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Phylogenetic group B2 expressed significant biofilm formation among drug-resistant uropathogenic *Escherichia coli*

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ABSTRACT

Biofilm is an important virulent marker attributed to the development of urinary tract infections (UTIs) by uropathogenic *E. coli* (UPEC). Drug-resistant and biofilm-producing UPEC are highly problematic causing catheter-associated or recurrent UTIs with significant morbidity and mortality. The aim of the current study was to investigate the prevalence of biofilm formation and phylogenetic groups in drug-resistant UPEC to predict their ability to cause disease. This prospective study was conducted at the Department of Microbiology, University of Karachi from January to June 2019. A total of 50 highly drug-resistant UPEC were selected for this study. UPEC isolates were screened to form biofilm by Congo-red agar (CRA) and microtiter plate (MTP) technique. The representative biofilm-producing isolates were analysed by scanning electron microscopy (SEM) monitoring. Phylogenetic analysis was done by PCR method based on two preserved genes; *chuA, yjaA* and TspE4-C2 DNA fragment. On CRA 34 (68%) UPEC were slime producers, while on MTP 20 (40%) were strong biofilm producers, 19 (38%) moderate and 11 (22%) were low to negligible biofilm producers. Molecular typing confirmed that phylogenetic group B2 was prevalent in drug resistant UPEC strains. Pathogenic strains belonged to phylogenetic group B2 and D were found to have greater biofilm forming ability as compare to non-pathogenic commensal strains that belonged to phylogenetic group A. Our results indicate that biofilm formation vary in drug resistant UPEC belonged to different phylogenetic groups. This study indicates possible link between in vitro biofilm formation and phylogenetic groups of UPEC, therefore this knowledge might be helpful to predict the pathogenic potential of UPEC and help design strategies for controlling UTIs.

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1. Introduction

Urinary tract infections (UTIs) affect approximately 150 million individuals annually worldwide with significant health care expenditures [1]. It is the third most detected bacterial infection and a crucial health problem following respiratory and gastrointestinal tract infections [1–3]. Being a prevalent etiologic agent *E. coli* is responsible for 90% community acquired and 50% nosocomial UTIs affecting all age groups [4,5]. *E. coli* has the ability to form microcolonies in lining of mucosa of urinary bladder and surface of urinary catheters [6]. Biofilm like intracellular bacterial communities (IBCs) permits UPEC to colonize in bladder and resist expulsion hence play important role in pathogenicity [7].

Biofilms are structurally and dynamically complex biological systems comprising sessile community of microorganisms that are usually concentrated at solid–liquid interface. These are typically surrounded by an extracellular polymeric substance referred as slime consists of exopolysaccharide, protiens and DNA that facilitate adherence to the abiotic or biotic surfaces and microbial aggregation [8,9]. Biofilm also makes organisms more virulent, resistant to antimicrobial drugs and host immune responses [10–12].

It is estimated that biofilm is accountable for 80% of all microbial infections and over 65% of nosocomial infections [7,13,14]. Biofilm also plays an important role in horizontal gene transfer (HGT) facilitated by highly dense cells in close proximity [15] which facilitate movement of resistance genes and virulence factors, especially under selective pressure of antibiotic (s) [10,16]. Bacteria in biofilm are metabolically less active, resistant to exogenous environmental stress, like antibacterial agents, due to mass transfer limitations in the biofilm matrix [17].

Clermont's original typing scheme generally divided *E. coli* isolates into four categories; group A, group B1, group B2, and group D [18]. These groups can be determined with the help of genetic markers (*chuA, yjaA* and TspE4.C2 DNA fragment). *chuA* encodes outer membrane hemin receptor gene that involves in heme transport. *yjaA* encodes for gene responsible for cellular response to hydrogen peroxide and acid stress and TspE4.C2 DNA encodes for putative lipase esterase gene [19,20].

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It is presumed that microbial biofilm is associated with bacterial pathogenicity and imparts a great impact in the progression of urological infections and treatment outcome [21]. Moreover, phylogenetic background also plays a significant role in virulence of *E. coli* strains [22]. To the best of our knowledge, the biofilm-forming ability of UPEC in association with phylogenetic background has not been reported from Pakistan. Therefore in this study we aimed to find out association between phylogenetic groups and biofilm-forming ability of UPEC from Pakistan. Understanding the relationship between biofilm formation and phylogenetic groups might prove critical to predict the pathogenic potential and to establish novel strategies for controlling UTIs.

2. Materials and methods

2.1. Bacterial strains

The study included 50 drug-resistant UPEC strains obtained from urine specimens (taken from patients with recurrent infections and suffering from urosepsis) with bacterial count $\geq 10^5$ CFU/mL submitted to clinical laboratory of tertiary care hospital in Karachi, Pakistan for routine culture and antimicrobial susceptibility testing. All UPEC were isolated on MacConkey's agar (Oxoid) and subjected to further confirmation by conventional biochemical tests [23].

2.2. Qualitative screening of biofilm formation on CRA

UPEC strains were grown on Congo-red agar (CRA) for slime production by method modified by Freeman et al. [24]. CRA was prepared by Brain Heart infusion agar with addition of sucrose (5 g/100 ml) and Congo-red (0.8 g/l). Aqueous solution of Congo-red was prepared separately, autoclaved at 121°C for 15 min, and added to agar when cooled down at 55°C. After inoculation of bacterial strains, plates were incubated for 24 to 48 hours at 37° C. CRA permits the detection of slime-producing bacteria by distinction of colour change of colonies. The colour of colonies was evaluated by six-colour reference scale for fine classification given by Arciola et al. [25]. Like; very black, black, and almost black colonies indicative of slime production considered as positive results, and Bordeaux, red, and very red classified as negative results for strains unable to produce slime.

2.3. Biofilm formation by micro titer plate (MTP) technique

Biofilm assay was performed by the method described by George A. O'Toole et al. [26]. UPEC isolates were inoculated in Luria Bertani (LB) broth overnight at 37°C. Aliquots of 20 µL of culture were inoculated in 180 µL LB broth in flat bottomed 96 well MTP in triplicate at 37°C, in static condition. After 48 h incubation, wells were gently washed, three times with water and dried at 65°C. Subsequently 200 µL of Crystal violet (0.1%) was added in each well of MTP and left for 30 min, at room temperature. MTP wells were washed three times and dried at room temperature overnight. Crystal violet was solubilized in 200 µL of 30% glacial acetic acid and absorbance was measured at 590 nm using microplate reader (Synergy, HTX Multimode reader). The optical density (OD) of the negative control wells was subtracted from the OD of each tested well by using formula BF = AB-CW, where BF = Biofilm formation, AB is the OD at 590 nm of stained bacteria, and CW is the OD 590 nm of control well having medium without bacteria.

2.4. Biofilm formation on glass slide

Biofilm forming potential of all UPEC isolates was evaluated on glass slides, as described earlier by Mirani & Jamil [27]. Cultures were refreshed and inoculated in 3 mL LB broth. Three glass slides were submerged in 50 mL LB broth and were autoclaved. Overnight broth cultures (1:100 dilution) were inoculated in 50 mL LB broth containing 3 glass slides and incubated at 37°C. 1st, 2nd, and 3rd slides were taken out after 24 h, 48 h and 72 h, respectively. Slides were washed and stained with Crystal Violet (1%), eluted in 5 ml of 95% ethanol and absorbance was taken at 590 nm by using Spectrophotometer (UV visible Spectrophotometer, UV Pharmaspec 1700, Shimadzu).

2.5. Scanning electron microscopy (SEM) analysis

Selected strains of UPEC (biofilm positive and negative isolates) on glass slides were observed at 24, 48 and 72 h by SEM monitoring as described by [28]. Biofilms were negatively stained on glass slides with 0.2% uranyl acetate, washed with 70% ethanol solution, air-dried and the sample was gold-coated, upto 300°A. Biofilm formation was observed under an SEM (Jeol JSM-6380A, Japan).

2.6. Statistical analysis

Mean OD of biofilm formation among UPEC at 24 h, 48 h and 72 h (on glass slides) was determined by a repeated measure ANOVA with a Greenhouse-Geisser correction using SPSS version 16. P-value <0.005 was considered as statistically significant.

2.7. Determination of phylogenetic groups in UPEC

DNA from purified UPEC cultures was isolated by colony boiling method [29]. Triplex PCR was performed for molecular typing of UPEC isolates, targeting three genetic markers: *chuA, yjaA* and TspE4.C2, as described by Clermont et al. [18], Primer sequences and product

Table 1. Primers for the amplification of phylogenetic grouping of UPEC.

PCR reaction	Primer ID	Target Primer Sequence	PCR Product (bp)
Triplex	*ChuA*.1	GACGAACCAACGGTCAGGAT	279
	ChuA.2	TGCCGCCAGTACCAAAGACA	
	YjaA.1	TGAAGTGTCAGGAGACGCTG	211
	YjaA.2	ATGGAGAATGCGTTCCTCAAC	
	TspE4C2.1	GAGTAATGTCGGGGCATTCA	152
	TspE4C2.2	CGCGCCAACAAAGTATTACG	

sizes are listed in Table 1. PCR program was run as follows: initial denaturation at 95°C for 10 min, 30 cycles of 5 s at 95°C, 10 s at 59°C, 30 s at 72°C and a final extension step of 7 min at 72°C. *E. coli* (ATCC 25,922) was used as positive control. PCR products were observed on 2% agarose gel and saved using digital camera in Gel-Doc EZ Imager system (Bio-Rad).

3. Results

3.1. Congo-red agar method

A total of 50, drug-resistant UPEC strains were assessed for their biofilm-forming ability in-vitro by slime production on CRA. Out of 50 strains 34 (68%) were found to be biofilm producers. Among these, 19 strains showed very black colonies, six isolates showed black while nine isolates showed almost black colonies. UPEC isolates unable to form black colonies are classified as no slime producers within 24–48 hours according to colour classification scheme described elsewhere [25].

3.2. Biofilm formation by micro titer plate (MTP) technique

Quantification of biofilm by standard MTP method has divided UPEC into three categories, strong, moderate and weak/negligible adherent based on crystal violet staining (OD at 590). The results indicated 20 (40%) as strong, 19 (38%) as moderate and 11 (22%) as weak or negligible biofilm producers (Table 3).

3.3. Biofilm formation on glass slides and scanning electron microscopy (SEM) analysis

Biofilm formation on glass slides gives more clear information about biofilm-forming ability of UPEC strains (Figure 1). All UPEC strains were evaluated by this method. Majority were found to be biofilm producers, only few isolates showed negligible biofilm formation at 24 h that were also found to be non-slime producers by CRA as well as by MTP method. OD at different time intervals (24, 48 and 72 h) showed considerable variability most of the UPEC strains achieved high level of biofilm formation at 48 h with the exception of some strains that achieved peak at 72 h (Figure 2).

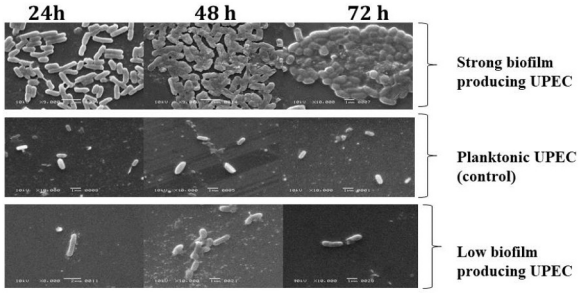

Figure 1. Scanning electron microscopy (SEM) analysis.

Biofilm producing UPEC can be differentiated from planktonic control *E. coli* by production of extracellular polymeric substance. Planktonic cells characterized by their intact structure throughout 24, 48 and 72 h while biofilm production characterized by cell to surface attachment, cell to cell interaction, cells aggregation and microcolonies formation leading to biofilm formation.

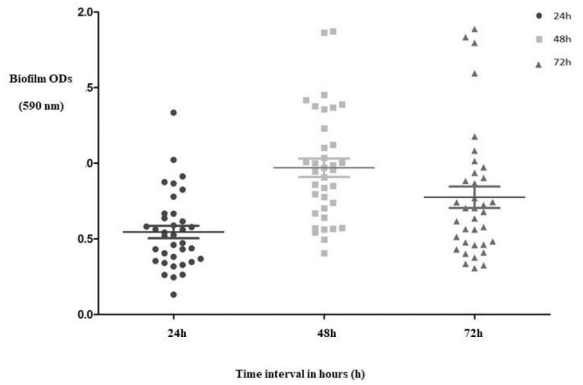

Figure 2. A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean OD of biofilm formation (on glass slides) differed statistically significantly between time points 24, 48 and 72 h ($p < 0.0001$) among *E. coli* ($n = 50$) isolated from urine specimen.

Biofilm formations on glass slides were selected for SEM analysis at time intervals of 24, 48 and 72 h to observe biofilm pattern while *E. coli* grown in broth was used as control for planktonic natural pattern. SEM is valuable tool for analysis of biofilm. Biofilm producing UPEC showed higher surface adherence in comparison to biofilm negative strains at 24 h. At 48 h SEM showed change in bacterial arrangements making community, while at 72 h this microcolony is marked by the presence of extracellular matrix while *E. coli* in planktonic form maintained its cell surface integrity at different time intervals (Figure 1) (Figure 3).

3.4. Phylogenetic groups

In order to classify 50 drug-resistant UPEC strains Clemont's original phylogenetic scheme was used which is based on 3 genetic determinants (*chuA, yjaA* and TspE4.C2 DNA fragment) (Figure 3). Among all the analysed UPEC strains the most pathogenic strains belonged to group B2 and to some extent

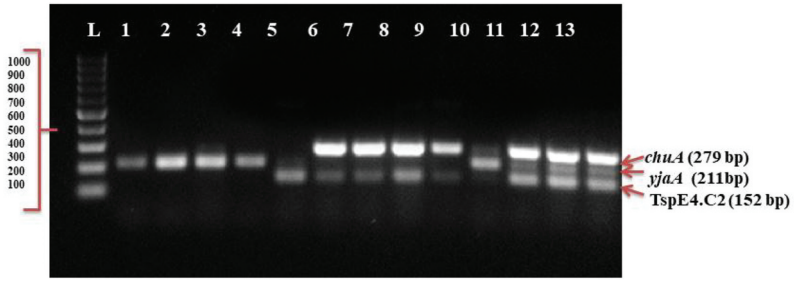

Figure 3. PCR amplification of *chuA, yjaA* and TSP.C2 genetic marker for UPEC strains.

Distribution of phylogroups in 50 UPEC isolates. L = molecular weight marker of 100 bp, Lane 1, 2, 3, 4 and 10 group A, Lane 5 group B1, Lane 6, 7, 8 and 9 group D and Lane 11 and 12 group B2 and Lane 13 *E. coli* ATCC 25,922 (positive control)

group D (Table 2). Non-pathogenic commensal UPEC were classified as group A and B1.

3.5. Association between biofilm formation and Clemont's phylogenetic scheme

Significant difference was examined in mean ODs of biofilm formation calculated at 48 h by MTP method in UPEC classified as per Clemont's original phylogenetic scheme. Majority of UPEC strains found in phylogenetic groups B2 and D were strong and moderate biofilm formers. Only one isolate belonging to phylogenetic group B1 showed strong biofilm-forming ability. Over all phylogenetic group A carried the highest number of low biofilm producers (Table 3).

4. Discussion

The bacterial adherence, aggregation and the growth on solid surfaces to form biofilm is an ancient survival strategy found in nature [30]. According to an estimation, less than 0.1% of the total microbial biomass present on earth is in the plankton form of growth while majority is in the aggregate condition, surrounded by an extracellular matrix as biofilm [31].

Table 2. UPEC phylogenetic grouping based on presence of DNA markers.

E. coli phylogenetic groups	No. of UPEC isolate (%)	Distribution on DNA markers (n)	*chuA* gene	*yjaA* gene	TSPE4. C2
Group A	15 (30%)	15	-	+	-
Group B1	1 (2%)	01	-	-	+
Group B2	24 (48%)	24	+	+	+
Group D	10 (20%)	10	+	-	+

According to a recent study, biofilm has now been considered as the default bacterial lifestyle, and it is thought that planktonic single cells are transitional lifestyle of bacteria [32]. Microbial biofilm is of great concern due to antibiotic treatments failure and host immunological defences [33].

As there is no standardized method for biofilm detection and because of multifactorial nature of biofilm we could not depend on single method. Therefore in the current study the ability of UPEC to produce biofilm was assessed by Congo-red agar, microtiter biofilm assay, glass slides in static uninduced condition and SEM analysis. Moreover, strain property, culture media and methodology have great impact on outcome of biofilm formation, in vitro conditions. CRA is a rapid method for screening of slime production in bacteria that gives clue about biofilm-forming ability of the strain. Congo red binds directly with polysaccharides and form colour complex [25]. Biofilm on glass slide has given the best results, especially in case of strains that are weak/non biofilm producers on CRA and MTP. This might be due to greater surface area available for biofilm growth on glass slides. SEM provides the facility to observe biofilm pattern closely in natural forms.

In successful biofilm development, the key event is the attachment to the surface leading to subsequent aggregation and mature biofilm formation. This enhances the stability to cause diseases and enhance its drug resistance capacity [34,35]. On glass slides, higher ODs of biofilm were obtained at 48 h while there is decrease in ODs at 72 h might be due to the process of biofilm shearing or erosion except for a few strains that showed higher ODs at 72 h [36]. At 72 h,

Table 3. Prevalence of biofilm formation (virulence) associated with UPEC phylogenetic groups.

Virulence mechanism	UPEC strains			
Prevalence of biofilm formation by MTP	**Phylogenetic groups**			
	A% (n = 15)	**B1% (n = 1)**	**B2% (n = 24)**	**D% (n = 10)**
Strong	13.33%(2)	100%(1)	62.5%(15)	20%(2)
Moderate	26.66%(4)	0%(0)	33.33%(8)	70%(7)
Low	60%(9)	0%(0)	4.16%(1)	10%(1)

Strong (OD ≥0.240), Moderate (OD 0.120–0.240) and Weak (OD≤0.120) based on crystal violet staining (OD 590). OD = optical density

biofilm was characterized by extracellular polymeric substance that is comparable with other findings [37]. Overall, all UPEC were able to form microcolonies on glass surface that showed different adhesion patterns clumps and chains were also visible. Frömmel et al. [38] had reported various adhesion patterns such as diffusely distributed bacteria, chain, clumps and micro-colonies while Gomes et al. [39], have reported that biofilm formed on glass showed a 37% higher elongation than those formed on silicon.

Phylogenetic background gives knowledge about ecological distribution and evolutionary history. Moreover phylogenetic analysis has also been reported to have important contribution in virulence of pathogens [40,41]. Generally variations in phylogenetic groups are associated with geographical region, site of infection and antibiotic resistance [42]. Moreover other factors include are health status of host, environmental and social conditions, dietary and host genetic factors and differences in sampling regions [43].

In this research study phylogenetic typing by Clermont's original phylogenetic typing scheme was evaluated. This method is highly congruent (2429 citations in September 2019 at www.ncbi.nlm.nih.gov) and still method of choice because of its simplicity and rapidity as described by recent studies [44–-44–46]. In the present study UPEC strains were mainly associated with phylogenetic groups, B2 and D; however, they have also been associated with phylogenetic group B1 [42]. These UPEC isolates might be considered community isolates with predisposing factors which assist UTIs particularly in immune compromised individuals [47]. While, commensal UPEC strain with ability to cause community-acquired UTIs have been associated with phylogenetic group B1, we identified only one UPEC isolate out of fifty that belonged to phylogenetic group B1. Phylogenetic group B2 was the most prevalent in UPEC strains as observed in other findings reported from Asia [40,41] and globally [48,49]. However, phylogenetic group A was second most prevalent group among drug-resistant UPEC strains that exhibit slight phylogenetic shift towards group A, such shift may possibly occur if resistance is more readily acquired or contained by certain phylogenetic group which may increase fitness for pathogen [50].

On combining all data together, majority of strong biofilm producers belonged to B2 phylogenetic group, while in group D isolates were found to be moderate biofilm producers. Overall, 68% of UPEC isolates belonged to B2 and D groups. It might be due to the presence of pathogenicity islands and expression of more virulence determinants like adhesion factors, cell surface hydrophobicity, siderophore and toxins production, etc. Moreover, *E. coli* phylogroup B2 is the most commonly associated with persistent infections. Majority of isolates from group A were low/negligible biofilm producers. Similar observation was seen in a previous study by Nielson et al. [7]. There seems to be a possible correlation between phylogenetic groups and biofilm phenotype. Chakraborty et al. also observed similar findings [51]. However in group A some strains also showed high to moderate biofilm capability.

Additionally data regarding antimicrobial resistance (accepted to be published in Pakistan journal of pharmaceutical sciences) indicated more than 90% to be multidrug resistant to three or more classes of antibiotics. However according to the susceptibility pattern, all strains were found sensitive to fosfomycin, imipenem and colistin and would be helpful to tackle these drug resistant biofilm-forming strains.

Drug-resistant UPEC strains are more likely to form biofilms that effect likelihood of risk biofilm-associated infections, dissemination of virulence factors and resistant determinants since the biofilm has potential role in recurrent and persistent infections.

5. Conclusion

The research work presented here indicated possible link between in vitro biofilm formation and phylogenetic typing in UPEC from Pakistan. Majority of drug-resistant UPEC strains belonging to phylogenetic group B2 and D (pathogenic strains) showed strong and moderate biofilm formation in vitro. Only one strain of UPEC belonging to group B1 and few strains in group A also showed strong biofilm-forming ability. Biofilm has the potential role in pathogenesis of recurrent UTIs, antibiotic resistance and also facilitates transfer of genetic material. In conclusion our findings contribute better understanding of biofilm-forming capabilities which is critical for designing novel strategies for controlling infectious diseases.

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Disclosure statement

All authors declare that there is no conflict of interest.

Ethical consideration

This study does not belong directly to any human or animal subject so the ethical approval is not required.

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