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Hemagglutination, serum resistance, cell surface hydrophobicity and ESBL production among Uropathogenic *Escherichia coli*

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Abstract

Background: Bacteria have adapted with increased pathogenesis and drug resistance mechanisms to overcome host defense systems**.** Uropathogenic *E. coli* (UPEC) responsible for symptomatic Urinary tract infections (UTIs) carry diverse virulence factors and extended-spectrum beta-lactamase (ESBL) production which play a pivotal role in pathogenesis and antibiotic resistance. This study aimed to determine the phenotypic expression of virulence factors; i.e., Hemagglutination, serum resistance, cell surface hydrophobicity, and resistance factor ESBL production among UPEC isolates. **Methods:** In this prospective study 50 drug-resistant UPEC strains were evaluated for hemagglutination by slide method, cell surface hydrophobicity by salt aggregation test, serum resistance by serum bactericidal assay, and ESBL detection by double disc synergy test. **Results:** Among 50 UPEC strains 46 (92%) showed mannose-resistant hemagglutination, 48 (96%) were found to be serum resistant, 40 (80%) isolates were positive for cell surface hydrophobicity, while 43 (86%) were ESBL producers. Most of the UPEC that expressed virulence factors were ESBL producers as well. **Conclusion:** Overall, this study indicated that drug resistant UPEC isolates have expressed several virulence factors with ESBL production. This explains how pathogens have increased their virulence and resistance repertoire resulting in treatment failures.

Keywords: virulence factors, Uropathogenic *E. coli*, ESBL

Introduction

UTIs are considered as the most common bacterial infections estimated to be 150 to 250 million cases per year worldwide.[1] UPEC are the most frequent etiologic agents responsible for 70-90% UTIs in community setup and 50% nosocomial UTIs with significant cause of morbidity and mortality globally.[2] Symptomatic UTIs account for major complications e.g. cystitis, pyelonephritis and bacteremia. The pathogenesis includes the colonization of the periurethral area followed by adherence of the bacteria to the epithelial cells of the human urinary tract.[3]

The expression of variety of virulence factors plays an important role in establishment of infection.

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The major virulence factors that take part in pathogenesis are: adhesins (type 1, P and F1C fimbriae), siderophore systems (IroN), cytotoxins (e.g. hemolysin), auto transporter proteins (Sat) and protectins (e.g. capsule, O antigen).[4]

Serologically, fimbriae are characterized by hemagglutination pattern of receptors specificity by mannose sensitive hemagglutination (MSHA) as well as mannose resistant hemagglutination (MRHA).[5] Vast variety of adhesins are reported in UPEC, Type 1 fimbriae and p fimbriae responsible for MSHA and MRHA respectively are most commonly found in UPEC. Besides this cell surface hydrophobicity (CSH) can increase the potential of bacteria to adhesion and penetration of host tissues. Other important virulence factors that help in colonization and entrance of UPEC in periurethral area is serum resistance (SR) specifically O and K antigen that provide resistance to phagocytosis and bactericidal action of serum .[6]

The development of antibiotic resistance has threatened human health and has increased rate of morbidity and mortality worldwide, especially multiple drug resistant bacteria (MDR) are the cause of longer hospital stay and severe economic loss.

Key phenomenon involves in beta-lactam antibiotic resistance is the production of beta-lactamases that degrade these antibiotics in periplasmic space and

preventing their contact with penicillin binding proteins.[7] Other β-lactamases that can degrade later classes of β-lactamS like 3rd generation cephalosporin are referred as extended spectrum β-lactamases (ESBLs). Beta-lactamases are paying substantial medical attention from clinicians and researchers due to their clinical, ecological as well as evolutionary curiosity. Moreover, ESBLs confer resistance to all beta-lactam (account for 50% of systemic antibiotics) and further leads to provide selective pressure for carbapenems consequently results in fewer treatment options.

Previous studies showed correlation between phenotypic virulence traits and drug resistance.[8] Virulence characteristics like MSHA, MRHA and cell surface hydrophobicity have a role to increase tendency of UPEC to adhere and form biofilms. Increased drug resistance is thought to be linked with biofilm as well. This increased drug resistance is connected to horizontal gene transfer within biofilm. The aim of the current research study was to determine the prevalence of virulence markers and correlation with ESBL production in UPEC strains.

Methods

This prospective study was conducted at the Department of Microbiology; University of Karachi from January to June 2019.The study was approved by ethical review board of University of Karachi via letter BASR/no./02753/sc dated: 15-07-2016. A total of 50 drug resistant strains were selected that were initially obtained from urine specimens with bacterial count ≥10^5 CFU/ml[9,10] submitted to clinical laboratory of tertiary care hospital in Karachi for routine culture and antimicrobial susceptibility testing. The isolates were further purified on MacConkey's agar (Oxoid) and subjected to confirmation by conventional biochemical tests. All UPEC isolates were preserved in 20% glycerol and kept in freezing condition for further studies.

UPEC strains were assessed for hemagglutination by slide method as described elsewhere.[11] Briefly Group O Rh +ve human erythrocytes suspension was made in saline after washing thrice. Freshly grown bacteria on blood agar were emulsified in phosphate buffer saline. On clean and grease free dry microscopic slide equal amount (40µl) of each of bacterial suspension, 3% RBCs suspension and 2% (w/v) D-mannose solution each was taken, slide was rotated and agglutination was observed. The presence of clumping of erythrocytes showed mannose resistant hemagglutination (MRHA) and absence of clumping of erythrocytes showed mannose sensitive hemagglutination (MSHA). UPEC strain showing positive result for MSHA used as positive control.

Salt aggregation test was performed to see cell surface hydrophobicity.[12] Bacterial suspension was made by suspending bacterial growth in phosphate buffer saline at pH 6.8 to get turbidity of McFarland's standard 6 (5x10^9 colonies/ml). Ammonium sulphate solution of 1M, 1.4M and 2M molar concentrations were prepared and 40µL of bacterial suspension was mixed with the same amount of ammonium sulphate solution on a glass slide. Slide was rotated for 1 min and observed under 10X magnification of microscope. The highest dilution that gave visible clumping regarded as salt aggregation test value. UPEC strains that showed visible clumping at 1.4 or less were considered hydrophobic.

Serum resistance was determined by serum bactericidal assay described by Benge *et al*., with slight modifications .[13] UPEC were grown overnight in 3mL of tryptone soya broth and then diluted with Phosphate buffered saline (PBS). Bacterial suspension (10µL) was mixed with 90µL of undiluted human serum and incubated at 37^{0}C for 180min. 10µL of resulting suspension was spread on tryptone soya agar and incubated at 37^{0}C for 24 hrs and checked for viable count. Strains of UPEC were interpreted serum sensitive if colony count dropped to 1% and resistant by serum killing if colony count was >90%. Strain showing serum resistance was used as positive control.

Strains of UPEC showed reduced susceptibilities to 3rd generation cephalosporins in our previous work, were screened for ESBL production. DDST was performed according to Jarlier *et al*.[14] The discs containing cephalosporin; cefotaxime (30ug) or ceftriaxone, (30ug) and aztreonam, (30ug) were placed next to a disc of amoxicillin (20ug) + clavulanic acid (10ug) on MHA plate 15–30 mm apart and incubated at 37 ◦C 18-24 hrs. The zone of inhibition around 3rd generation cephalosporin augmented toward clavulanic acid disc considered as positive for extended spectrum beta-lactamase.

Results

In this research study UPEC were characterized for the phenotypic expression of various virulence factors such as hemagglutination, serum bactericidal assay and cell surface hydrophobicity (Fig.1) and the detection of ESBL enzyme.

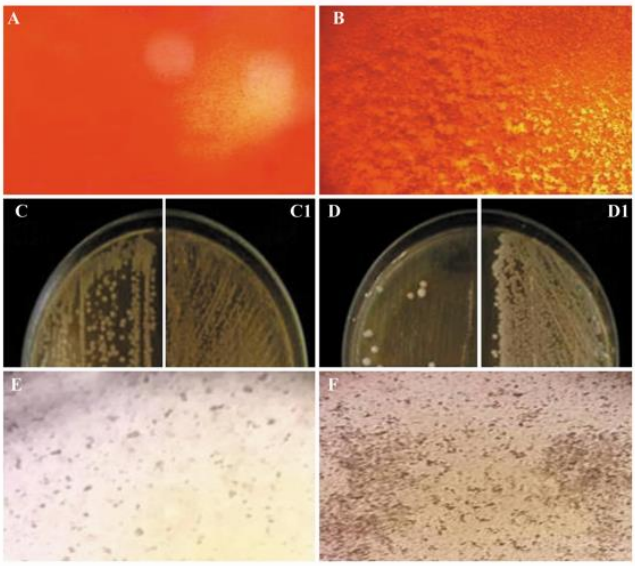

Fig1.A = hemagglutination negative, B = hemagglutination positive strain of UPEC (Microscopic view under 10X) C = test strain showing serum resistance after incubation with human serum, C1 = control strain incubated without serum .D = test strain showing serum sensitivity in which colony count drop notably, D1 = control strain incubated without human serum.E = salt aggregation negative, F = salt aggregation positive UPEC strain (Microscopic view under 10X).

In order to investigate the adherent property of UPEC we performed hemagglutination assay. Among 50 strains 46 were MRHA that agglutinate erythrocytes in presence of D-mannose while only 4 strains were MSHA.

Serum resistance (SR) helps UPEC to overcome host defense mechanisms. In order to find out serum resistance in UPEC the strains were subjected to serum bactericidal assay. Out of 50 UPEC strains 48 (96%) showed resistance to bactericidal action of the serum with a colony count of >90%.

Cell surface hydrophobicity (CSH) through which UPEC interact with surrounding cell surface was analysed by salt aggregation test .Out of 50 UPEC 40 isolates (80%) were positive for cell surface hydrophobicity having salt aggregation test value (SAT value) 1.4M while, 10 (20%) strains were not hydrophobic.

According to our investigations UPEC isolates showed high prevalence of virulence factors like serum resistance was the most common virulence factor and MRHA was second most common virulence factor followed by cell surface hydrophobicity.

Out of 50 UPEC strains 43 (86%) were ESBL producers while 7 (14%) did not show ESBL production. CSH, SR and MRHA were found in combination in 68% of UPEC in which 54% of strains were ESBL producers moreover UPEC strains showing combination of SR+MRHA, CSH+SR and CSH+MRHA were also found to be ESBL producers (Fig.2)

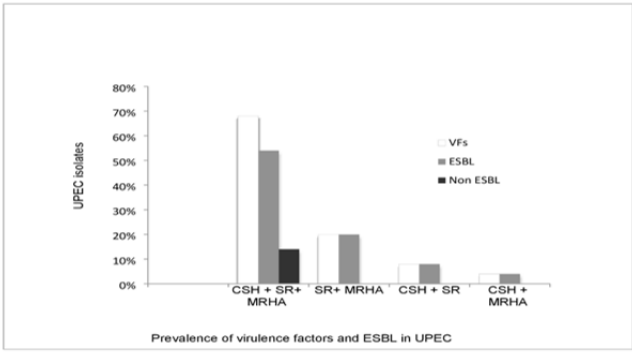

Fig2. Prevalence of virulence factors and ESBL in UPEC. VF= virulence factors, ESBL= extended spectrum beta-lactamase, CSH= cell surface hydrophobicity, SR= serum resistance, MRHA= mannose resistant hemagglutination

Discussion

UPEC isolates take advantage of expression of variety of virulence factors as well as drug resistant mechanisms for the colonization and establishment of UTIs. Virulence factors act in concert. Certain virulence factors help bacteria in adherence, persistence and tissue damage while others confer resistance to host defence mechanisms. Our results have shown high prevalence of tested virulence factors and ESBL expression in these isolates.

Hemagglutination is an indirect indication of adherence which is mediated by fimbriae and recognized as the key step in biofilm formation linked to the pathogenesis of UTIs. Mannose resistant hemagglutination is generally due to P, X, FIC and Dr fimbriae that involve in adherence to the fibronectin on uroepithelial cells thus take part in persistence. While mannose sensitive hemagglutination is mediated by type 1 fimbriae.[15] In the present study MRHA is found in majority of UPEC isolates. Similar results are also observed in a recent study of Kaira *et al.*, .[16]

Cell surface hydrophobicity is one of the important factors which contributes in virulence and increases the propensity of bacteria to adhesion and to penetrate in the host tissues.[17] Hydrophobic microorganisms are often more invasive and cause diseases that are difficult to treat. Considering that medical implants such as catheters, mechanical heart valves or pacemakers are constructed from hydrophobic materials (silicon, stainless steel, teflon, etc.), hydrophobic microorganisms are relatively easily adhering to them. In this regard crystalline surface

layers, S layers play key role (in interaction to various surfaces such as mucosal epithelial cells).[15] In the current study 40 (80%) strains showed cell surface hydrophobicity, a recent study of Kaira *et al*., showed 34 (27.64%)[16] and previous study by Mittal *et al*., showed 61% of strains were hydrophobic .[18]

Normal human serum has ability to kill bacteria by lytic activity of complement system but on the other hand certain bacteria have developed strategy to combat it such as *E. coli* capsular polysaccharides, surface proteins as well as lipopolysaccharides play critical role to confer serum resistance by escaping from phagocytosis.[19] In the present research work we found 48 (96%) strains were serum resistance other studies showed varying degree of serum resistance among UPEC like Sharma *et al*., in 2007 reported 68%, Sabitha *et al*., in 2014 reported 63.5% while, in contrast Raksha *et al*., has reported 32.7%.[6] The presence of multiple virulence factors makes an organism more virulent. In this study we investigated the virulent and resistant features of UPEC strains prevaling in Karachi. Overall this study showed high prevalence of ESBL in drug resistant strains of UPEC. A multicentre based study in China showed that ESBL-producing *E. coli* is the major pathogen responsible for symptomatic UTI.[20] Prevalence of ESBL producing strains have also been reported in our neighboring countries like Iran 67%, Bangladesh 43% and India 42%.[21] According to Pakistani study emergence of ESBL production in *E. coli* was jumped from 33% in 2005 to 60% in 2010.[22] The majority of UPEC strains showing multiple urovirulent factors in current study were found to be ESBL producers as well. It has also been reported to exhibit multiple drug resistance phenotype most commonly include cephalosporin, fluoroquinolones and aminoglycosides. ESBL production further leads to provide selective pressure for carbapenems thereby limiting the therapeutic options.

Extended antibiotic resistance is causing huge burden on economic and public health and affecting the quality of life. Different studies have shown that highly virulent strains have also become drug resistant therefore better understanding of pathogenesis is required by exploring the virulence factors. Gram negative bacteria carrying ESBLs is a global concern that demands urgent attention due to limited therapeutic options specifically ESBL producing *E. coli* is predominant organism that poses signficant threat to human health. In addition, inappropriate antibacterial treatment has also contributed to the emergence of antibacterial-resistance in bacteria. Hence, ESBL production in pathogens greatly limited the repertoire of antibiotics accessible for effective treatment of infections. It is therefore important to assess the virulence and drug resistance potential of common pathogens from time to time.

Conclusion

The study of virulence factors and detection of ESBL enzyme in UPEC isolates by phenotypic method indicates correlation between them and highlighted the need to further evaluate these isolates at molecular level. Both of these factors are important contributors which provide substantial advantage for survival of pathogen that could be responsible for chronicity of UTIs. ESBL producing UPEC have shown multiple drug resistant features therefore selection of appropriate antibiotic therapy is important to tackle them efficiently.

Conflicts of Interest: None declared.
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A. Conception/Study/Designing/Planning
B. Active Participation in Active Methodology
C. Interpretation/ Analysis and Discussion