



Original Research Article

Evaluation of cell surface hydrophobicity and biofilm formation as pathogenic determinants among ESBL producing uropathogenic *Escherichia coli*

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ABSTRACT

Background: The attachment of the bacteria to the host cell and ability to invade the cell are regarded as important steps in the infectious process. The hydrophobicity of the microbial surface plays a critical role in the adherence of bacteria to the surface. The ability of biofilm formation can increase survival chance of microorganism, as cell growing in biofilm are highly resistant to the components of the immune system and many antimicrobial agents. Infection caused by ESBL- producers are associated with severe adverse outcomes and may be related to increased virulence of these strains.

Materials and Methods: A total of 100 urinary *E. coli* were selected for the study, of which 50 strains were from ESBL producers and 50 from non-ESBL-producing uropathogenic *E. coli* (UPEC) strains. The urinary *E. coli* isolates that were resistant to at least one of the three indicator cephalosporins (cefotaxime, cefpodoxime and ceftazidime) were tested for ESBL production by quantitative E-strip method. All the 100 urinary *Escherichia coli* strains were tested for cell surface hydrophobicity (CSH) by salt aggregation method and Biofilm production by tissue culture plate method.

Results: Among ESBL producers, 19 (38%) were CSH positive and 34 (68%) were biofilm producers. However among non-ESBL producers, 05 (10%) were CSH positive and 12 (24%) were biofilm producers. Statistically significant difference (<0.001) was seen in the occurrence of CSH and biofilm production between ESBL and non ESBL producing UPEC isolates.

Conclusion: In the present study, it was found that the ESBL producing isolates had a higher ability to form biofilm and CSH; both of them are among the important virulence factors associated with cell surface adherence which is the first step in bacterial infection.

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1. Introduction

The pathogenic *Escherichia coli* strains are common cause of extra intestinal infections such as urinary tract infection, neonatal meningitis, as well as bacteraemia.^{1,2} The strains that cause extra-intestinal infections possess virulence factors (VF) that enhance the ability to cause systemic infection.³⁻⁵ In *Escherichia coli* isolates virulence results

from the cumulative impact of one or several special properties, or VFs, which distinguish potential pathogens from harmless intestinal strains.

At some point of life, at least 12% of men and 10-20% of women experience an acute symptomatic UTI^{6,7} and an even greater number develop asymptomatic bacteriuria (ABU).

A vital step in early stages of infection by many bacterial pathogens is the initial adherence to the mucosal surfaces of respiratory, gastrointestinal or urinary tract epithelium and

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in some instances colonization alone may be sufficient to cause disease⁸. The hydrophobic interaction plays a role in the adherence of microorganisms to a wide variety of surfaces.^{4,6–8} The fimbrial adhesions found in pathogenic *E. coli* are predominantly composed of hydrophobic aminoacids, these increase the surface hydrophobicity and reduce the cell surface charge.^{9–12} Bacterial cell adhesins can be ranked on the basis of their hydrophobicity with recognized pathogenic *E. coli* showing a greater surface hydrophobicity than non pathogens.^{9,12} The hydrophobicity of the microbial surface plays a critical role in the adherence of bacteria to the wide variety of surfaces.^{10,13} And it is also a significant determinant of adhesion and biofilm formation on polystyrene surfaces.^{11,14} The ability of biofilm formation can increase survival of microorganism, as cells growing in biofilm are highly resistant to the components of the human immune system and many antimicrobial agents.^{12,15–17}

Also, the infection caused by ESBL- producers is associated with adverse outcomes and may be related to increased virulence of the strains.^{15,18,19} Some studies have reported an association between ESBL- production and the higher expression pathogenicity factors.^{8,20}

Such infections are extremely difficult to eradicate and the fact that cell surface hydrophobicity (CSH) and biofilm formation play a significant role in a wide range of microbial infections.

Hence, this study was performed to evaluate the CSH and Biofilm formation as two cell surface determinants involved in pathogenicity of among ESBL- and non-ESBL-producing *Escherichia coli* isolated from patients with urinary tract infection.

2. Materials and Methods

The prospective study was conducted in the Department of Microbiology, Shyam Shah Medical College, Rewa, Madhya Pradesh, India. The study material and data collections were carried out with the approval from institutional ethical committee and informed consent were taken from all the subjects willing to participate.

The Clean -catch mid-stream urine samples collected in (uricol, Hi-Media) sterile disposable container were immediately transported to the laboratory within one hour. The semi quantitative calibrated loop technique was employed to culture urine on blood agar and Mac Conkey agar. The inoculated plates were incubated over night at 37°C. The significant growth of bacteria on culture media (colony count $\geq 10^5$ CFU/ml) was identified and sensitivity testing was done by standard procedures.^{21,22} Throughout the study, standard strains including *Escherichia coli* ATCC 25922 and *Klasiella pneumonia* ATCC 700603 were used as the controls.

2.1. Detection of extended spectrum β - lactamases

The strains which showed resistance to at least one of the indicator cephalosporins were confirmed with E-strip test (AB Biomeurix Solna, Sweden).^{22,23}

The E test ESBL strip carries two gradients; on the one end, Ceftazidime (0.5 to 32 ug/ml) and on the opposite end, Ceftazidime (0.125 to 8 g/ml) plus Clavulanic acid (4 ug/ml). Briefly, after overnight growth on brain heart infusion agar, the organisms were suspended in saline to turbidity equal to that of a 0.5 McFarland turbidity standard. This suspension was then used to inoculate Mueller Hinton agar, the E test strips were placed on the plates and incubated overnight in air at 37°C. The MICs on both ends of the strip were interpreted as the point of intersection of the inhibition eclipse with the E-test strip edge.^{6,19,24,25}

2.2. Salt aggregation test for detection of Cell Surface Hydrophobicity (CSH)

Bacteria were tested for their hydrophobic property by using different molar concentrations of ammonium sulphate. Those which aggregated with salt particles and formed clumps were considered hydrophobic. The colonies of *E. coli* were inoculated into 1 ml of PBS pH 6.8 and turbidity were matched with Mc Farland tubes 6 and 7 to get a colony count of 5×10^6 colonies/ml. Different molar concentrations of ammonium sulphate were prepared. Forty microliter of 0.2 M PBS pH6.8 was taken in first column of VDRL slide. And 40 ul of 1M, 1.4 M, and 2M concentration of ammonium sulphate were taken in each well of other columns of VDRL slide. Forty microliter of *E. coli* suspension was added to each of these wells. The clumps formed in different molar concentrations were observed under inverted microscope at 20X magnification.²⁶ The results were recorded on three scales namely, weak 0.1M, 0.4 M, Moderate 1.0M, 1.4 M & Strong CSH ≥ 2.0 M.

2.3. Detection of Biofilm formation by Tissue Culture Plate (TCP) method

A volume of 200 ul of *Escherichia coli* was inoculated in microtitre plate and incubated for 18 hours at 37°C. The wells were washed four times with PBS (pH 7.2) to remove free floating 'planktonic' bacteria. Biofilms formed by adherent sessile organisms were fixed with 200 μ l 99% methanol in each well and stained with 0.1% (w/v) Crystal violet for five minutes. Excess stain was rinsed off by placing the plate under running tap water. The plates were air dried and dye attached to well was dissolved in 200 μ l of 33% glacial acetic acid. Optical Density (OD) of each well was determined by using ELISA Reader (Biotek ELX 800-MS) at 490 nm (OD_{490nm}). These OD values were considered as an index of bacteria adhering to surface and forming biofilm.^{23,24,27,28} Experiment was performed in triplicate, the data was then averaged. The

bacterial adherence was recorded as weak/non (OD <0.120), Moderate (OD 0.12-0.24) and strong (OD >0.24)

2.4. Statistical analysis

The results were analyzed with descriptive statistics wherever appropriate. Fisher's exact test was used to evaluate the statistical significance of differences in the result. A p-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS v 16.0 software.

3. Results

A total of 100 urinary *E.coli* isolates included in the study were screened for ESBL production by using three indicator cephalosporins, ceftazidime (30 µg), cefotaxime (30 µg) and cefpodoxime (30 µg). The zone diameter of < 22 mm for ceftazidime, < 27 mm for cefotaxime and <17 mm for cefpodoxime was recorded as resistant. The strains which showed resistance to at least one of these cephalosporins were further confirmed for the production of ESBL enzyme by E-strip method. The ratio of Ceftazidime / Ceftazidime-Clavulanate MIC equal to or greater than 8 were considered positive for ESBL enzyme production.

All the 100 urinary *Escherichia coli* strains were tested for the presence of cell surface hydrophobicity and the ability of biofilm production of which 50 strains from confirmed ESBL positive strains and 50 strains from non-ESBL-producing group. Among 100 *E.coli* isolates 37 were cell surface hydrophobicity positive and 46 *E.coli* strains were biofilm producers

Out of 50 ESBL producing *E.coli* isolates, 29 (58%) strains were positive for cell surface hydrophobicity, of which 12 (24%) were strongly positive and 16 (32%) were moderately positive. In 50 Non-ESBL *E.coli* strains, only 8 (16%) were positive for cell surface hydrophobicity as depicted in Table 1.

Among 10 *E.coli* strains tested for biofilm formation, 46 strains were biofilm producers, in which 34 (68%) strains were from ESBL producers and 12 (24%) strains were non-ESBL producers. A total of 54 *E.coli* strains were non/weak biofilm producers, of which ESBL producing *E.coli* contributed 16 (29.6%) isolates and non-ESBL producing *E.coli*, 38 (70.4%) isolates. Among 50 ESBL producing *E.coli* strains, 12 (24%) were strongly biofilm producers and 22 (44%) moderately biofilm producers. Comparatively, only 12 (24%) non-ESBL *E.coli* strains positive for biofilm production, of which 5 (10%) were strongly positive for biofilm production and 7 (14%) were moderately positive for biofilm production. In ESBL producing *E.coli* strains, the mean OD of biofilm formation was 0.194 ± 0.12 with a OD range of 0.072 to 0.312 and in non-ESBL strains of *E.coli*, the mean OD was 0.128 ± 0.094 with the OD range of 0.034-0.224 as mentioned in Table 2.

Among ESBL producing *E.coli*, 21 strains were having both CSH and biofilm producing virulence ability. However, among non-ESBL *E.coli* only 02 strains were having both the virulence factors.

Statistically significant difference was seen in the occurrence of cell surface hydrophobicity ($p= 0.0019$) as well as biofilm production ($p<0.0001$) between ESBL and non-ESBL producing uropathogenic *Escherichia coli* isolates (Table 3).

4. Discussion

In our study, the salt aggregation was recorded in 24 out of 100 strains as they have shown clumping in ≤ 1.4 M salt solution. Similarly, Raksha et al²⁹ found that a total 58 (26.36%) among 220 cases and 9 (18%) strains among 50 controls were cell surface hydrophobic. In another study by Sharma et al,³⁰ out of 152 *E.coli* strains tested for virulence factors, 42 strains (27.6%) were hydrophobic and observed 67 (44.0%) strains of *E.coli* with multiple virulence factors

A significant difference in the CSH ($p=0.0019$) of the ESBL producing *Escherichia coli* isolates was seen in comparison with non-ESBL producing isolates. This finding was in accordance with the study carried out by Sharma et al³⁰ as they also showed significant association between multiple virulence factors and ESBL production in extra-intestinal *Escherichia coli*.

Among 50 ESBL positive isolates, 34 (68%) were positive for Biofilm formation, however only 12 (24%) strains of *Escherichia coli* among non-ESBL group showed Biofilm formation. Another study by Mathur et al³¹ recorded that 22 (14.4%) isolates were strong biofilm producers, 60 (39.4%) moderate and 70 (46%) non biofilm producers.

The first step in bacterial infection is adherence and colonization of the bacteria to the surface. CSH is an importance adherence and colonization factor. It's a complex interaction between components of the surface of bacteria and surrounding environment. Pompilio et al³² found a positive correlation between CSH and Biofilm formation in *Stenotrophomonas multophila*. Lee et al³³ showed a positive correlation between Biofilm formation and presence of ESBL bla_{PER-1} among the *Acinetobacter baumannii*. A correlation between ESBL type PER in *Acinetobacter* species and *Pseudomonas aeruginosa* with poor clinical outcome has been reported by Vahaboglu et al.³⁴ In our region data is lacking regarding the presence of virulence factors in pathogenic organisms and their association with drug resistance like extended spectrum beta lactamases. The studies on more number of phenotypic virulence factors and their virulence gene is necessary to compare the pathogenicity of ESBL and non-ESBL producing isolates.

In this study we found that ESBL producing isolates had a higher ability to form biofilm in comparison with

Table 1: Detection of cell surface hydrophobicity among ESBL and non- ESBL producing *E.coli* isolates

ESBL Production	Aggregation at different molar concentrations					Salt Aggregation	CSH Positive
	2.0 M	1.4 M	1.0 M	0.4M	0.1 M		
Positive N=50	12	07	09	01	00	29	19 (38%)
Negative N=50	03	02	03	00	00	08	05 (10%)
Total N=100	15	09	12	01	00	37	24 (24%)

Table 2: Detection of Biofilm formation among ESBL and non- ESBL producing *E.coli* isolates

ESBL Production	Biofilm formation			Biofilm Positive
	Strong OD value (>0.24)	Moderate OD value (0.124-0.24)	Weak/non biofilm OD value (<0.12)	
Positive N=50	12	22	16	34 (68%)
Negative N=50	05	07	38	12 (24%)
Total N=100	17	29	54	46 (46%)

Table 3: Occurrence of virulence factors among ESBL and non- ESBL producing *E.coli* isolates.

Virulence Factors in <i>E.coli</i>	ESBL Producing <i>E.coli</i>	Non-ESBL Producing <i>E.coli</i>	-Value
Cell surface hydrophobicity	19	05	0.0019
Biofilm formation	34	12	<0.0001

non ESBL producing isolates, Also ESBL producing isolates had a significantly higher CSH; both of them are among the important virulent factors associated with cell surface. It has been suggested that a number of chromosomal gene rearrangement occurs upon acquisition of the ESBL plasmid. It is possible that higher mortality and severity of infection caused by ESBL producing isolates is due to the expression of several virulence genes simultaneously, rather than gaining new virulence genes.²⁰

There is hardly any report regarding CSH and biofilm formation in clinical isolates of *E.coli* producing and non-producing ESBLs. However, study on the gene expression is necessary to compare the pathogenicity of ESBL and non ESBL isolates. Due to highly emerged isolates producing different types of ESBL and the importance of ESBL producing *E.coli*, study on other virulence factors both genetically and phenotypically is recommended.

5. Source of Funding

None.

6. Conflict of Interest

The authors declare no conflict of interest.

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