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Correlation Between *in vitro* Biofilm Formation and Virulence Properties of Extra-Intestinal Pathogenic *Escherichia Coli* (Expec)

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Abstract: About 65 *Escherichia coli* strains isolated from extra-intestinal sites were included in this study to determine their biofilm formation capability and virulence properties. About 87% ExPEC isolates were motile and 12.3% were found to be non-motile. Only 12.30% isolates were strong biofilm former (SBF >1.5) whereas 18.46% isolates were non biofilm former. About 71% isolates were capable to express curli but curli expression is lower in isolates from pus and peritoneal fluid. About 41.53% isolates have moderate capability (CSH value 21-50%) to adhere to hydrocarbons whereas 20% didn't show any hydrocarbon adherence capability. Most of the isolates showed moderate cell aggregation (60-80%) potential with *S. maltophila* but 15.4% isolates were found to be non-aggregative. Forty-one (63.07%) strains were resistant to 2 or more of the most commonly clinically used antibiotics. 44.6% isolates were haemolytic, 35.3% showed colicin activity, 47.7% were MRHA positive, 49.2% had SAT value ≤2.0 M and 9.2% were protease producer. About 77% (50 out of 65) of the isolates harbor type 1 gene. *csgA* gene was found in 61.5% (40) isolate, *papC* was found in 64.6% (42), *afa* and *sfa* genes were found in 67.7% (44) each. Biofilm formation may contribute to the pathogenic potential of ExPEC.

Keywords: *E. Coli*, Biofilm, ExPEC, Adhesion, Virulence

Introduction

Bacterial biofilms are complex communities of microorganisms embedded in a self-produced matrix andadhering to inert or living surfaces (Rijavec *et al*., 2008). Biofilms are architecturally complex assemblies of microorganisms that form on biotic or abiotic surfaces or at interfaces. Biofilms are characterized by interactions between microorganisms embedded in a matrix of extracellular polymeric substances created by the microbial populations and exhibit altered phenotypes with respect to growth rate and gene transcription (Taj *et al*., 2012). Biofilm production is considered as a marker of clinically relevant infection and persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections (Murugan *et al*., 2011). A role for bacterial biofilms in pathogenesis is well established for a number of infections and opportunistic pathogens (Lassaro *et al*., 2009). Biofilm mediates interaction between bacteria and host tissue through adhesion, which is pre-requisite for invaion and tissue-specific colonization (Ghanbarpour and Salehi, 2010). Biofilms are not only resistant to antibiotics but also to a variety of disinfectants which emphasizes that their characterization is an important aspect of infection control (Mathur *et al*., 2006). Biofilm also facilitates co-existence of otherwise competing bacterial species (Tirumalai and Prakash, 2012).

Extra-intestinal Pathogenic *E. Coli* (ExPEC) is a group of *E. coli* strains which are genetically diverse species that causes a variety of extra-intestinal infections (Fakruddin *et al*., 2012b) which fulfill many or all of the proposed criteria for biofilm-associated infections (Norouzi *et al*., 2010). Many of the key virulence factors are surface structures involved in adherence and motility which are critical for biofilm formation (Holden and Gally,

2004). Uropathogenic *E. coli* strains are frequently isolated from biofilms formed in the lumen of catheters, where they resist antibiotic treatment and shear forces (Uhlich *et al*., 2006). Based on these in vivo and *in vitro* observations, it is reasonable to predict that the pathotypes causing these infections possess genetic repertoires that enable formation of stable cell-cell interactions and biofilms under appropriate environmental conditions (Naves *et al*., 2008a). If true, the establishment of an *in vitro* biofilm system that reflects the *in vivo* biofilm formation of *E. coli* pathotypes would enable development of drugs directed against this virulence strategy (Reisner *et al*., 2006).

The present work was undertaken to assess the biofilm formation potential of *E. coli* isolated from extra-intestinal infections and to assess the correlation of biofilm formation with virulent properties.

Materials and Methods

Source of Isolates and Strains

A total of 65 clinical isolates of *Escherichia coli* strains, of which 25 were from infected urine, 12 from peritoneal fluid, 12 from blood, 10 from pus and 6 from Cerebrospinal Fluid (CSF) were included in the study. 15 *E. coli* strains isolated from stool of healthy individuals were also included. All the isolates were previously identified and characterized as *E. coli* (Fakruddin *et al*., 2012a). *Streptococcus pyogenes*, Nonpathogenic *E. coli* ATCC-35218, *E. coli* ATCC-25922, *E. coli* K-12 (Col^-), *E. coli* K-12 Col V^+, *Pseudomonas aeruginosa* ATCC-10145 were also included in the study as control. All the isolates were identified and preserved by stab culture in soft agar base and stored at 4-8°C.

Determination of Motility

This was done according to the method adapted from Sperandio *et al*. (2002). Overnight cultures of LB broth were used to assay motility in plates containing 0.4% (w/v) agar. Diameter of motility halos was to be measured. Bacterial strains were propagated in Luria-Beftani (LB) broth medium containing 5 g NaCl per liter. The pH was adjusted to 7.0, when motility assay was done in LB soft agar (0.4%). The plates were incubated at room temperature overnight in a very strict static condition.

Biofilm Formation Assay

Biofi1m formation assays were performed following the method of Danese *et al*. (2000) with some modifications. All strains were grown overnight in tubes containing 5 mL Luria-Bertani (LB) (Sigma, St. Louis, USA) at 37°C. Aliquots of 10 µL culture from overnight broth were inoculated into 1 mL LB broth (dilution 1: 100) and then was inoculated into non cell-treated, 96 Well polystyrene microtitre plates (Nunclon™, Denmark) and incubated overnight at 30°C without shaking. The edge of the plate was covered with parafilm to avoid evaporation. The Optical Densities (ODs) of the overnight growths were read at a wave length of 630 nm. The broth was then removed and the wells rinsed once with 200 µL of PBS and air dried. The wells were then stained with 120 µL of 0.1% Safranin for 30 min at 30°C; the colorant was then discarded and the wells rinsed three times with 200 µL of PBS. The adhered safranin was solubilized in 120 µL of Dimethyl Sulfoxide (DMSO). After 5 min at room temperature the ODs of the attached and stained bacteria and control wells were read at 490 nm wavelength. The assays were performed in triplicate. Biofilm measurements were made using the formula SBF = AB-CW/G in which SBF is the specific biofilm formation, AB is the OD_{490} nm of the attached and stained bacteria, CW is the OD_{490} nm of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values) and G is the OD_{630} nm of cells growth in broth (Niu, Gilbert, 2004). The SBF values were classified into two categories: Strong biofilm producers (SBF index>1.00) and weak biofilm producers (SBF index <1.00).

Curli Expression

Expression of curli was visualized by growth of test strains on Congo Red (CR; Sigma) medium containing 2% agar. In this medium 0.004% congo red and 0.001% coomassie blue were added after sterilization of the medium. The strains that express the gene appear as pink colony on the CR medium. The plates were incubated at 28°C for 2 days (Norouzi *et al*., 2010).

Microbial Adhesion to Hydrocarbons

Microbial surface hydrophobicity was assessed with xylene (Merck) according to Rosenberg and Gutnick (1980). All isolates including standard strain were grown into nutrient broth (50 mL) in a 250 mL erlenmeyer flask with shaking in 200 rpm. Cells were harvested by centrifugation (10000×g, 15 min), washed twice in sterile phosphate-buffered saline (pH 7.1) and suspended in the same buffer to an initial Optical Density (OD) of about 1.0 (A0) at 600 nm. Next, 300 µL of xylene was added to 3 mL of microbial suspension and vortex for 2 min. After 10 min the OD of the aqueous-phase was measured (A1) at 600 nm. The degree of hydrophobicity was calculated as [1-(A1/A0)] 100 [%].

Cell Aggregation Assay

Overnight cultures of *E. coli* and *Stenotrophomonas maltophilia* were re-suspended in fresh 0.1×LB. Two mixtures of *E. coli* with 10-fold less and with the same amount of *S. maltophilia* cells were prepared. Pure *E. coli* and the two mixtures were added into 5-mL test

tubes (Fisher Scientific, Pittsburgh, PA) (3 ml/tube) to form a culture column and set static on the bench for 24 h. Cells were allowed to adhere together to form aggregates, which gradually settled down due to gravity. As a result, the cell density in the top layer of the culture column decreased. *E. coli* cell aggregation was reported as the relative decrease in cell density (percentage) due to aggregates formation and settling. *E. coli* cell density in both pure and mixtures was measured by the plate count method using selective agar (Abdallah *et al*., 2009).

Sensitivity to the Bactericidal Effect of Normal Serum

The sensitivity of *E. faecalis* to the bactericidal effect of human normal serum was tested as described by Pelkonen and Finne (1987). Bacteria grown in BHI for 18 h were diluted in PBS (10^9 bacteria/mL) and 175 µL of the bacterial suspension and 175 µL of PBS were pipetted into the wells of microtiter plates. One hundred microliters of serum (final concentration 36%) were added to the wells and the plates were incubated at 37°C. The absorbance at 630 nm was measured at 0, 30, 60, 90, 120 and 180 min. The plates were shaken before each measurement, to avoid the influence of bacterial sedimentation on the final absorbance. The strains were classified as resistant, intermediate or sensitive.

Virulence Properties

Detection of Haemolytic Strains

The haemolytic activity was observed on washed blood agar plates according to Sharma *et al*. (2007). About 65 *E. coli* clinical isolates and 15 *E. coli* fecal isolates were screened for haemolytic property. *Streptococcus pyogenes* was used as positive control.

Colicin Production Test

Colicin production was determined by method described by Fernandez-Beros *et al*. (1990). The colicin negative *E. coli* K-12 and Colicin V positive *E. coli* K-12 Col V^+ strains were used as control.

Haemagglutinin Test

Slide haemagglutination of erythrocytes was performed as described by Klosowska and Plotkin (2006).

Mannose sensitivity Test

The haemagglutination positive strains were used for mannose-sensitivity assay. The ability of D-mannose to inhibit haemagglutination was tested by using this sugar to pre-treat either human type O erythrocyte or bacteria (Najar *et al*., 2007).

Measurement of Bacterial Cell Surface Hydrophobicity

Salt Agglutination Test (SAT) was used to measure the bacterial cell surface hydrophobicity (Nalina and Rahim, 2006). An *E. coli* strain with a SAT value of 3M was used as negative control.

Protease Production

Protease production by *E. coli* was tested by observing hydrolysis of casein when grown on milk agar medium (Mansour *et al.*, 2014). *Pseudomonas aeruginosa* NCTC-6750 was used as positive control strains.

Antibiotic Susceptibility Testing

All the clinical isolates of *E. coli* were tested for antibiotic resistance by the standard agar disc diffusion technique described by (Bauer *et al*., 1966) on Mueller Hinton agar using commercial discs (Oxoid, UK). The following antibiotics with the disc strength in parentheses were used: Tetracycline (Tet, 30 µg), Streptomycin (Str, 10 µg), Ceftriaxone (Cef, 30 µg), Ampicillin (Amp, 25 µg), Chloramphenicol (Clr, 20 µg), Gentamycin (Gen, 30 µg), Penicillin (Pen, 10 µg), Ceftazidime (Caz, 30 µg), Polymixin B (Pol, 300 IU) and Nalidixic acid (Nal, 30µg). A control strain of *E. coli* ATCC 25922 was included in each plate. Antimicrobial breakpoints and interpretation were taken from the CLSI standards (CLSI, 2006).

DNA Extraction from Target Organisms

DNA from working cultures of target organisms were extracted by phenol/chloroform and ethanol precipitation method (Wilson, 1997). Bacterial cells were grown overnight in nutrient broth at 37°C, aerated by shaking at 120 rpm in a shaking incubator. Bacterial cells were harvested by centrifuging the culture at 10000 rpm for 5 min. The supernatant was discarded and cell pellet was taken. The cell pellet was washed twice with sterile physiological saline for removing residual culture medium from the cells and was subjected to treatment with DNA extraction solution I (Tris HCl + EDTA + sucrose) for 30 min at 37°C on a water bath in order to disrupt cells. Then de-proteinization was done using DNA extraction solution II (proteinase K + SDS + NaCl) at 55°C for 1 h on a water bath. Phenol: Chloroform: Isoamylalcohol mixture was used to precipitate proteins. The cell extract was mixed gently with the solvent.The nucleic acids were separated in the aqueous layer by centrifugation at 10000 rpm for 5 min. The aqueous solution of DNA was then removed using micropipette. The DNA was then concentrated by ethanol precipitation in the presence of Sodium acetate. After centrifuging and washing with 70% ethanol solution the final pellet was taken and suspended in TE buffer. This suspension was then stored at 4°C for further use (Fakruddin *et al*., 2012b).

Quantification and Purity of DNA

Quantification of genomic DNA was done using 1.0% agarose gel electrophoresis in 1X TAE buffer followed by

staining with ethidium bromide. The concentration of extracted DNA was also estimated by visual comparison of the band with 100 bp marker DNA. The purity and concentration of the extracted DNA was also checked by measuring absorbances on T60 UVVIS spectrophotometer at 260 and 280 nm. Purity was analyzed by absorbances ratios i.e., 260/280 nm (Sahasrabudhe and Deodhar, 2010).

Detection of Adhesion Genes

DNA samples stored at -20°C were subjected to PCR assay for the amplification of *type 1*, *pap C*, *afa*, *asgA* and *sfa* genes specific for adhesion and virulence properties of Enterotoxigenic *E. Coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. Coli* (EHEC) and Enteroinvasive *E. Coli* (EIEC) (Table 1).

Statistical Analysis

All the statistical analysis was carried out with SPSS17 software. Independent Sample T Test was used to check the comparison of CSH between ESBL- and non-ESBL-producing isolates. Mann-Whitney Test was used to compare biofilm formation of clinical isolates with or without ESBL. Data were expressed as mean values ± Standard Deviation (SD) and $p \leq 0.05$ was considered to be statistically significant.

Results

Determination of Motility

56% of the urinary isolates were highly motile (>10 cm zone diameter) whereas others are less motile but no urinary strains were found to be non-motile. More than 91% of the blood isolates were found to be motile. Isolates from peritoneal fluid, pus and CSF are less motile than urine and blood isolates. In general, about 87% ExPEC isolates were motile of which 43.08% were highly motile. About 12.3% ExPEC isolates were found to be non-motile. In contrary, only about 46% fecal *E. coli* isolates were motile (Table 2).

Biofilm Formation Assay

In biofilm formation assay, only 12.30% ExPEC isolates were Strong Biofilm Former (SBF>1.5) whereas 18.46% isolates were non biofilm former. About 44.5% ExPEC isolates were found to be moderate biofilm former whereas 24.61% were weak biofilm former. Urine and blood isolates have relatively more biofilm forming capability. Only 33.3% fecal isolates were found to be weak biofilm former and no fecal isolates were moderate or strong biofilm former (Table 3).

Curli Expression

In curli expression assay, 84% urine isolates expressed curli whereas 75% blood isolates did so. Isolates from CSF also able to express curli (66.67%), but curli expression is lower in isolates from pus (50%) and peritoneal fluid (58.33%). About 71% ExPEC isolates were capable to express curli whereas only 20% fecal isolates were able to express curli (Table 4).

Microbial Adhesion to Hydrocarbons

ExPEC isolates vary significantly in terms of their ability to adhere to hydrocarbons. About 41.53% ExPEC isolates have moderate capability (CSH value 21-50%) to adhere to hydrocarbons whereas 16.9% have strong capability. Abuot 20% ExPEC isolates didn't show any hydrocarbon adherence capability. Urine and blood isolates have better hydrocarbon adherence capability than isolates from peritoneal fluid, pus and CSF. Only 26.67% fecal isolates have weak adherence capability and 73.33% are non-adherent to hydrocarbon (Table 5).

Cell Aggregation Assay

In cell aggregation assay, most of the ExPEC isolates showed moderate cell aggregation (60-80%) potential with *S. maltophila*. About 27.7% isolate had 60-80 and 26.25% had 40-60% cell aggregation. About 15.4% of the ExPEC isolates were found to be non-aggregative. In contrast, most of the stool isolates (53.3%) were found to be non-aggregative. Only 6.67% stool isolates had 60-80% cell aggregation potential. Urine isolates had lower cell aggregation potential than peritoneal fluid, pus, blood and CSF isolates (Table 6).

Virulence Properties (Hydrophobic, Cell Surface Adhesion, Haemolysin)

Haemolysin Production

It was found that 29 (44.6%) clinical isolates of *E. coli* were haemolytic. Among the *E. coli* isolates from urine, blood, pus and peritoneal fluid, 15 (60.0%), 5 (41.67%), 4 (33.3%) and 5 (50.0%) strains, respectively, were haemolytic. While only four of the 15 faecal *E. coli* strains produced haemolysin.

Colicin Biosynthesis

Of the 65 clinical isolates of *E. coli*, 23 strains (35.3%) showed colicin activity when grown on trypticase soy agar (+0.6% yeast extract) medium. Of the colicin positive strains, 13 (52.0%) were isolated from urine, 6 (50.0%) from blood and 4 (33.3%) from peritoneal fluid. The colicin positive *E. coli* strains were further tested for colicin V biosynthesis. Among the clinical *E. coli* isolates, only the urinary and blood isolates produced colicin V; 6 (24.0%) urinary strains and 2 (16.7%) blood isolate showed colicin V activity. 7 (46.0%) of the control strains produced colicin, of which none was colicin V producer.

Mannose-resistant Haemagglutination (MRHA) test

The tests showed 31 (47.7%) clinical isolates of *E. coli* were MRHA positive, compared to 4 (26.7%) strains positive among the controls. Among the clinical *E. coli* isolates, 13 (52.0%) urinary strains and 5 (41.7%) blood strains were MRHA positive. None of the *E. coli* strains isolated from peritoneal fluid and pus gave MRHA positive reaction. In total 50 strains produced either hemolysin or MRHA or both. Of these, 22 strains produced both haemolysin and MRHA, 15 strains produced only haemolysin and 13 strains were MRHA positive but haemolysin negative.

Measurement of Cell-Surface Hydrophobicity

Salt Aggregation Test (SAT) showed that 32 (49.2%) clinical isolates of *E. coli* aggregated with ammonium-sulphate solution of ≤2.0 M concentration, whereas, 9 out of 15 (60%) of control strains had SAT value ≤2.0 M. Source-wise analysis showed that 18 (72%) urine isolates; 5 (41.7%) peritoneal isolates and 9 (75%) blood isolates had SAT value ≤2.0 M. A total of 29 strains had SAT value >2.0 M. whereas, 4 out of 15 (20%) of control strains had SAT value >2.0 M. Source-wise analysis showed that 14 (56%) urine isolates; 7 (58.7%) peritoneal isolates and 8 (66.7%) blood isolates had SAT value >2.0 M. A total of 24 strains had SAT value ≤1.0 M. whereas, 7 out of 15 (46.7%) of control strains had SAT value ≤1.0 M. Source-wise analysis showed that 15 (60%) urine isolates; 4 (33.3%) peritoneal isolates and 5 (41.7%) blood isolates had SAT value ≤1.0 M.

Protease Production

About 6 (9.2%) clinical isolates of *E. coli* were protease positive, compared to 3 (20.0%) strains positive among the controls. Among the clinical *E. coli* isolates, 2 (13.3%) urinary strains, 1 (8.33%) peritoneal strains, 1 (10%) pus strains and 2 (16.7%) blood strains were peotease positive.

Analysis of Virulence Factors of E. Coli Isolates

The results showed that isolates of *E. coli* from various sources possess several virulence factors that solely or collectively contribute to their virulence. Of the 25 *E. coli* isolates from urine, 60% produced haemolysin, 52% produced Mannose-Resistant Haemagglutinin (MRHA), 52% produced colicin, 24% produced colicin V, 13.3% produced protease and 69% had cell surface hydrophobicity. Comparison between *E. coli* isolates from different sources with respect to their virulence factors have been summarized in Table 7.

Antibiotic Sensitivity

The clinical isolates of *E. coli* were tested for their susceptibility to 10 different antibiotics. It was found that none of the *E. coli* strain was susceptible to all of the antibiotics. Forty-one (63.07%) strains were resistant to 2 or more of the most commonly clinically used antibiotics. About 89.2% strains was resistant to ampicillin, 83% strains were resistant to tetracycline, 80% strains were resistant to streptomycin, 90.8% strains were resistant to penicillin, while resistance to chloramphenicol was 81.5% (Table 8). The third-generation cephalosporin (ceftriaxone and ceftazidime) and polymyxin B showed most effectiveness. Other drugs that appeared to be clinically useful were the first-generation cephalosporin, nalidixic acid and gentamycin.

Sensitivity to the Bactericidal effect of Normal Serum

All ExPEC *E. coli*isolates were found to be resistant to the bactericidal action of normal serum.

Detection of Adhesion Genes

Type 1 gene was found to be most prevalent in the ExPEC strains as about 77% (50 out of 65) of the isolates harbor type 1 gene. *csgA* gene was found in 61.5% (40) isolate, *papC* was found in 64.6% (42), *afa* and *sfa* genes were found in 67.7% (44) each. All the genes were more prevalent in urine and blood isolates than isolates from peritoneal fluid, pus and CSF. Of the 25 urine isolate, 21 had type 1 gene, 18 had *csgA* gene, 19 had *papC* gene, 21 had *afa* gene and 22 had *sfa* gene. Only 2 stool isolate had type 1 gene and 1 isolate contained *afa* gene. No other gene was found in stool isolates (Table 9).

Table 1. Primer sequences and product size of the genes targeted

Gene	Primers	Sequences	Product size (bp)	Reference
Type 1	Forward	5'-CGA CGC ATC TTC CTC ATT CTT CT-3'	700	Soto *et al*. (2007)
	Reverse	5'-TTT CGA TTG TCT GGC TGT ATG-3'		
csgA	Forward	5'-ACT CTG ACT TGA CTA TTA CC-3'	220	Olivier *et al*. (1998)
	Reverse	5'-AGA TGC AGT CTG GTC AAC-3'		
papC	Forward	5'-·GAC GGC TGT ACT GCA GGG TGT GGC G-3'	328	Uhlin *et al*. (1985)
	Reverse	5'-ATA TCC TTT CTG CAG GGA TGC ATA-3'		
afa	Forward	5'-GCT GGG CAG CAA ACT GAT AAC TCT C-3'	750	Garcia *et al*. (1994)
	Reverse	5'-CAT CAA GCT GTT TGT TCG TCC GCC G-3'		
sfa	Forward	5'-CTC CGG AGA ACT GGG TGC ATC TTA C-3'	410	Stins *et al*. (1994)
	Reverse	5'-CGG AGG AGT AAT TAC AAA CCT GGC A-3'		

Table 2. Motility assay of ExPEC and fecal *E. coli* isolates

	Motility				
Source of strain	++++	+++	++	+	Non-motile
Urine (25)	14 (56%)	6 (24%)	3 (12%)	2 (8%)	-(0%)
Peritoneal fluid (12)	2 (16.67%)	7 (58.3%)	1 (8.3%)	1 (8.3%)	1 (8.3%)
Blood (12)	9 (75%)	-(0%)	-(0%)	1 (8.3%)	2 (16.67%)
Pus (10)	-(0%)	7 (70%)	1 (10%)	-(0%)	2 (20%)
CSF (6)	3 (50%)	-(0%)	-(0%)	-(0%)	3 (50%)
ExPEC (65)	28 (43.08%)	20 (30.76%)	5 (7.7%)	4 (6.15%)	8 (12.3%)
Stool (15)	-(0%)	-(0%)	4 (26.7%)	3 (20%)	8 (53.33%)

(+++= >10 cm; ++ = 8-10 cm; ++= 5-8 cm; += 2-5 cm)

Table 3. Specific biofilm formation of ExPEC isolates

	Specific Biofilm formation				
Source of strain	<0.5	0.5-1.0	1.0-1.5	>1.5	*Non-biofilm former*
Urine (25)	8 (32%)	4 (16%)	6 (24%)	5 (20%)	2 (8%)
Peritoneal fluid (12)	4 (33.33%)	3 (25%)	1 (8.3%)	-(0%)	4 (33.33%)
Blood (12)	3 (25%)	4 (33.33%)	2 (16.67%)	1 (8.3%)	2 (16.67%)
Pus (10)	-(0%)	4 (40%)	3 (30%)	1 (10%)	2 (20%)
CSF (6)	1 (16.67%)	-(0%)	2 (33.33%)	1 (16.67%)	2 (33.33%)
ExPEC (65)	16 (24.61%)	15 (23.07%)	14 (21.54%)	8 (12.30%)	12 (18.46%)
Stool (15)	5 (33.33%)	-(0%)	-(0%)	-(0%)	10 (66.67%)

Table 4. Curli expression by ExPEC isolates

	Curli expression	
Source of strain	+	-
Urine (25)	21 (84%)	4 (16%)
Peritoneal fluid (12)	7 (58.33%)	5 (41.67%)
Blood (12)	9 (75%)	3 (25%)
Pus (10)	5 (50%)	5 (50%)
CSF (6)	4 (66.67%)	2 (33.33%)
ExPEC (65)	46 (70.77%)	19 (29.23%)
Stool (15)	3 (20%)	12 (80%)

Table 5. Adhesion capability of ExPEC isolates to hydrocarbon

	CSH			
Source of Strain	Weak (0-20%)	Moderate (21-50%)	Strong (>50%)	*Negative*
Urine (25)	4 (16%)	12 (48%)	5 (20%)	4 (16%)
Peritoneal fluid (12)	3 (25%)	5 (20%)	1 (4%)	3 (12%)
Blood (12)	2 (25%)	7 (58.33%)	2 (16.67%)	1 (8.3%)
Pus (10)	3 (30%)	2 (20%)	2 (20%)	3 (30%)
CSF (6)	2 (33.33%)	1 (16.67%)	1 (16.67%)	2 (33.33%)
ExPEC (65)	14 (21.53%)	27 (41.53%)	11 (16.9%)	13 (20%)
Stool (15)	4 (26.67%)	-(0%)	-(0%)	11 (73.33%)

(*CSH was determined based on the difference of the OD of bacterial before and after adsorption to hydrocarbon ×100, weak (0-20%), moderate (21-50%) and strong CSH >50%)

Table 6. Cell aggregation capability of ExPEC isolates

	Cell aggregation				
Source of Strain	>80%	60-80%	40-60%	20-40%	Non- aggregative
Urine (25)	2 (8%)	4 (16%)	5 (20%)	9 (36%)	5 (20%)
Peritoneal fluid (12)	-(0%)	3 (25%)	5 (41.67%)	2 (16.67%)	2 (16.67%)
Blood (12)	1 (8.3%)	4 (33.33%)	3 (25%)	3 (25%)	1 (8.3%)
Pus (10)	-(0%)	5 (50%)	4 (40%)	-(0%)	1 (10%)
CSF (6)	-(0%)	2 (33.33%)	-(0%)	3 (50%)	1 (16.67%)
ExPEC (65)	3 (4.6%)	18 (27.7%)	17 (26.15%)	17 (26.15%)	10 (15.4%)
Stool (15)	-(0%)	1 (6.67%)	2 (13.3%)	4 (26.67%)	8 (53.33%)

Table 7. Comparison of virulence phenotypes in extra-intestinal *E. coli* isolates

	Percentage of *E. coli* isolates				
Virulence property	Control (stool)	UTI	Septicemia	Peritonitis	Pus
Haemolysin$^+$	26.7	60.0	41.7	33.30	0
MRHA+	33.3	52.0	50.0	33.30	0
Colicin+	40.0	24.0	16.7	24.00	0
ColicinV+	31.0	69.0	72.0	75.00	75
Hydrophobic+	12.0	76.0	50.0	77.00	58
Protease+	20.0	13.3	16.7	8.33	10

Table 8. Antibiotic susceptibility of the ExPEC isolates

Source of strain	Tet	Str	Cef	Amp	Clr	Gen	Pen	Caz	Pol	Nal
Urine (25)	21 (84%)	19 (76%)	16 (64%)	22 (88%)	20 (80%)	19 (76%)	23 (92%)	11 (44%)	17 (68%)	15 (60%)
Peritoneal fluid (12)	9 (75%)	8 (66.7%)	5 (41.7%)	11 (91.7%)	9 (75%)	7 (58.3%)	10 (83.3%)	4 (33.3%)	7 (58.3%)	5 (41.7%)
Blood (12)	11 (91.7%)	12 (100%)	10 (83.3%)	11 (91.7%)	11 (91.7%)	9 (75%)	12 (100%)	8 (66.7%)	9 (75%)	7 (58.3%)
Pus (10)	9 (90%)	10 (100%)	6 (60%)	10 (100%)	9 (90%)	5 (50%)	9 (90%)	3 (30%)	5 (50%)	7 (70%)
CSF (6)	4 (66.7%)	3 (50%)	2 (33.3%)	4 (66.7%)	4 (66.7%)	2 (33.3%)	5 (83.3%)	1 (16.7%)	3 (50%)	3 (50%)
ExPEC (65)	54 (83%)	52 (80%)	39 (60%)	58 (89.2%)	53 (81.5%)	42 (64.6%)	59 (90.8%)	27 (41.5%)	41 (63%)	37 (56.9%)
Stool (15)	13 (86.7%)	11 (73.3%)	9 (60%)	12 (80%)	12 (80%)	9 (60%)	11 (73.3%)	8 (53.3%)	12 (80%)	9 (60%)

(Tet = Tetracycline; Str = Streptomycin; Cef = Ceftriaxone; Amp = Ampicillin; Clr = Chloramphenicol; Gen = Gentamycin; Pen = Penicillin; Caz = Ceftazidime; Pol = Polymixin B; Nal = Nalidixic acid)

Table 9. Presence of adhesion and virulence genes in ExPEC isolates

Source of Strain	*type 1*	*csgA*	*papC*	*afa*	*sfa*
Urine (25)	21 (84%)	18 (72%)	19 (76%)	21 (84%)	22 (88%)
Peritoneal fluid (12)	9 (75%)	7 (58.3%)	8 (66.7%)	9 (75%)	6 (50%)
Blood (12)	11 (91.7%)	8 (66.7%)	8(66.7%)	7 (58.3%)	9 (75%)
Pus (10)	6 (60%)	5 (50%)	7 (70%)	6 (60%)	5 (50%)
CSF (6)	3 (50%)	2 (33.3%)	0	1 (16.7%)	2 (33.3%)
ExPEC (65)	50 (76.9%)	40 (61.5%)	42 (64.6%)	44 (67.7%)	44 (67.7%)
Stool (15)	2 (13.33%)	0	0	1 (6.7%)	0

Discussion

The research work was aimed at determining any possible correlation between biofilm forming capability of ExPEC and their virulence properties. A number of biofilm formation capability and related properties and virulence traits of 65 previously isolated ExPEC strains has been assessed. Furthermore, attempts were taken to find out the role of motility and presence of adhesin genes in biofilm formations.

The potential biofilm formers had been tested for the motility assay using LB soft agar. About 88% of the ExPEC strains were found to be motile. No significant correlation was seen between motility and biofilm production with the strains though motile isolates tend to form biofilm better. Some non-motile strains also formed biofilm questioning any strict correlation between motility and biofilm formation. Therefore, it could be stated that, motility might be required for reaching the target, but not essential to form biofilm.

Isolates from urine and blood showed better biofilm forming capability than form other sites. This result is in accordance with Golia *et al*. (2012) who concluded that biofilm formation is more prevalent in uropathogenic *E. coli*. Isolates from stool of healthy individual showed very poor biofilm forming capability and only 33% isolate showed weak biofilm formation.

There may be a connection between biofilm formation and the survival of pathogenic *E. coli* within the host (Lassaro *et al*., 2009). To assess the correlation, virulence properties of the ExPEC isolates were determined.

In this study, 44.6% extra-intestinal *E. coli* isolates were haemolytic and 60% (15/25) of the *E. coli* strains isolated from urine were haemolytic ($p<0.001$) and 41.6% (5/12) of the septicemic *E. coli* strains were haemolytic ($p>0.001$). Production of haemolysin was found statistically significant only in urinary and blood isolates ($p<0.001$ and >0.001 respectively). Only the urinary and blood isolates produced colicin V. About 6 (24%) urinary strains and 2 (16.7%) blood isolates showed colicin V activity. Possession of mannose resistant haemagglutinin was found significant for the urinary isolates ($p<0.001$) while, for the blood isolates the p value was >0.05. Urinary and the peritoneal isolates were the most hydrophobic and Blood isolates

have relatively high SAT values. In general, virulence factors are more prevalent in urinary and blood isolates. Biofilm formation capability of urinary and blood isolates were also better than other isolates. From these results, it can be presumed that biofilm formation has correlation with possession of virulence factors. In a previous study, Rijavec *et al*. (2008) could not find any correlation between biofilm formation and virulence properties of uropathogenic *E. coli*. Again, according to Naves *et al*. (2008b) mannose-resistant haemagglutination was the only phenotypically expressed surface virulence factor more frequently found in the strong biofilm group.

ExPEC acquired multi-drug resistance is one of the major source of illness and death, therby increasing healthcare cost (Akond *et al*., 2009). Antibiotic susceptibility pattern of the isolates revealed multi-antibiotic resistance in the isolates but resistance to third generation antibiotics were comparatively lower. Most of the strong biofilm formers with virulence traits were more antibiotic resistant than other isolates. Golia *et al*. (2012) suggested that screening of biofilm can be considered as virulence marker in drug resistant *E. coli* isolates. Murugan *et al*. (2011) remarked that biofilm production in *E. coli* may promote colonization and lead to increased rate of infections like UTI and such infections may be difficult to treat as they exhibit multi drug resistance.

The molecular based analysis of the strains revealed that potential biofilm producers possessed certain adhesin and virulence genes. *Type 1*, *afa* and *sfa* genes were most prevalent in the isolates. This results support the findings of Mihaylova *et al*. (2012) who found type 1 gene as most prevalent in uropathogenic *E. coli*. *csgA* and *papC* were also detected in a good number of isolates. According to Naves *et al*. (2008b), five virulence-associated genes were more common ($p<0.05$) in strong biofilm producers: *PapC* and *papG* alleles, *sfa/focDE*, *focG*, *hlyA* and *cnf1*. Presence of *papC* and *sfa* was also high in the ExPEC isolates. Martinez-Medina *et al*. (2009) also observed that adhesion and invasion indices, motility, type 1 flagellin and presence of virulence genes such as *sfa* are frequent characteristics of strong biofilm producing *E. coli*. Our findings are in concordance with these findings. Most urinary and blood isolates were strong biofilm former and all the five adhesion and virulence associated genes targeted in this study were prevalent in those isolates.

Very little evidences exists describing correlation between biofilm formation, virulence properties and antibiotic resistance of ExPEC. This study can be considered as a footstep in elucidating such correlation. Findings of this study indicate that biofilm formation can be regarded as indication of virulence and drug-resistance of ExPEC isolates and biofilm formation has strong correlation with these virulence properties. To combat ExPEC infection, interrupting exo-polysaccharide production and biofilm formation may therefore represent effective strategies (2012). More detailed study including molecular level should be conducted to determine correlation between biofilm formation and specific virulence factors to elucidate the underlying mechanisms.

Conclusion

Biofilm production ability of extra-intestinal pathogenic *E. coli* (ExPEC) strains could be an additional trait involved in their pathogenesis. More detailed study is needed to elucidate the correlation between biofilm production and virulence traits of ExPEC such as haemolysin production, cytotoxin production, possession of aerobactin iron-acquisition system. Further investigations to detect ExPEC specific genetic determinants involved in biofilm formation and to analyze the genetic regulatory processes are essential to fully understand ExPEC pathogenesis.

Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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