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 12.3% 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. maltophilia 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 and adhering 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 invasion 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,
Determination of Motility

Pseudomonas aeruginosa biofilm formation assay previously identified and characterized as peritoneal fluid, 12 from blood, 10 from pus and 6 from urine. 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 stool 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-Bertani (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

Biofilm 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) medium (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 (Nunc™, 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$_{650}$ nm of the attached and stained bacteria, $CW$ is the OD$_{650}$ nm of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values) and $G$ is the OD$_{650}$ nm of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values).

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 $\left[1 - \frac{A1}{A0}\right] \times 100\%$.

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
tube (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 (10^8 bacterium/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), Amoxicillin (Amp, 25 µg), Chloramphenicol (Clr, 20 µg), Gentamycin (Gen, 30 µg), Penicillin (Pen, 10 µg), Cefazidime (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. 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).

**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 (CHS value 21-50%) to adhere to hydrocarbons whereas 16.9% have strong capability. About 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. maltophilia*. 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 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%) 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 protease 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 TTC ATT CTT CT-3' | 700 | Soto et al. (2007) |
| | Reverse | 5'-TTT CGA TGG TCT GGC TGG ATG-3' | 220 | Olivier et al. (1998) |
| csgA | Forward | 5'-ACT CTG ACT TGA CTA TTA CC-3' | | |
| | Reverse | 5'-AGA TGC AGT CTG GTC GTC AAT C-3' | | |
| papC | Forward | 5'-GAC GGC TGT ACT GCA GGG TGG GGC G-3' | 328 | Uhlin et al. (1985) |
| | Reverse | 5'-ATA TCC TTC TTT CTG CAG GGA TGC ATC-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 TGC 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 GCC A-3' | | |
Table 2. Motility assay of ExPEC and fecal E. coli isolates

| Source of strain | ++++ | +++ | ++ | + | Non-motile |
|------------------|------|-----|----|---|-----------|
| Urine (25)       | 14 (56%) | 6 (24%) | 3 (12%) | 2 (8%) | 0 (0%) |
| Peritoneal fluid (12) | 2 (16.7%) | 1 (8.3%) | 1 (8.3%) | 1 (8.3%) | 1 (8.3%) |
| Blood (12)       | 9 (75%) | 7 (58.3%) | 1 (8.3%) | 1 (8.3%) | 2 (16.67%) |
| Pus (10)         | 0 (0%) | 7 (70%) | 1 (10%) | 0 (0%) | 2 (20%) |
| CSF (6)          | 3 (50%) | 0 (0%) | 0 (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

| 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%) | 4 (33.33%) |
| Blood (12)       | 3 (25%) | 4 (33.33%) | 2 (16.67%) | 2 (16.67%) |
| Pus (10)         | 0 (0%) | 4 (40%) | 3 (30%) | 1 (10%) | 2 (20%) |
| CSF (6)          | 1 (16.67%) | 0 (0%) | 2 (33.33%) | 2 (33.33%) |
| ExPEC (65)       | 16 (24.61%) | 15 (23.07%) | 14 (21.54%) | 8 (12.3%) |
| Stool (15)       | 5 (33.33%) | 0 (0%) | 0 (0%) | 10 (66.67%) |

Table 4. Curli expression by ExPEC isolates

| Source of strain | + | - |
|------------------|---|---|
| Urine (25)       | 21 (84%) | 4 (16%) |
| Peritoneal fluid (12) | 7 (58.33%) | 3 (25%) |
| Blood (12)       | 9 (75%) | 5 (20%) |
| Pus (10)         | 5 (50%) | 3 (25%) |
| 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

| 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%) | 2 (8%) |
| Pus (10)         | 3 (30%) | 2 (20%) | 2 (20%) | 2 (10%) |
| CSF (6)          | 2 (33.33%) | 1 (16.67%) | 1 (16.67%) | 2 (16.67%) |
| ExPEC (65)       | 14 (21.53%) | 27 (41.53%) | 11 (16.9%) | 13 (20%) |
| Stool (15)       | 4 (26.67%) | 0 (0%) | 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

| 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%) | 1 (8.3%) |
| Pus (10)         | -0% | 5 (50%) | 4 (40%) | 3 (50%) | 1 (10%) |
| CSF (6)          | -0% | 2 (33.33%) | 3 (50%) | 1 (10%) | 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%) |

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

| Virulence property | Control (stool) | UTI | Septicemia | Peritonitis | Pus |
|--------------------|----------------|-----|------------|-------------|-----|
| Haemolysin         | 26.7           | 60.0| 41.7       | 33.3        | 0   |
| MRHA+              | 33.3           | 52.0| 50.0       | 33.3        | 0   |
| Colicin+           | 40.0           | 24.0| 16.7       | 24.0        | 0   |
| ColicinV+          | 31.0           | 69.0| 72.0       | 75.0        | 75  |
| Hydrophobic+       | 12.0           | 76.0| 50.0       | 77.0        | 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%) | 5 (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%) | 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 (13.3%) | 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 \textit{E. coli}. Again, according to Naves \textit{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, thereby increasing healthcare cost (Akond \textit{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 \textit{et al}. (2012) suggested that screening of biofilm can be considered as virulence marker in drug resistant \textit{E. coli} isolates. Murugan \textit{et al}. (2011) remarked that biofilm production in \textit{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. \textit{Type 1}, \textit{sfa} and \textit{sfa} genes were most prevalent in the isolates. This results support the findings of Mihaylova \textit{et al}. (2012) who found \textit{type 1} gene as most prevalent in uropathogenic \textit{E. coli}. \textit{csgA} and \textit{papC} were also detected in a good number of isolates. According to Naves \textit{et al}. (2008b), five virulence-associated genes were more common ($p<0.05$) in strong biofilm producers: \textit{PapC} and \textit{papG} alleles, \textit{sfa/focDE}, \textit{focG}, \textit{hlyA} and \textit{cnf1}. Presence of \textit{papC} and \textit{sfa} was also high in the ExPEC isolates. Martinez-Medina \textit{et al}. (2009) also observed that adhesion and invasion indices, motility, \textit{type 1} flagellin and presence of virulence genes such as \textit{sfa} are frequent characteristics of strong biofilm producing \textit{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 exopolysaccharide 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 \textit{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.

**References**

Abdallah, F.B., K. Chaieb, T. Zmatar, H. Kallel and A. Bakhrouf, 2009. Adherence assays and slime production of \textit{Vibrio alginolyticus} and \textit{Vibrio parahaemolyticus}. Braz. J. Microbiol., 40: 394-398. DOI: 10.1590/S1517-83822009000200033

Akond, M.A., S.M.R. Hassan, S. Alam and M. Shirin, 2009. Antibiotic resistance of \textit{Escherichia coli} isolated from poultry and poultry environment of Bangladesh. Am. J. Environ. Sci., 5: 47-52. DOI: 10.3844/ajessp.2009.47.52

Bauer, A.W., W.M. Kirby, J.C. Shiris and M.Turck, 1966. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Path., 45: 493-496.
CLSI, 2006. Performance standards for Antimicrobial Susceptibility Testing. Proceedings of the 16th Informational Supplement, CLSI document M100-S16.

Danese, P.N., L.A. Pratt, S.L. Dove and R. Kolter, 2000. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within Escherichia coli biofilms. Mol. Microbiol., 37: 424-432. DOI: 10.1046/j.1365-2958.2000.02008.x

Fakruddin, M., R.M. Mazumdar, A. Chowdhury and K.S.B. Mannan, 2012a. Comparative analysis of virulence factors of Escherichia coli from non-enteric infections. J. Med. Sci., 12: 37-44. DOI: 10.3923/jms.2012.37.47

Fakruddin, M., S. Islam, M.M. Ahmed, A. Chowdhury and M.M. Hoque, 2012b. Development of multiplex PCR (polymerase chain reaction) method for detection of salmonella spp. and vibrio parahaemolyticus from shrimp samples of Bangladesh. Asian J. Bio. Sci., 5: 76-85. DOI: 10.3923/ajbs.2012.76.85

Fernandez-Beros, M.E., V. Kissel, H. Lior and F.C. Cabello, 1990. Virulence-related genes in col V plasmids of Escherichia coli isolated from human blood and intestine. J. Clin. Microbiol., 28: 742-746.

Garcia, M.I., A. Labigne and C. Le Bouguenec, 1994. Nucleotide sequence of the afimbrial-adhesin-encoding afa-3 gene cluster and its translocation via flanking IS1 insertion sequences. J. Bacteriol., 176: 7601-7613

Ghanbarpouri, R. and M. Salehi, 2010. Determination of adhesion encoding genes in Escherichia coli isolates from Omphalitis of chicks. Am. J. Anim. Vet. Sci., 5: 91-96. DOI: 10.3844/ajasv.2010.91.96.pdf

Golia, S., V. Hittinahalli, S.K. Karjigi and K.M. Reddy, 2012. Correlation between biofilm formation of uropathogenic Escherichia coli and its antibiotic resistance pattern. J. Evol. Med. Dental Sci., 1: 166-175. DOI: 10.14260/jems/26

Holden, N.J. and D.L. Gally, 2004. Switches, cross-talk and memory in Escherichia coli adherence. J. Med. Microbiol., 53: 585-593. DOI: 10.1099/jmm.0.05491-0

Klosowska, K. and B.J. Plotkin, 2006. Human insulin modulation of Escherichia coli adherence and chemotaxis. Am. J. Inf. Dis., 2: 197-200. DOI: 10.3844/ajidsp.2006.197.200.pdf

Lassaro, M.A., N. Salinger, J. Zhang, Y. Wang and Z. Zhong et al., 2009. F1C Fimbriae play an important role in biofilm formation and intestinal colonization by the Escherichia coli commensal strain Nissle 1917. App. Env. Microbiol., 75: 246-251. DOI: 10.1128/AEM.01144-08

Mansour, A.M.A., H.M. Zaki, N.A. Hassan and N.A.M. El-Nashar, 2014. Phenotyping, virulence characteristics of Aeromonas species and the effects of essential plant oils as antimicrobial agents against pathogenic isolates from different sources. Am. J. Inf. Dis., 10: 21-35. DOI: 10.3844/ajidsp.2014.21.35

Martinez-Medina, M., P. Navés, J. Blanco, X. Aldeguer and J. Blanco et al., 2009. Biofilm formation as a novel phenotypic feature of adherent-invasive Escherichia coli (AIEC). BMC Microbiol., 9: 202. DOI: 10.1186/1471-2180-9-202

Mathur, T., S. Singhal, S. Khan, D.J. Upadhyay and T. Fatma et al., 2006. Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. Indian J. Med. Microbiol., 24: 25-29. DOI: 10.4103/0255-0857.19890

Mihaylova, M., S. Kostadinova and M. Marhova, 2012. Distribution of virulence determinants and biofilm-forming among clinical urinary isolates. J. Biosci. Biotech., SE/ONLINE: 45-51.

Murugan, S., P.U. Devi and P.N. John, 2011. Antimicrobial susceptibility pattern of biofilm producing Escherichia coli of urinary tract infections. Cur. Res. Bacteriol., 4: 73-80. DOI: 10.3923/crb.2011.73.80

Najar, A.G., M.M. Nejad and S. Mansouri, 2007. The comparison between virulence factors of Escherichia coli isolated from urinary tract infections and faecal flora. Res. Pharm. Sci., 2: 99-103.

Nalima, T. and Z.H.A. Rahim, 2006. Effect of Piper betle L. leaf extract on the virulence activity of Streptococcus mutans- An in vitro study. Pak. J. Biol. Sci., 9: 1470-1475. DOI: 10.3923/pjbs.2006.1470.1475

Navés, P., G. del Prado, L. Huelves, M. Gracia and V. Ruiz et al., 2008a. Correlation between virulence factors and in vitro biofilm formation by Escherichia coli strains. Microbial. Pathogenesis, 45: 86-91. DOI: 10.1016/j.micpath.2008.03.003

Navés, P., G.D. Prado, L. Huelves, M. Gracia and V. Ruiz et al., 2008b. Measurement of biofilm formation by clinical isolates of Escherichia coli is method-dependent. J. App. Microbiol., 105: 585-590. DOI: 10.1111/j.1365-2672.2008.03791.x

Norouzi, F., S. Mansouri, M. Moradi and M. Razavi, 2012. Distribution of virulence determinants and biofilm formation among clinical urinary isolates of Escherichia coli. Asian J. Bio. Sci., 5: 76-85.

Olivier, V., R. Longin, C. Prigent-Combaret, C. Dorel and M. Hooreman et al., 1998. Isolation of an Escherichia coli K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new ompR allele that increases curli expression. J. Bacteriol., 180: 2442-2449.
Pelkonen, S. and J. Finne, 1987. A rapid turbidmetric assay for the study of serum sensitivity of *Escherichia coli*. FEMS Microbiol. Lett., 42: 55-57. DOI: 10.1111/j.1574-6968.1987.tb02298.x

Reisner, A., K.A. Krogfelt, B.M. Klein, E.L. Zechners and S. Molin, 2006. *in vitro* biofilm formation of commensal and pathogenic *Escherichia coli* strains: impact of environmental and genetic factors. J. Bacteriol., 188: 3572-3581. DOI: 10.1128/JB.188.10.3572-3581.2006

Rijavec, M., M. Muller-Premru, B. Zakotnik and D. Zgur-Bertok, 2008. Virulence factors and biofilm production among *Escherichia coli* strains causing bacteraemia of urinary tract origin. J. Med. Microbiol., 57: 1329-1334. DOI: 10.1099/jmm.0.2008/002543-0

Rosenberg, M. and D. Gutnick, 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett., 9: 29-33. DOI: 10.1111/j.1574-6968.1980.tb05599.x

Sahasrabudhe, A. and M. Deodhar, 2010. Standardization of DNA extraction and optimization of RAPD-PCR conditions in Garcinia indica. Int. J. Botany, 6: 293-298. DOI: 10.3923/ijb.2010.293.298

Sharma, S., G.K. Bhat and S. Shenoy, 2007. Virulence factors and drug resistance in *escherichia coli* isolated from extra-intestinal infections. Indian J. Med. Microbiol., 25: 369-73. DOI: 10.4103/0255-0857.37341

Soto, S.M., A. Smithson, J.A. Martinez, J.P. Horcajada and J. Mensa et al., 2007. Biofilm formation in uropathogenic *escherichia coli* strains: Relationship with prostatitis, urovirulence factors and antimicrobial resistance. J. Urol., 177: 365-368. DOI: 10.1016/j.juro.2006.08.081

Sperandio, V., A.G. Torres and J.B. Kaper, 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): A novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *Escherichia coli*. Mol. Microbiol., 43: 809-821. DOI: 10.1046/j.1365-2958.2002.02803.x

Stins, M.F., N.V. Prasadara, L. Ibric, C.A. Wass and P. Luckett et al., 1994. Binding characteristics of S fimbriated *Escherichia coli* to isolated brain microvascular endothelial cells. Am. J. Pathol., 145: 1228-1236.

Taj, Y., F. Essa, F. Aziz and S.U. Kazmi, 2012. Study on biofilm-forming properties of clinical isolates of Staphylococcus aureus. J. Infect. Dev. Ctries., 5: 403-409.

Tirumalai, P.S. and S. Prakash, 2012. Antibiotic resistance in co-culture biofilm of Listeria monocytogenes J0161. Am. J. Microbiol., 3: 7-17. DOI: 10.3844/ajmsp.2012.7.17

Uhlich, G.A., P.H. Cooke and E.B. Solomon, 2006. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. Appl. Environ. Microbiol., 72: 2564-2572. DOI: 10.1128/AEM.72.4.2564-2572.2006

Uhlin, B.E., M. Norgren, M. Baga and S. Normark, 1985. Adhesion to human cells by *Escherichia coli* lacking the major subunit of a digalactoside-specific pilus-adhesin. Proc. Nat. Acad. Sci. USA, 82: 1800-1804. DOI: 10.1073/pnas.82.6.1800

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. App. Env. Microbiol., 63: 3741-3751.