	5 (8.6)
41 – 50	16 (16.7)	30 (15.6)	8 (13.8)

51 – 60	14 (14.6)	30 (15.6)	12 (20.7)
61 – 70	16 (16.7)	29 (15.1)	10 (17.2)
71 – 80	5 (5.2)	12 (6.3)	5 (8.6)
81 - 90	4 (4.2)	7 (3.6)	3 (5.2)

The antibiotic pattern showed resistance to Nalidixic acid (80.8%), Ceftazidime (49.5%), Cefepime (47.6%), Gentamicin (20.2%), Norfloxacin (30.8%).

Discussion:-

Uropathogenic *E. coli* are definitely associated with urinary tract infection, a most prevalent infectious disease. Various virulence factors facilitates extra intestinal survival of UPEC, enables to colonize the urinary tract and causes infection which may lead to renal failure in severe cases. Virulence factors of UPEC might possess strategies to delay or suppress the activation of components of the innate host response in the urinary tract and the association among them in a single strain determines the degree of pathogenicity of UTI. (Azman Karimian et al., 2012)

The aim of the study was to characterize the surface adhesins (pili/ fimbriae) of *E. coli*, responsible for adhering to uroepithelium, a basic factor that protects the bacteria from urinary discharge and promotes their ability to multiply and invade renal tissue (Soto et al.,2011). The two principle pili (type 1 and P) were found in patients with UTIs. S fimbrial adhesion recognizes the surface sialic acid expressed on the receptors of kidney epithelial and endothelial cells mediating the adherence. Recently, it was identified that sialic acid also present in uroplakin3 (UP3) expressed on the bladder luminal surface suggesting its role in cystitis (Monique Ribeiro et al., 2008)

Table 5:- Prevalence of fimH, pap, sfa virulence in various studies.

Virulence genes			**Research studies**
fimH (%)	**pap (%)**	**sfa (%)**	
97.5	27.8	27.8	Monique Ribeiro TIBA et al., 2013
86	36	23	Usein et al., 2001
65.9	63.7	56	Soto et al.,2011
79.67	50.4	---	Azam Karimian et al., 2012
92.3	46.2	27.9	Our study

In this study *fimH* 92.3% out of 208 *E.coli* followed by *pap* 46% and 27.9% *sfa* genes (Table 2). Previous studies have established that *fimH* was most frequent in isolates from a variety of forms of UTI (Mabbett et al., 2009, Cheng et al., 2010, Wang et al., 2013). This study denotes the increased prevalence of virulence 92.3%, 46.2 and 27.9% for *fimH, pap*, and *sfa* respectively with *fim H* being the highest compared to other studies (Table 5).

A study identified 97.5% *fimH*, 32.7% *pap*, 27.8% *sfa* showing that *fimH* is the most prevalent and detected in 100% of pap gene positive isolates (Sanchez et al., 2013). The above study was similar to our study with highest rate of 92.3% *fimH* and all 46% of *pap* is positive for *fimH*. UPEC strains isolated from neurogenic bladder patients harbours different virulence genes implicates in the initiation and the development of the infectious process represented by adhesins *(fimH)* and other secretory toxin genes (Mladin et al., 2009). Differences in prevalence of UPEC virulence genes indicate that the virulence properties of UPEC strains are closely dependant on geographic areas and weather climate of each regions.

This study shows less percentage of 46.2% *pap* and 27.9% *sfa* compared to the study done by AV Shetty et al., 2014 the prevalence of *pap* gene was highest 60.87% (14/23) followed by *sfa* and *afa* 39.1% (9/23) genes each. But higher percent with the study on children with UTI showed 27.1, 14.6% - *pap, sfa* respectively (Farshad and Emamghorashi, 2009).

A study in Iran 86.7% UPEC strains contained virulence genes, of which 34 (22.6%), 52 (34.6%), 39 (26%), and 5 (3.5%) were found to carry one, two, three, and four virulence genes, respectively. 27.8% *pap* from pyelonephritis and 6.4% from cystis indicating its high prevalence than other genes (Farzaneh Firoozeh et al.,2014) . Our study with 46.2% pap and 75 (36%) isolates with only *fimH*, 4 (1.9%) with *pap* and 1 (0.5%) with *sfa* alone with the combinations of 44% possess pap and *fim H*, 31.8% pap and *sfa, 61.4% fim H and sfa carry two genes.* All three adhesive genes were present in 25 (12.5%) of the isolates.

Adhesins are the significant virulence markers to adhere to uroepithelium (Eden CS *et al.,* 1976; Sandberg T *et al.,* 1988). Type 1 fimbriae are present in > 90% of commensal and pathogenic *E. coli* helping the bacterium to adhere mucosal epithelium, tissue matrix and biofilm formation (Agarwal J *et al.,* 2012) and may involve in colonizing the lower urinary tract (Nataro JP, Mobley HL., 2004; Wullt B., 2003). P fimbriae present in 40 – 60% of UPEC isolates and may associate with increased host inflammatory response. No surface factors were found in certain *E.coli* which is correlating our result that 3.4% isolates had none of the studied three adhesion genes. There also various other genes for adhesion in the urinary tract (Agarwal J *et al.,* 2012, Wullt B., 2003) and conserved virulence factors called as syndrome specific VF's (Johnson et al., 2005)

Another study done by Ahmed et al., 2015 determined 22% and 14% , Tarchouna et al., 2013 in 41% and 34%, in Santo et al., 2006 32.0% and 19.0% of *pap* and *sfa* respectively. The *pap* gene was commonly found in patients with age groups (1-10 years) and (21-30 years) in our study it's for age groups (41 – 50, 61 -70 years), *sfa* gene was found among patients with age (31-40 years) in our study its (51 -60 years). While age group (1-10 years) has not this gene (0.0%) this does not correlate our study that all three genes were present in all age groups Ahmed et al., 2015.

In Ahmed et al., 2015, 43 (76.79%) infections occur in female patients and 13 (23.21%) infections in male patients out of 56 UPEC. Similar observation has been documented in Hilla/Iraq (Abdul et al., 2011); our study also determined 130 (62.5%) female and 78 (37.5%) male regarding the relative occurrence of the gender groups. Females are more susceptible than male. This may be explained on the basis that these pathogens may be emerging from gastrointestinal flora through faecal contamination and may gain entry into the female urethra because of the close proximity of the anus to the urethra in females (Awaness et al.,2000).

Our study results are comparative with other studies with highest prevalence of *fimH* coding type 1 fimbriae but the prevalence of *pap* and *sfa* varied among the studies. This may be due to the different in climatic, public health environment and distribution of population etc., in different regions and this was the first study to analyse the surface adhesins in our region. The virulence factors are the results of different genes which can be detected by PCR which is more accurate and sensitive (Le Bougue´nec et al.,1992, Codruta-Romanita et al.,2001, Licznar et al.,2003).

Conclusion:-

The presence of surface virulence factors in *E.coli* enhances the potential to adhere and more no. of different adhesins indicates its potential to cause severe infection. Theoretical information of virulence studies can be applied practically in the areas of proteome analysis, DNA arrays etc., paves the way for molecular diagnosis and epidemiology.

The structural form of same type of virulent protein may differ between one another in an individual *E.coli* though the function is same. It may or may not express against the host as it depends on differences in invivo and invitro conditions, innate host and antibiotic defence. Advanced studies are necessary to target the new site for antimicrobial intervention and also for prevention which can be improved by utilising receptor analogues, vaccine development with mono/ polyvalent antigens.

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