International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

Study of Virulence Genes of Uropathogenic *E.coli* in Central India

Pallavi Wanjari[1], Dr. Y. S. Thakar[2], Dr. Vinay Tule[3]

[1, 2]Vishakha Clinical Microbiology Laboratory, Nagpur, Maharashtra, India

[3]Eugenics lab, Nagpur, Maharashtra, India

Abstract: ***Uropathogenic Escherichia coli, the most frequent pathogens in acute urinary tract infections, which possess various virulence factors. Four virulence factors associated with uropathogenic Escherichia coli were investigated in 232 clinically isolated E.coli. Conventional PCR systems were used to detect genes encoding for haemagglutination (Pap gene: p fimbriae), adhesion to epithelial cells (Sfa: S fimbrial adhesion, Afa: afimbrial adhesion), Haemolysin production (Hly) and Serum resistance (Tra t). One or more virulence markers were detected in as many as 223 (96.12%) isolates. The genes for Afa+Sfa (69.64%) and Hly (69.40%) was commonest genotypic virulence marker followed byTra t (61.64%) gene and Pap gene (53.45%) was relatively less common genotypic virulence marker found in the present study. Only single virulence marker was detected in 24(10.34%), two virulence markers simultaneously in 76(32.76%), three virulence markers together 79(34.05%) and all four were present in 44 (18.97%) strains. Thus Presence of virulence genes of E.coli appears alone with less frequency and simultaneously with other virulence markers with more frequencies. In the pathogenesis of UTI though one virulence marker may be essential, most of the time multiple virulence markers may operate simultaneously.***

Keywords: Uropathogenic *Escherichia coli*, virulence marker, genotype, Adhesion, UTI

1. Introduction

Escherichia coli are common inhabitant of the gastrointestinal tract of humans and animals. Some strains of *E. coli* can diverge from their commensal cohorts, taking on a more pathogenic nature. These strains acquire specific virulence factors (via DNA horizontal transfer of transposons, plasmids, bacteriophages, and pathogenicity islands), which confer an increased ability to adapt to new niches and allow the bacteria to increase the ability to cause a broad spectrum of diseases.(1)*E. coli*, is responsible for more than 85 percent of all UTIs, according to a 2012 report in the journal Emerging Infectious Diseases(2), regardless of whether symptoms are evident, as in cystitis and pyelonephritis, or not evident, as in asymptomatic bacteriuria.Urinary tract infections aren't usually serious, but they can be dangerous if the bacteria make their way into the kidneys.

Uropathogenic *Escherichia coli* (UPEC) contain several virulence factors that facilitate its colonization and invasion of host cells. Surface virulence factors (adhesins) as the main attachment factor, P fimbriae is particularly associated with pyelonephritis and is encoded by pap genes. Another adhesion that acts as a virulence factor is S fimbrial adhesion, which is coded by sfa genes., A fimbrial adhesions (Afa) of *E. coli*, coded by Afa genes, have been reported in cases of pyelonephritis and recurring cystitis. Other important virulence factors of UPEC strains are the toxins that act as secretory virulence factors are alpha-hemolysin (Hly A), which is encoded by the Hly gene and serum resistance which is ability to survive killing by components of serum, due to the outer membrane protein TraT encoded by Tra t genes. (3).

In view of these, the present study was conducted on urinary isolates of *E.coli* to ascertain the frequency of occurrence of four common virulence markers genes and their association with each other.

2. Literature & Review

E.coli infection can lead to Urinary tract infection, respiratory illness, pneumonia and other illnesses like meningitis. *E.coli* refers to wide range of bacteria that can cause various diseases, like diarrhea, urinary tract infection. Some strains of *E.coli* can cause of acute kidney failure.*E. coli* bacteria are a common cause of urinary tract infections, for example, cystitis. The exit of the urinary tract is near the anus, and so the bacteria can spread from the GI tract to the urinary tract.(4)

Urinary tract infections (UTIs) are a major public health concern in developing countries. Most UTIs are caused by *Escherichia coli*, accounting for up to 90% of community-acquired UTIs(5).*E. coli* has acquired genes encoding diverse virulence factors (VFs) that enable them to cause infection in both normal and compromised hosts (6). *E.coli* (UPEC) strains are responsible for approximately 80 % of community acquired and 30 % of nosocomial-acquired urinary tract infections (UTIs).(7). Virulence factor (VF) genes were detected by use of a novel polymerase chain reaction (PCR) assay. Compared with probe hybridization, the PCR assay's specificity was 100% and sensitivity 97.1%.(8). Attachment of P fimbriae is also associated with increased host inflammatory response. It is present in 40-60 % of UPEC isolates (9). G. Kallenius and his coworkers found 91% positive cases for cell adherence. The most important secretory virulence factor is Alpha - haemolysin (HlyA), which is encoded by the *Hly* gene. Also, cytotoxic necrotizing factor 1 (*CNF1*) is reported in a third of pyelonephrogenic strains. (3) The cytotoxic necrotizing factor 1 (CNF1) secreted by most haemolytic *E.coli and some other Escherichia coli* strainsisolates is known as necrotoxigenic *E. coli*. In human CNF-1 producing *E.coli*

isolates have been implicated in extraintestinal infection, specially UTI and meningitis. NTEC strains causes 80% of uncomplicated UTI.(10). The difference between the cases and controls, for haemolysin production, was highly significant (P<0.001)(11).Mohsen Tabasi and co workers(12) reported 85% haemolysin positivity. Adhesion for haemagglutination that acts as a virulence factor is S fimbrial adhesion, which is coded by *Sfa*genes. Also, Afimbrial adhesions (*Afa*) of *E. coli*, coded by *Afa*genes, have been reported in cases of pyelonephritis and recurring cystitis.(3). The UTI-causing *E. coli* strains typically agglutinate human erythrocytes despite the presence of mannose (mannose-resistant haemagglutination [MRHA]) and adhere to human uroepithelial cells. Moreover, adherence to uroepithelial cells is usually unaffected by mannose (mannose-resistant adherence) and is more common among strains exhibiting MRHA than among those exhibiting only mannose-sensitive haemagglutination (HA). HA preparations are cytotoxic toward tissue culture cells.(13)

Recent studies have shown that HA and erythrocyte hemolysis can help bacteria to adhere to host cells and colonize them to cause UTI.(13)

Haemagglutination was found in total 63.5% *E. coli* isolates positive by PoojaTomar and her co-workers (14). Serum resistance virulence factors have important roles in the development of UTIs ability due to the outer membrane protein TraT encoded by *traT* genes. The gene *traT* is a surface exclusion protein that mediates resistance to serum(3).

The *tra t* and i*ss* genes were responsible for serum resistance virulence marker but in investigation of R. Bollmann it found that the *traT* genes were present with higher frequency than the *iss* determinants.(15) . Sharma et al (16) found 86% serum resistance *E.coli* and nearly 63% and 60% were observed by Sabitha Baby (17) and Prachi Shaw (18) respectively.

3. Material and Methods

The present study was conducted for a period of Nov 2015 to Nov 2018. The total 232 isolates of *E. coli* obtainedfrom patients of urinary tract infection (UTI) and those who had pyuria were included in this study. The strains were stocked on nutrient agar slants at 4^0C and processed within one week of isolation.

Detection of genes:
The genes responsible for particular phenotypic markers for four virulence markers were detected which include:
hly gene for Haemolysin
pap gene for Haemagglutination
Sfa and Afa genes for Adhesion
Tra t gene for Serum resistance.

1) Isolation of DNA
For the detection of the genes the DNA of E.coli was isolated by boiling method. (19)Two to three isolated colonies of *E.coli* were taken in 500 µl of sterile distilled water in eppendorf tube and mixed properly. Then it was boiled in water bath for 10 min. then the tube was cooled at room temperature and centrifuged at 1000 rpm for 5 minutes and stored the supernatant in deep freeze.

2) Primer preparation:-(20)
The primers sequences, both forward and reverse sequences for above virulence gene markers as shown in table No.2.1 were obtained from Xcelris labs limited, Ahmedabad. The primers were appropriately diluted as per the instructions of the manufacturer for use in the PCR reactions.

3) PCR reaction mixture preparation:-
Separate tubes were used for detection of separate genes. For PCR reaction mixture 2X PCR Taq Mixture and molecular biology grade water were supplied by HI MEDIA Ltd., Mumbai and stored at -20ºC until used. PCR Taq mixture contains Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentration. The PCR reaction mixtures and all reagents were thawed at room temperature. The PCR reaction mixture for 25µl reaction was prepared. For one reaction 12.5 ul of PCR Taq mixture was taken in 0.5 ml size eppendorftube. The concentrations of different primers were added into the 25 µl reaction mix as per Table 3. The volume was made upto of 25 µl by adding required quantity of Molecular biology grade water.This reaction mixture tube was placed in Thermal cycler (HIMEDIA) were amplification carried out as 94^0C for 60sec one cycle, 94^0Cfor 30ssec, 65^0Cfor 30sec and 50^0Cfor 30sec.repeated for 30 cycles and 72^0C for one cycle . After PCR completion the reaction mixture was stored in deep freeze before electrophoresis was carried out.

4) Gel Electrophoresis
Total of 100 ml of 1X TAE buffer (ML010 HIMEDIA) was taken, out of which 50ml TAE buffer was taken in a conical flask.To this 0.75mg agarose powder (MB002 HIMEDIA) was added, boiled the agarose powder for melting. Then 2 µl Ethidium bromide (EtBr) was added to the melted agarose at about lukewarm temperature.

It was then poured to casting tray with comb of Electrophoresis unit placed in it.

The gel was allowed to settle for about 30 minutes at room temperature.

Then the gel was placed into the electrophoresis unit.

Remaining 1X TAE buffer was added to electrophoresis running tank. The samples were loaded in different wells formed by the comb (15 µl PCR reaction mixture as processed above). Loading dye (3 ul) was also added in each well to monitor progress during electrophoresis. Current was started and electrophoresis was done for 30 min at 100 volts. The resulting bands were visualized on UV transilluminator.

DNA ladder (100bp) (MBT049 Hi Media) was also loaded in agarose gel every time in first well to monitor band size of the sample. One negative control sample (PCR reaction mixture without any DNA) was also run everytime.

4. Results

The study comprises of serially isolated *E.coli* obtained from 232 Urine specimens of patients of UTI. Of the total urine specimens 89 (38.36%) were from males, 123 (53.02%) from females and 20 (8.62%) from children. The number of *E.coli* isolated from female (53.02%) is significantly high.

Of the virulence markers Hly (69.40%) and Afa+Sfa (69.40%) were commonest followed by Tra t (61.64%) and Pap (53.45%) (Table No. 2)

Of all 223 strains, single genetic virulence marker was seen in only 10.34% while in all remaining isolates they were found together. Ther frequency is shown in table No. 3.

The distribution of individual virulence markers, either alone or in combination is shown in Table No.4

Of the two genetic markers Sfa and Afa S fa was more common than Afa. Sfa and Afa virulence markers were simultaneously detected in 11.2% and both were simultaneously negative in 30.60% isolates. Their comparative isolation is shown in table No. 5.

5. Discussion

It is well known that *E. coli* is the commonest organism responsible to cause UTI in human beings (21). The same *E. coli* also comprises of normal flora of human intestine. It is always possible that these organisms can easily gain an access to the urinary tract. It is equally essential to ascertain whether these commensal organisms can cause infection or not and a useful means to ascertain it is to find out whether the *E. coli* in urinary tract possess the virulence potentials or not i.e. the virulence markers. In the present study a comprehensive analysis of four important virulence markers vizhaemolysin, haemagglutination, adhesion and serum resistance was done by genotypic methods.

The analysis of various genotypic virulence markers viz Hly gene (for hemolysin) Pap gene (for heamagglutination), Afa+Sfa gene (for adhesion) and Tra t gene (for serum resistance) were detected in present study. Of these four genotypic virulence markers analysed Afa+Sfa (69.40%) and Hly (69.40%) were commonest genotypic virulence markers followed by Tra t (61.64%) gene and Pap gene (53.45%) was relatively less common genotypic virulence marker found in the present study. F. A. Oliveira (22) obtained Tra (76%), Pap (25%), Hly (5%), Afa (6%) and Sfa (26%) genes. Renate Bollmann(15) detected Tra t gene and iss gene responsible for serum resistance in 73.7% samples.

Since *E.coli* also possesses multiple virulence markers, the detection of genotypes of these virulence markers also displayed variation in the present study. It was observed that frequency of only single virulence marker was seen in 10.34% and any two virulence markers combination were obtained in 32.76%, any three in 34.05% and in 18.97% all four virulence markers were detected.

Adhesion and hemolysin are important and commonest virulence markers of uropathogenic *E. coli*.(23)&(24).Sfa and Afa genes represent S-fimbrial adhesions and A-fimbrial adhesions. In the present study both either individually or simultaneously were detected in 161 (69.40%) urinary strains.(table no.5) Afa was more common, positive in 117 (50.43%) while Sfa was positive in 70 (30.17%). Both were simultaneously positive in only 26 (11.20%) strains. Detection of both these genes helps in better detaection of adhesion. In earlier reports Afa and Sfa genes was found in 20% and 34% strains by MounaTarchouna et al (25) and 26% Sfa gene and 6% Afa gene positively was reported by F. A. Oliveira et al.(22).

Thus the results of the present study confirm the role of various virulence markers genes in uropathogenic *E.coli*. The gene responsible for four virulence marker detected in the present study, individually or together determine virulence of about 96.12% of *E.coli* responsible for causing UTI's .Afa and Sfa genes for Adhesion to uroepithelial is the most frequently appearing virulence markers followed by Hly gene for haemolycin and serum resistance Tra t gene while Pap gene for heamagglutination are relatively infrequent and probably just add to their pathogenic potential. More than one virulence markers can occur in the uropathogenic *E.coli* and relationship between their virulence and presence need to be further evaluated. There is may further add to their pathogenicity and can be considered as a surrogate marker of virulence of *E.coli*.

6. Future Scope

1) Since more than one virulence marker occur in uropathogenic *E.coli*. There individual relationship with virulence need to be further evaluated.
2) Likewise the role of individual virulence marker in causation of pyelonephritis and also complications of UTI need to be ascertained.
3) In asymptomatic bacteriuria further studies can be done as to presence of virulence markers and whether they are associated in the proportion of cases which further develop symptomatic infection.
4) More studies are also required to determine the presence of virulence markers in infections other than urinary tract caused by *E.coli*.Detection of virulence markers from *E. coli* isolates obtained from birds and animals can also provide the clue for transmission across humans and the animals or birds.
5) Since more than one gene can confer the virulence of one type, some more efforts could be made to search for more genes which determine the virulence markers.
6) Targeting of virulence markers for therapy as well as for vaccine preparations are yet other prospective approaches for treatment and prevention of such infections caused by E. coli.

Table 1: Concentration of primers used in PCR reaction mixture

PRIMER	Sequence	Product Size	Amount to be added
Pap3	GCAACAGCAACGCTGGTTGCATCAT	336bp	1.2 µl
Pap4	AGAGAGAGCCACTCTTATACGGACA		1.3 µl
sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410bp	1.2 µl
sfa2	CGGAGGAGTAATTACAACCTGGCA		1.2 µl
afa1	GCTGGGCAGCAAACTGATAACTCTC	750bp	1.3 µl
afa2	CATCAAGCTGTTTGTTCGTCCGCCCG		1.3 µl
hly1	AACAAGGATAAGCACTGTTCTGGCT	1177bp	1.1µl
hly2	ACCATATAAGCGGTCATTCCCGTCA		1.1 µl
tra T F	GGTGTGGTGCGATGAGCACAG	290bp	1.3 µl
tra T R	CACGGTTCAGCCATCCCTGAG		1.3 µl

Table 2: Prevalence Of Individual Genotypic Virulence Markers in Urine Samples

Total Genotypic VMs	Urine (%), N=232
Hly	161(69.40%)
Pap	124(53.45%)
Afa+Sfa	161(69.40%)
Tra t	143(61.64%)

Table 3: Frequency of Genotypic virulence markers, either alone or simultaneously together, detected in Urine (N=232) specimens

Combination of Genotypic VMs	URINE N=232	%
A. ONLY ONE VM	24	10.34
B. ANY TWO VMs	76	32.76
C. ANY THREE VMs	79	34.05
D. ANY FOUR VMs	44	18.97
TOTAL A+B+C+D	223	96.12

Table 4: Profile of Various Combinations of Genotypic Virulence Marker in Urine Sample

GENOTYPIC VM	URINE N=232	%
A. ONLY ONE		
HLY	1	0.43
TRA	3	1.29
PAP	9	3.88
SFA/AFA	11	4.74
TOTAL A	24	10.34
B. ANY TWO VMs		
HLY+TRA	15	6.47
HLY+PAP	6	2.59
HLY+SFA/AFA	20	8.62
TRA+PAP	8	3.45
TRA+SFA/AFA	6	2.59
PAP+SFA/AFA	21	25.00
TOTAL B	76	32.76
C. ANY THREE VMs		
HLY+PAP+SFA/AFA	12	5.17
HLY+TRA+PAP	20	8.62
HLY+TRA+SFA/AFA	43	18.53
TRA+PAP+SFA/AFA	4	1.72
TOTAL C	79	34.05
D. ALL FOUR VMs		
HLY+TRA+PAP+SFA/AFA	44	18.97
TOTAL A+B+C+D	223	96.12

Table 5: Positivity of SFA and AFA genetic virulence markers

SFA/AFA	POS	NEG	TOTAL
POS	26(11.20%)	44(18.96%)	70(30.17%)
NEG	91(39.22%)	71(30.60%)	162(69.82%)
TOTAL	117(50.43%)	115(49.56%)	232(100%)

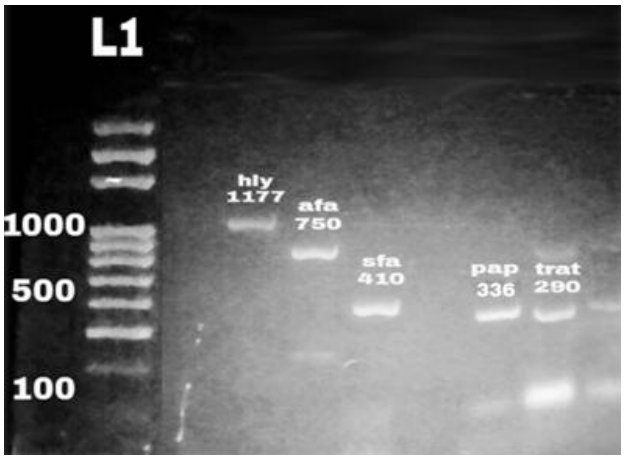

Figure 1: Polymerase chain reaction products shown on agarose gel

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Author Profile

Mrs. Pallavi Shital Wanjari works as Research Associate inVishakha Clinical Microbiology Laboratory Nagpur. She submitted her Ph.D. thesis entitled "Study of Biological Virulence Factors of Uropathogenic *EscherichiaColi* and Detection of the Virulence Genes.**"**In RTM Nagpur University, January 2019. Under guidance of Dr. Vinay Tule, Eugeniks laboratory, Nagpur. She did M.Sc in Biotechnology from RTM Nagpur University. She served as Ad-Hoc lecturer for UG and PG in Biotechnology and also institute for laboratory medicine. She has two paper published in international journal and presented three poster in National and International conferences.

Dr. Yagnesh Shirish Thakar owns **Vishakha Clinical Microbiology Laboratory**. He is specialized in Microbiological and molecular diagnosis of Infectious diseases. He is Consultant Microbiologist, **Care Hospital**, Nagpur. He did MBBS from Govt. Medical College, Nagpur and MD in Microbiology from Govt. Medical College, Nagpur. He did M.A. in Public Administration from Nagpur University. He is Former Professor at Peoples Medical College, Bhopal and Indira Gandhi Govt. Medical College, Nagpur. He has 30 Research Articles published in National & International Journals and authored a book on Microbiology for MBBS students. He co-authored three manuals on Clinical Microbiology and Immunology. He authored a manual on Hospital Infection Control Methods. He is Past President of Maharashtra Chapter of Indian Association of Medical Microbiologists and Past President of Vidarbha Association of Medical Microbiologists. He is associated with Masonic Lodge, Lions and PEACE Foundation.

Dr. Vinay Tule owns Eugeniks Genetic Laboratory, Nagpur. He is Post-Doctoral training: Ducan Guthrie institute of medical genetics, Glasglow, Scotland, UK (1990, 1995). PhD: Nagpur University, Nagpur (1987) and M.Sc.: Department of Microbiology, Nagpur University, Nagpur (1983). He is recognized as supervisor for PhD by Nagpur University in the field of Biotechnology, Biochemistry, and Microbiology. He has attended more than 27 Conference/ seminar/ workshop/ symposium and more than 12 lectures are delivered on invitation at different colleges and conferences. Awards and Honours in his name includes Best paper award at biotech symposium, Nagpur, 2013. Best net trophy in AOC-I-C olf tournament, Nagpur in 2013. Best Rotrian award, Rotary club Nagpur West. Best Rotrian couple award, Rotary club Nagpur West. Most active new Rotrian, Rotary club Nagpur West. Best Director award, Rotary club Nagpur West. Best past President award, Rotary club Nagpur West. Best most active past President award, Rotary club Nagpur West. Best alumni award from Somlwar High School Alumani Association. He has more than 10 research paper published in National and International Journals.