ORIGINAL RESEARCH PAPER

Microbiology

EXPRESSION OF TYPE 1 FIMBRIAE ENHANCES VIRULENCE OF UROPATHOGENIC E. COLI BY BIOFILM FORMATION

KEY WORDS: E. coli, Biofilm, Urinary Tract Infection, fimH

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ABSTRACT

BACKGROUND: Many bacterial infections are associated with biofilm formation. It is one of the important virulent factors of *E. coli* in urinary tract, causing recurrent and drug resistant infections. Fecal *E. coli* colonize the urethra and spread up the urinary tract to the bladder and kidney. Type 1 fimbriae are surface located adhesion organelles of *E. coli* that are directly associated with adherence to the urinary tract. The present study was aimed to study biofilm production in *E. coli* isolated from urinary tract infection and to correlate it with expression of *fimH* gene and compare its sequences.

METHOD: Total 150 urine samples were processed for isolation and identification of uropathogens. *E. coli* isolates were further processed for detection of biofilm by TCP method and screened for the presence of *fim*H gene by PCR using specific primers. The PCR products were purified and sequenced bidirectionally by Sanger dideoxy sequencing system using ABI 3500 Genetic analyzer.

RESULTS: From the total 98 urine samples with significant bacteriuria, 77 *E. coli* were isolated out of which, 40 were positive for *in vitro* biofilm production. Among them 11 were classified as strong biofilm producers and 29 as moderate. The *fimH* gene from *E. coli* isolates was amplified using specific primers and appeared as a band of about 508bp on agarose gel. It was noted that the *fimH* gene was detected in moderate and strong biofilm forming *E. coli* while absent in non biofilm isolates. The sequences showed 99% similarity with *fim H* gene of *E coli.*

CONCLUSION: The high binding ability of *fimH* could result in increased bacterial binding to target cells and increased pathogenicity of *E. coli*.

INTRODUCTION

Type 1 fimbriae are adhesion organelles expressed by many Gram negative bacteria. They facilitate adherence to mucosal surface and inflammatory cells as well as solid surfaces *in vitro*. This adherence results in biofilm formation on the surfaces thus contributing to the virulence of the strain as it leads to recurrent and drug resistant infections. *E. coli* is the most frequent pathogen responsible for up to 80% of UTIs [1].

Uropathogenic *E. coli* (UPEC) have special virulence factors, including pilli or fimbriae, which mediate attachment to uroepithelial and vaginal cells, resistance to human serum bactericidal activity, haemolysin production and increased amount of k capsular antigen [2]. The bacterial attachment permits bacteria to resist mechanical elimination by the flow of urine thus increasing persistence of *E. coli*. UPEC also produce different types of adhesions, including type 1 fimbriae, which are essential for recognition and attachment to urinary tract receptors [3]. Among adhesions of UPEC, the adhesive subunit of type 1 fimbriae, *fimH* is a major determinant, which has high tropism for urinary tract receptor, thus, *fimH* adhesion is important in colonizing different niches of *E. coli* [4]. Surface attachment is mediated by *fimH* adhesion, placed at the tip of the type 1 fimbriae, which prevents bacterial washout by urine flow and starts bacterial invasion [5,6]. Recurring UTIs increase the risk for pyelonephritis and long-term renal damage. The emergence of drug-resistant uropathogens presents a further clinical challenge [7]. The present study was aimed for *in vitro* qualitative estimation of biofilm production in *E coli* isolated from urinary tract infection and to correlate it with expression of Type 1 fimbriae by amplification of *fimH* gene by PCR using specific primers. The isolated genes were sequenced and compared.

MATERIAL AND METHODS

Clinical isolates

A total of 150 clean catch midstream urine samples were collected from patients in sterile containers visiting OPDs and IPDs of tertiary health care organization from Amravati district and immediately transported to the laboratory for further analysis.

Quantitative Estimation of UTI

A measured amount of urine i.e.0.01ml was inoculated by using calibrated loop method on Hi-chrome UTI agar (Hi-Media, India) and the plates were incubated at 37° C for 24 h. After incubation colonies were counted. CFU $>10^5$ was considered as significant bacteriuria.

Detection of biofilm formation by UPEC using Tissue culture plate Method (TCP)

TCP method described by Christensen *et al,* 1995 is considered the Gold-standard method for biofilm detection [9]. Isolated UPEC were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture plates (Sigma-Aldrich, Costar, and USA) were filled with 200 µL of the diluted cultures. The plates were incubated at 37°C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using ELISA reader at wavelength 570 nm. OD value <0.120 was considered non biofilm forming, 0.120-0.240 was considered moderate and >0.240 as strong biofilm producing.

DNA Extraction

Three types of strains of UPEC i.e. strong, moderate and non-biofilm forming were selected for DNA extraction. Genomic DNA templates for PCR amplification were gained from overnight growth of UPEC on Luria-Bertani broth (Hi Media, India). Bacterial culture (1.5 ml) was collected in eppendorff tube and centrifuged. Pellet was resuspended in 700µl of cell lysis solution at room temperature, incubated for 5 minutes and centrifuged at 10,000 rpm for 10 minutes. Supernatant (500µl) was collected in a fresh vial and 1ml of alcohol was added and mixed till DNA precipitated out. DAN was pelleted out by centrifuging and washed twice with 70% alcohol. DNA was dissolved in 100 µl of TE buffer (pH 8).

Polymerase Chain Reaction

The isolated DNA from UPEC was subjected to screening for

the presence of the *fimH* gene by PCR. The nucleotide sequence of the primers for amplification of the *fimH* gene is shown in Table 1.

Table – 1 Primers Used For *Fimh* Amplification

Gene	Size (bp)	Description	Primer sequence (5'-3')
fimH	508bp	Type 1 fimbriae	F-TTGAGAACGGAT AAGCCGTGG R-GCAGTCACCTGC CCTCCGGTA

PCR was performed in a 25µl reaction mixture containing 1.5U of Taq DNA polymerase, 2.5µl of 10× reaction buffer, 2µl of 2.5mM dNTP mixture, 10pmol of the forward and reverse primer and 0.5µl of DNA template. The PCR conditions were an initial denaturation at 94°C for 5 minute followed by 35 cycles of 94°C for 1min, 58°C for 1 minute and 72°C for 1 minute and then a final extension at 72°C for 5 minute. PCR products were analyzed by agarose gel (1%) electrophoresis using 200bp ladder.

DNA Sequencing

The PCR products were purified and sequenced bidirectionally by Sanger Dideoxy sequencing systems using ABI 3500 Genetic analyzer using ABI Big dye Tm terminator cycle sequencing kit at Central Instrumentation Centre at Sant Gadge Baba Amravati University, Amravati.

RESULTS

Quantitative estimation of urine samples

A total of 150 urine samples were analyzed during the period of study. Out of 150 urine samples, 98 samples had significant bacteriuria with the count more than 10^5 cfu/ml, 40 had insignificant growth and 12 samples no growth was observed.

Bacterial isolates from urine samples

Out of 98 urine samples with significant count, *Escherichia coli* was isolated in 77, *Klebsiella pneumoniae* in 10, *Enterococcus faecalis* in 9, *Staphylococcus aureus* and *Micrococcus* 1 in each sample (Fig 1).

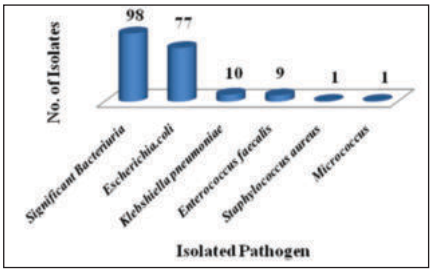


Fig 1. Bacterial isolates from urine sample Biofilm formation

Quantitative Tissue Culture Plate method of biofilm screening was done for 77 isolates of E. *coli* out of which 40 isolates were positive for biofilm formation (Fig 2).

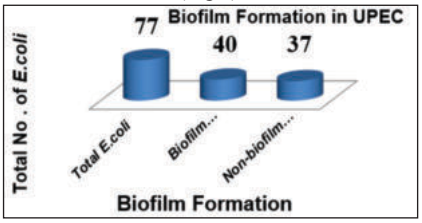


Fig 2. Number of biofilm forming and non-biofilm forming UPEC

They were categorized as strong, moderate and weak biofilm forming as explained in methodology. Wells with strong biofilm forming UPEC were stained dark as compared to wells with moderate and weak biofilm forming UPEC (Photo plate 1). Last horizontal and vertical rows were kept as controls containing only trypticase soy broth.

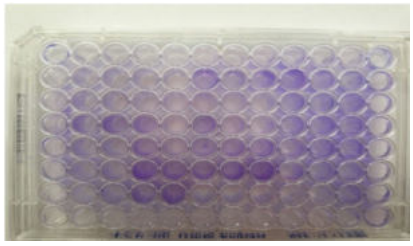


Photo plate 1: Tissue Culture Plate showing Biofilm formation by UPEC

Absorbance of the stained TCP was read by ELISA reader and the OD values <0.120 was considered as non biofilm forming, 0.120-0.240 as moderate and >0.240 as strong biofilm producing. Greater the absorbance more is the density of the biofilm formed on the walls of TCP wells indicating enhanced adherence of UPEC. Thick biofilm will also contain more number of bacteria embedded within the biofilm matrix. Below graph represents comparative range of the OD values of strong, moderate and weakly adherent UPEC isolates. The adherence was noted as high as 0.57 nm indicating maximum biofilm formation (Fig 3).

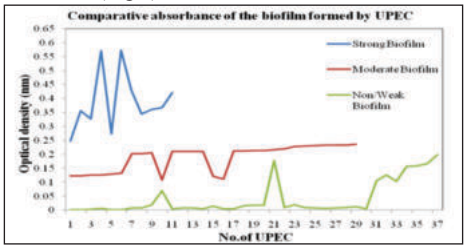


Fig 3: Absorbance of the biofilms formed by UPEC in the wells of tissue culture plate

Out of the total 40 biofilm forming UPEC, 11 isolates were classified as strong biofilm producing and 29 were moderate biofilm producing (Fig 4).

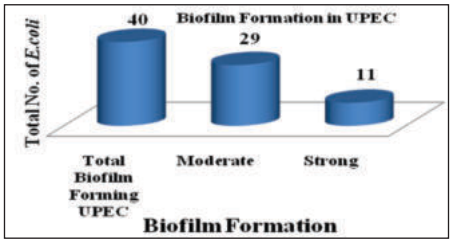


Fig 4. Total number of moderate and strong biofilm forming UPEC Polymerase Chain Reaction

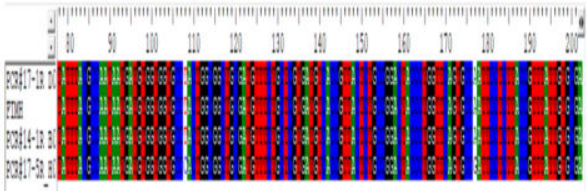
The *fimH* gene was amplified using specific primers and appeared as a band of about 508 bp on Agarose gel. The *fimH* gene was found in all the selected strong and moderate biofilm forming UPEC. But the gene was absent in the non-biofilm forming isolates of UPEC (Photo plate 2).

Photo plate 2: Polymerase Chain Reaction Amplification of the *fimH* Gene

Lane 1- Empty
Lane 2- Ladder
Lane3- Moderate biofilm forming UPEC
Lane 4-Non biofilm forming UPEC
Lane 5- Strong biofilm forming UPEC

Sequencing

Of total nine cultures, four strong biofilm forming and five weak biofilm forming strains were selected and the *fim*H gene was sequenced partially. The sequences were trimmed and blast search was conducted using nBlast. The sequences showed 99% similarity with *fim*H gene of *E coli*. The sequences were aligned with *E coli* strain k 12 sub strain MGI 655's *fim*H sequence. The *fim*H gene sequences of all weak biofilm forming UPEC and the standard *E coli* strain k 12 sub strain MGI 655's *fim*H sequence were 100% similar. All strong biofilm UPEC *fim*H sequences were also similar. For final sequence alignment, two representative sequences of weak UPEC, one of strong UPEC and that of standard strain was used. The alignment is shown in figure 5. The major difference were found in strong biofilm strain of *E coli* at base position number 108, where T was replaced by C and base position 177, where A present in weak biofilm forming strain was replaced by G. The sequences were submitted to NCBI. Accession numbers for weak biofilm forming strain is MZ456240 and for strong biofilm strain is MZ456241. The sequences were aligned using Bio Edit software [21].



#17-1R DO and #14-1R BO: weak biofilm forming UPEC
#17-5R HO: strong biofilm forming UPEC
FIMH: *E coli* strain k 12 sub strain MGI 655

Fig 5. Sequence alignment of *fim*H gene of weak and strong biofilm forming UPEC with standard strain of E coli

DISCUSSION

Overall the study demonstrated that, *E. coli* is the most frequent isolate of urinary tract infection and have high propensity to form biofilm. From 150 urine samples, 98 had significant count out of which 78% isolates were *E. coli*. *E. coli* is the most common etiological agent involved in UTI, accounting for up to 80% of UTIs [10]. Bacterial adherence mediated by fimbriae is an essential prerequisite for colonization of the urinary tract. Uropathogenic *Escherichia coli* express a number of different adhesive organelles, including P, type 1, S, and F1C fimbriae [11]. Type 1, or mannose-sensitive, fimbriae are produced by >80% of all uropathogenic *E. coli*.

In the present investigation, adherence of all UPEC was studied using quantitative Tissue Culture Plate method. It was found that, 11 (14%) isolates displayed strong adherence, 29 (37%) moderate and 37 (48%) were weakly adherent to the walls of tissue culture plate. In the study conducted by Sharma *et al* (2009), the significant production of biofilm was 54% in uropathogenic *E. coli.* The study conducted by Matija *et al (*2009) demonstrated that 53% strains of UPEC were biofilm producing. In the present study the biofilm forming UPEC were 52%.

The expression of type 1 fimbriae by *E. coli* is a virulence factor for pathogenesis of the urinary tract [14, 15] in the sense that they can produce a biofilm in within the urinary tract. The fimH protein is the receptor-recognizing element of type 1 fimbriae. *Fim*H is located at the organelle tip in a short fibrillum and perhaps additionally intercalated along the fimbrial shaft. The components of the fimbrial organelle are encoded by the chromosomally located *fim* gene cluster. The *fim*H protein is produced as a precursor of 300 amino acids and is processed into a mature form of 279 amino acids. *Fim* H is exported, from the periplasm and across the outer membrane, is dependent on a fimbriae-specific export and assembly system constituted by the *Fim*C and *Fim*D proteins [11].

Expression of *fimH* gene of *E. coli* promotes adhesion, colonization and lead to increased rate of urinary tract infections. The onset of UTI results from the ability of UPEC attachment to urinary tract epithelial cells by specific adhesions including type 1 fimbriae [16]. The *fimH* gene from all the UPEC isolates was amplified using specific primers. The gene was a band of about 508 bp on electrophoresis (fig 4). It was interesting to note that; *fimH* gene was present in all the 40 strong and moderate biofilm forming UPEC. But the gene was absent in the non-biofilm forming isolates of UPEC, thus demonstrating that *fim*H is required for the adherence of UPEC. Almost 52% UPEC were positive for *fim*H gene.

In the study conducted by Hojati *et al* (2015) the *fimH* gene was found in 130 isolates (92.8%) of the UPEC strains. Of 130 isolates positive for the *fimH* gene, 62 (47.7%) and 68 (52.3%) belonged to hospitalized patients and outpatients, respectively. The study indicated that more than 90% of *E. coli* isolates harbored the *fimH* gene. In the study conducted by Nam *et al* (2013), all virulence genes tested (including adhesins, iron acquisition, and protectin), except toxin genes, were detected among the canine UPEC isolates. Schembri *et al* (2000) reported that UTI *fimH* variants possess adaptations that enhance biofilm formation and suggests a novel role for *fimH* in UTIs associated with medical implants such as catheters. The study conducted by Krogfelt *et al* (1990), stated that type 1 fimbriae of *Escherichia coli* is surface organelle which mediate binding to D-mannose-containing structures. Investigation of *FimH* gene occurrence was performed by Samah *et al* (2020) in *E coli* isolated from clinical and environmental samples. The biofilm formation was detected in 66.6% strong, 33.30% moderate and weak biofilm producers not exist in clinical isolates, while 41% strong, 34.70% moderate and 8.60% for weak biofilm formation in environmental isolates. PCR assay of *FimH* showed that 95.23% were positive results for clinical isolates and 73.9% positive for environmental.

Uropathogenic *E. coli* had the higher occurrence rate for fimbriae gene (*FimH*) compared with environmental isolates, stronger biofilm formation by clinical *E. coli* isolates than by environmental isolates. There was a significant correlation between biofilm formation and probability of *FimH* occurrence in both clinical and environmental isolates. Swaine *et al* studied the Positive selection identifies an *in vivo* role for *Fim*H during urinary tract infection in addition to mannose binding. They performed *in silico* analysis of *Fim*H gene sequences from 279 *E. coli* strains identified specific amino acids evolving under positive selection outside of its mannose-binding pocket. They found that mutating two of these residues (A27V/V163A) had no effect on phase variation, pilus assembly, or mannose binding in vitro. However, compared to wild-type, this double mutant strain exhibited a 10,000-fold reduction in mouse bladder colonization 24 h after inoculation and was unable to form IBCs even though it bound normally to mannosylated receptors in the urothelium. In contrast, the single A62S mutation altered phase variation, reducing the proportion of piliated cells, reduced mannose binding 8-fold, and decreased bladder colonization 30-fold in vivo compared to wild-type. They concluded that positive selection analysis of *Fim*H has separated mannose binding from in vivo fitness, suggesting that IBC formation is critical for successful infection of the mammalian bladder, providing support for more general use of *in silico* positive selection analysis to define the molecular

underpinnings of bacterial pathogenesis.

CONCLUSION

We can conclude that type 1 fimbriae are present among biofilm forming UPEC to the extent that the *fimH* gene was detected in all strong and moderate biofilm forming UPEC and absent in non-biofilm forming UPEC. The high binding ability of *fimH* could result in increased bacterial binding to target cells and increased pathogenicity of *E. coli*. Thus *fimH* could be used to design vaccine for prevention of *E. coli* infections by blocking the bacterial attachment and colonization. In addition, *fimH* could be used as a tool for the extension of rapid detection-based assays as well as to differentiate between biofilm and non biofilm forming pathogens. Sequencing of the isolated *fim*H gene matched with 99% with *E coli fim*H gene and showed little difference in base position in strong and weak biofilm formation.

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