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Phenotypic and molecular characterization of virulence factors of extra-intestinal pathogenic *Escherichia coli* isolated from patients of Peshawar, Pakistan

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Abstract: Extra-Intestinal *Escherichia coli* (ExPEC) are important cause of Urinary Tract Infections (UTIs) and systemic infections. The purpose of this study was to investigate numerous ExPEC bacterial isolates for phenotypic virulence characteristics including hemolytic activity and resistance pattern and to observe their association with genetic traits via Polymerase Chain Reaction (PCR). A total of 367 ExPEC isolates were collected from patients admitted in Khyber Teaching Hospital (KTH) Peshawar, Pakistan. Standard techniques were used for identification of isolates, determination of hemolytic potential and antimicrobial susceptibility testing. PCR was used for screening of virulence genes using specific primers. A total of 367 ExPEC isolates were characterized, among which 62.7, 24.3, 7.1 and 6% were isolated from urine, pus, sputum and wound specimens, respectively. Majority of the isolates (82.8%) were hemolysin positive. Multi drug resistance pattern was shown by 41% of the isolates and harbored at least one virulence gene (71.7%), of which *sat* was the most prevalent (64.3%). The highest resistance was found to cefotaxime (99.2%), ampicillin (97.5%) and aztreonem (89.6%). 15 different virulence genes combinations were observed in the current study. A total of 16 virotypes (15 of positive virulence genes and one of no virulence gene) were observed in the current study. The current investigation showed a high prevalence of *sat* and *hlyA* genes among ExPEC isolate, suggesting a role of these genes in the pathogenesis of ExPEC.

Keywords: *Escherichia coli*, ExPEC, virulence genes, virotypes, antimicrobial resistance.

INTRODUCTION

Escherichia coli, a well-documented commensal in intestinal tract of humans and certain pathotypes are documented pathogens causing various kinds of infection. The disease causing potential of *E. coli* is determined by certain risk factors like toxins, capsules, invasions, adhesions, hemolysins, cytotoxic necrotic factors. The disease causing *E. coli* are designated based on clinical signs and virulence factors as either InPEC, causing disease in intestinal tract or ExPEC, causing disease in extra-intestinal sites of the body. The InPEC, mainly diarrhea causing, is a leading cause of mortality in children; however, pathogenic ExPEC isolates are implicated in infections such as UTIs, meningitis and septicemia (Nataro *et al*., 1998; Bandyopadhyay *et al*., 2012; Kaper *et al* 2004).

Various studies reflect the difference in virulence profile and phylogeny of *E. coli* isolates isolated from UTIs, infections of blood stream, central nervous system (CNS) and respiratory tract from commensals and diarrheagenic isolates. Recently uropathogenic *E. coli* (UPEC), neonatal-meningitis causing *E. coli* (NMEC) and sepsis causing *E. coli* (SPEC) have been collectively termed ExPEC as they shared common genetic virulence traits to tackle host defenses and to cause various diseases in humans and animals (Johnson and Kuskowski, 2003; Johnson and Russo, 2002; Smith *et al*., 2007). Although various virulence factors and their role in pathogenicity of ExPEC have been described, yet there are many such isolates that show resemblance with non-ExPEC in their virulence profile. Even so, the classification of ExPEC is proposed on the basis of the detection of virulence-associated genes (VAGs) (Johnson and Russo, 2005). In humans UTIs (including cystitis and pyelonephritis) are found to be the second most common type of infections that account for approximately 150-250 million cases per annum globally. It has been observed that more than half of the women have at least single episode of UTI in their lifetime and 20-40% of them are prone to get recurrent UTI (Johnson and Russo, 2005; Asadi *et al*., 2014).

According to the previous investigations, the UPEC isolates possess widespread virulence factors associated pathogenesis of *E. coli* in urinary tract in which the most prevalent and important factors are toxins. They often secrete α-hemolysin (HlyA) toxin which is primarily associated with highly virulent isolates of ExPEC (Dhakal *et al*., 2008; Firoozeh *et al*., 2014). The role of hemolysin

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is to release iron from the lysed red blood cells (erythrocytes) thereby increasing the bacterial pathogenicity (Naveen and Mathai, 2005). It is considered to be a principal pore-forming toxin belonging to the RTX family and reported in 25-52% of the ExPEC isolates (Caprioli *et al*., 1983, Caprioli *et al*., 1984). Many more toxins are secreted by *E. coli* including families of cytotoxic necrotizing factor (*CNF*), SPATE (serine protease autotransporters of Enterobacteriaceae) and cytolethal distending toxin (*CDT*). *CNF1* and *CNF2* are known to be dermonecrotic toxins and *CNF1* is found regularly in UPEC, NMEC isolates and also in bacteremia cases. The SPATE subfamily of auto-transporters is currently composed of *sat*, *tsh*, *vat*, *espP*, *epeA*, *pic*, *espC* and more. UPEC is a leading cause of UTIs that produces *sat*. It is cytotoxic on vero kidney cells, HK-2 human bladder and HEp-2 cell lines. Like *pet* or *espC*, *sat* contact with culture cells results in cell elongation and detachment from their support (Guyer *et al*., 2000). It causes kidney and bladder cells vacuolation that is dependent on protease activity of *sat* and also on internalization. Like *sat*, *vat* induces the formation of intracellular vacuoles in cell culture known as vacuolation. So far no substrates have been identified for *vat* (Wang *et al*., 2003).

Cytolethal distending toxins are now considered to have a preserved and extremely dispersed family of reputed virulence factors produced by a large group of more than 30 γ and ε *Proteobacteria*, as a consequence of which inflammatory disease and chronic infection occurs, usually affecting mucocutaneous tissue.

The sequence of *cdt* genes of *E. coli* (O86:H34) reported that the three closely linked genes named as *cdtA*, *cdtB* and *cdtC* are responsible for cdt activity. The *cdtA*, *cdtB* and *cdtC* genes among different bacterial species producing this toxin show variable sequence homology and even within the same species, several CDTs have been identified (*E. coli*). CdtA and CdtC subunits are encoded by the most divergent genes whereas CdtB subunit is encoded by the most conserved gene (Roesch *et al*., 2003). Additionally, emergence of antimicrobial resistance in virulent isolates increases their persistence capability that is a serious threat to public health (Hilbert, 2011). Phenotypic measurement *In-vitro* does not accurately determine the *In-vivo* expression of the virulence factor. Also, determining the virulence genotype does not show the *In-vivo* expression (Naveen and Mathai, 2005).

However, phenotypic determination of highly resistant and virulent ExPEC is required to find rapid methods to routinely determine virulence factors and to design new drugs to treat all the infections. To the best of our knowledge no work has been reported on ExPEC SPATE and CDT toxins and this is the very first report from Pakistan, in which we have examined the molecular prevalence of toxin genes in pathogenic ExPEC isolates, their hemolytic activity, phenotypic antibiotic resistance and correlation with clinical manifestation.

MATERIALS AND METHODS

Samples collection

A cross sectional study was conducted prospectively between October, 2014 and October, 2016 in KTH and Center of Biotechnology and Microbiology, University of Peshawar (COBAM, UOP), Pakistan. All non-duplicate clinical samples including urine, pus, sputum and infected wound swabs were aseptically collected from patients of different age groups and transported immediately to the microbiology laboratory for culture and susceptibility testing. Urine specimens were obtained from patients suffering from UTIs, pyelonephritis and/or cystitis. Specimens of pus and wound were obtained from patients with surgical site infections (SSIs) while sputum samples were taken from patients with pulmonary infections. Patients undergoing antibiotic therapy and/or suffering from enteric infections were excluded from the study.

Patient demographics

All of the patient's necessary information including history of infection and treatment summary were obtained from hospital and laboratory records with the approval of ethical committee. Personal information of the patients and their medical records were kept confidential.

Bacterial culture and Identification

All microbiological specimens collected aseptically were transported to the microbiology laboratory of KTH and were inoculated without any delay. Urine samples were plated on Cystine Lactose Electrolyte-Deficient medium (CLED) and MacConkey agar (Oxoid, Basingstoke Hampshire, UK), using calibrated loops and then incubated at 37°C for 24 hours. All positive urine cultures were further evaluated for significant growth; $\geq 10^5$ colony-forming units per milliliter (CFU/ml) were considered clinically significant and were further identified to species level according to the standard microbiological methods (Hilbert, 2011). Specimens like pus, wound swabs and respiratory specimens like sputum were inoculated onto 5% sheep's blood agar, chocolate agar and MacConkey agar plates (Oxoid, Basingstoke Hampshire, UK). The plates were incubated at 37°C aerobically and examined after 24 and 48 hours (Cheesbrough, 2006).

Pure and well isolated colonies were analyzed for their morphology and further subjected to Gram staining. Bacterial identification was done primarily by different conventional biochemical tests including urease, Triple-Sugar Iron (TSI), Indole, motility, Methyl-red, Voges-Proskauer and Citrate (IMViC). Later on pure cultures of

bacteria were further subjected to different biochemical tests using Analytical Profile Index (API) 10S strips (Biomerieux, France) for confirmation (Alnahass *et al*., 2016).

Antimicrobial susceptibility testing

All isolates were subjected to antimicrobial-susceptibility testing against selected antibiotic discs by Kirby Bauer disc diffusion technique as per Clinical and Laboratory Standard Institute (CLSI 2017) guidelines. Briefly, bacterial suspension was adjusted to 0.5McF and spread on Mueller-Hinton Agar (Oxoid, Basingstoke Hampshire, UK). The selective discs were then applied including: AMP (10µg), AMC (20/10µg), SAM (10/10µg), TZP (100/10µg), FEP (30µg), CTX (30µg), CAZ (30µg), ATM (30µg), MEM (10µg), IPM (10µg), CN (10µg), TOB (10µg), AK (30µg), DO (30µg), CIP (05µg), LVX (5µg), NA (30µg), SXT (1.25/23.75µg), C (30µg), FOS (200µg) (CLSI, 2017).

The results were interpreted after overnight incubation at 37˚C by measuring the inhibition zone diameter according to CLSI protocols. The antibiotic testing quality control was assured by utilizing *E. coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 700603.

Hemolysin Tube Assay

All isolates were investigated for hemolysin production by tube assay as described previously (Chouduri *et al*., 2015). Briefly, human intact erythrocytes suspension was prepared from 10ml of blood collected from healthy donor and centrifuged at 5000rpm for 5 minutes. The supernatant was discarded and erythrocytes were washed thrice with 0.9% sodium chloride (NaCl) and finally suspended in normal saline to prepare 3% erythrocytes suspension. Bacterial isolates from the stock were sub-cultured on MacConkey agar plates to get isolated colonies. After overnight incubation at 37°C, 2 - 3 colonies were inoculated in Tryptone Soy Broth (TSB) and incubated at 37°C for 24 hours. Then 2ml of bacterial culture was centrifuged at 1000rpm for 10 min and the supernatant was incubated with equal amount of 3% washed erythrocytes suspension on water bath at 37˚C for 3 hours. The contents were observed at regular intervals each hour with mild shaking. The mixture was then centrifuged at 5000rpm for 10 minutes and the concentration of released hemoglobin was determined at OD_{530nm}. Triton-X-100 (0.1% v/v) was used as positive control, showing 100% hemolysis, while washed erythrocytes solution with normal saline with no bacterial culture supernatant was used as negative control. All isolates were categorized into four groups; strong (OD: ≥1), moderate (OD: ≥0.5-<1), weak (OD: >0.025-<0.5) and non-hemolytic (OD ≤0.025) (Croxall *et al*., 2011).

Culture Maintenance and Genomic DNA extraction

All the isolates were cryo-preserved in triplicates in 20% glycerol supplemented with TSB (Oxoid, Basingstoke Hampshire, UK) and stored at -80°C. The frozen isolates were sub cultured on blood agar and incubated overnight at 37˚C. Chromosomal DNA was extracted by using GeneJet genomic DNA purification kit (Thermo Scientific, Waltham, MA). The DNA integrity and quality was determined by gel electrophoresis in 1% agarose (w/v) and purified DNA was stored at -20°C (Wang *et al*., 2013).

Bacterial confirmation by PCR

E. coli isolates were confirmed by amplifying *uspA* and *uidA* genes for universal stress protein and beta-D-glucouronidase, respectively (Chen and Griffiths, 1998; Bashir *et al*., 2012). The final PCR reaction was done in 27µl final reaction volume containing; 12.5µl Taq master mix (Bioron, Germany), 0.5µl of 50pM forward primer, 0.5µl of 50pM reverse primer, 2µl of DNA template and PCR grade water. Amplification was carried out by using specific primer sequences [table 1] under the following conditions; 1 cycle of initial denaturation at 94˚C for 5 mins followed by 30 cycles of denaturation at 94˚C for 30sec, annealing for 1min, extension at 72˚C for 1min and 1 cycle of final extension at 72˚C for 7 mins. Amplicons were visualized by electrophoresis using 1.5% agarose (w/v) dissolved in 1X Tris-Acetate Ethylene diaminetriacetic acid (TAE) buffer with EtBr stain (fig. 1a-1e).

Molecular Characterization of Virulence Genes

All isolates were screened for toxin genes encoding α-hemolysin subunit A (*hlyA*), cytotoxic necrotizing factor 1 (*cnf*1), secreted autotransporter toxin (*sat*), vacuolating autotransporter toxin (*vat*) and cytolethal distending toxin subunit B (*cdtB*) (Ali and Rashki, 2015). Uniplex PCR amplification was carried out for each toxin gene in a 27µl final reaction volume by using already reported specific primers except for *hlyA* which was designed in this study by NCBI Primer Blast Tool with an amplicon size 263bp using specific sequence of *E. coli* ATCC 25922 from position 1764379 to 1764641 as template [table 1]. The PCR reaction was performed under following cycling conditions: 1 cycle of initial denaturation at 94°C for 5mins followed by 30 cycles of denaturation, annealing and extension and 1 cycle of final extension at 72˚C for 7mins [table 1]. All testing was done with *E. coli* ATCC 25922 as positive control and nuclease free sterile water as negative control.

Gel Electrophoresis

The amplified products were electrophoresed for 40 to 60 minutes at 110 volts on 1.5% agarose gel suspended in 1X TAE buffer. Gels were stained with EtBr solution. Bands were visualized and photographed by gel documentation system (Bio Rad Milan, Italy) using GeneSnap software. The amplicon sizes were determined by comparing them with a 100-bp DNA ladder (Thermo scientific, USA) (Ali and Rashki, 2015).

STATISTICAL ANALYSIS

All variables were expressed as percentages. The chi-square test was used to compare the proportion of bacterial isolates with patient sex and age; and comparison of antimicrobial resistances. P-values of <0.05 were considered to be statistically significant. Odds ratios (OR) and 95% confidence intervals were used to determine association between toxin genes and antibiotic phenotypic resistance, an OR<1 and >1 values were considered as negative and positive associations, respectively. All statistical studies were conducted using SPSS v.15.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

A total of 367 isolates of ExPEC were obtained in this study. The majority of ExPEC isolates were female patients (59.7%) and the rest were from male patients (40.3%). The overall distribution of ExPEC isolates among different age groups and gender is shown in table **2**. Furthermore, considering age distribution, patients of age-group 41-60 years were highly infected (31.3%) followed by patients of age-group 21-40 years (25.9%), 11-20 years (20.7%) and patients above 60 years (15.5 %) while only 6.5% of patients with age group 0-10 years was found to have ExPEC infection (table 2). The highest number of ExPEC isolates was isolated from urine samples (62.7%) followed by pus (24.3%), sputum (7.1%) and wounds accounted for 6% of isolates (table 2).

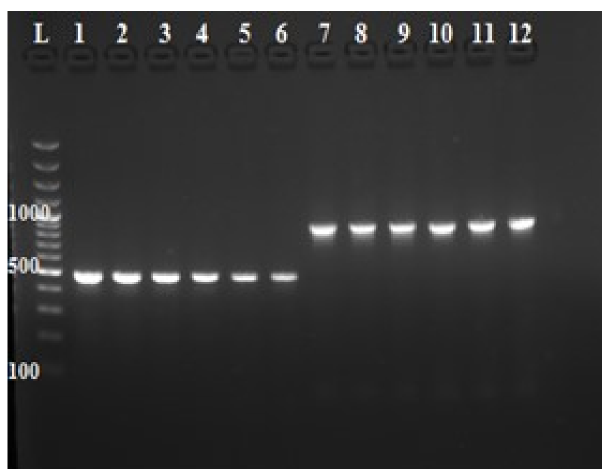

Fig. 1a: Gel electrophoresis of PCR products of *uid*A and *usp*A genes, L: 100-bp DNA ladder (Thermo scientific), Lane 2-6: ExPEC isolates positive for *uidA* gene with amplicon of 486bp, Lane 8-12: ExPEC isolates positive for *uspA* gene with amplicon of 884bp, Lane 1: Positive control for *uidA* gene, Lane 7: Positive control for *uspA* gene.

ExPEC distribution among different age groups

The infection rate among female of age group 41-60 years was high (32%) followed by age-group 21-40 years (26.9%), age group 11-20 years (17.3%) and above 60 years (15.1%) while the incidence rate of ExPEC in females of age-group 0-10 years was 8.7%. The highest number of ExPEC isolation rate among male patients was in age group 41-60 years (30.4%).

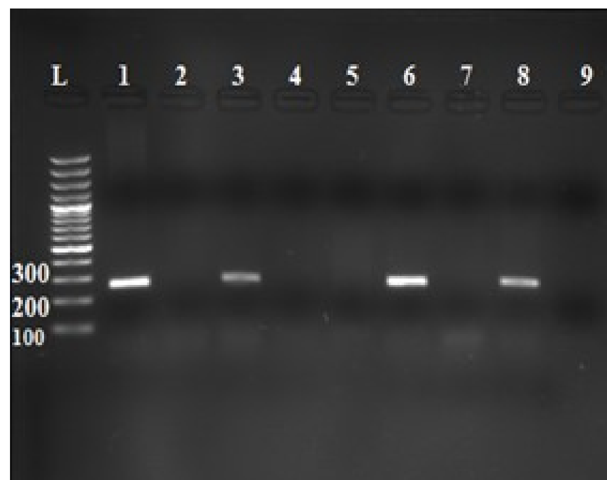

Fig. 1b: Gel electrophoresis of PCR products of *hlyA* gene; L: 100-bp DNA ladder (Thermo scientific), Lane 1: Positive control, Lane 9: Negative control, Lane 3,6,8: ExPEC isolates positive for *hlyA* gene with amplicon of 263bp, Lane 2,4,5,7:ExPEC isolates negative for *hlyA* gene

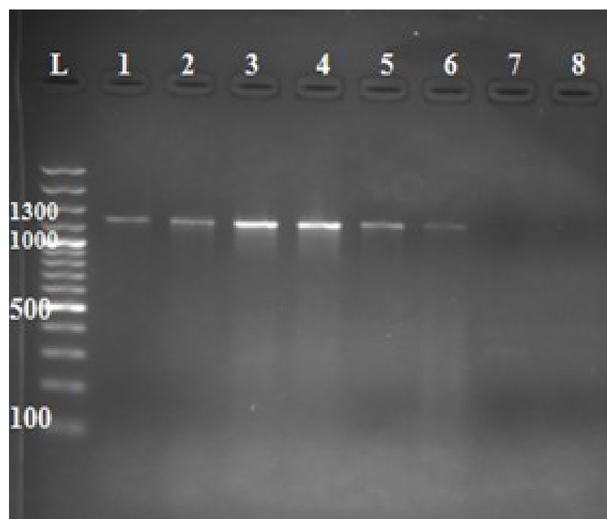

Fig. 1c: Gel electrophoresis of PCR products of *cnf1* gene, L: 100-bp DNA ladder (Thermo scientific), Lane 1: Positive control, Lane 8: Negative control, Lane 2-6: ExPEC isolates positive for *cnf1* gene with amplicon of 1295bp, Lane 7: ExPEC isolates negative for cnf1 gene.

Hemolytic strength

The hemolytic activity was observed in 304 (82.8%) isolates. Among the hemolytic isolates (n=304), 11.4% of ExPEC isolates were strongly hemolytic, 43.3% were moderate, 28.1% were weak and 17.2% of the isolates were non-hemolytic (table 2).

Fig. 1d: Gel electrophoresis of PCR products of *sat* gene, L: 100-bp DNA ladder (Thermo scientific), Lane 1: Positive control, Lane 9: Negative control, Lane 2-8: ExPEC isolates positive for *sat* gene with amplicon of 410bp.

Fig. 1e: Gel electrophoresis of PCR products of *vat* gene, L: 100-bp DNA ladder (Thermo scientific), Lane 1: Positive control, Lane 7: Negative control, Lane 2-4: ExPEC isolates positive for *vat* gene with amplicon of 217bp, Lane 5,6: ExPEC isolates negative for vat gene.

Prevalence of Toxin Genes

The targeted toxin genes were detected in 263 (71.7%) of ExPEC isolates and was not detected in 104 (28.3%) isolates. The most prevalent toxin gene was *sat* 236 (64.3%), followed by *hlyA* 73 (19.9%), while *vat* and *cnf*1 were positive in 45 (12.3%) and 44 (12%) isolates, respectively. None of the isolates were positive for *cdtB* gene as shown in Table 2 and presented in fig. 1a-1e. The toxin genes distribution was higher in ExPEC isolates from female compared to male patients. All toxin genes were frequently detected in patients of age group 41-60 years except *sat* gene that was found mostly in patients with age group 21-40 years.

Phenotypic antibiotic resistance

The most frequent resistance was found against; CTX (99.2%), followed by AMP (97.5%), ATM (89.6%), CIP and LVX (83.4%), while moderate rate of resistance was observed for FEP (78.2%), CAZ(73.3%), AMC (60.8%). However, 51%, 50.1%, 46%, 40.3%, 33.8%, 30.5%, 25.3%, 14.4% and 12.3% of isolates were resistant to TZP, TOB, DO, CN, AK, IPM and MEM, C, SAM and FOS, respectively. For statistical analysis, isolates of intermediate susceptibility were considered to be sensitive. A total of 151 (41%) isolates showed resistance to more than three classes of antibiotics and were thus considered as multidrug resistant (MDR) isolates. Table 3 summarizes the distribution of resistance phenotypes among ExPEC isolates (n=367).

Frequency of different Virotypes among ExPEC

A total of 16 different toxin genes combinations were observed in the studied isolates (n=367). In this study, the majority of ExPEC isolates were positive for *sat* (42%), followed by those isolates which harbored none of the tested toxin genes (28.3%), 7.1% of the isolates were positive for *hlyA/cnf1/sat* genes combination; *hlyA/sat* genes combination was detected in 6.3% of isolates, 5.2% of ExPEC isolates were positive for *sat/vat* genes combination, *hlyA*, *vat* and *hlyA/sat/vat* toxin genes was positive in 2.7, 1.9 and 1.4% of the isolates, respectively. The *cnf1/vat* and *hlyA/cnf1/vat/sat* genes combination was positive in 1.1% of isolates, other genes combination frequency was <1%. The frequency distribution of different toxin genes (virotypes) combination is presented in Table 4.

Virotypes association with antibiotics resistance

The statistical correlation of associations between antibiotic resistance phenotypes and toxin genes were calculated. A varied statistical association (P>0.05) was observed between the presence of the different toxin genes and antimicrobial resistance phenotypes. Overall, a negative association was observed between all studied genes and virotypes with phenotypic antibiotic resistance (odds ratio was <1, meaning negative correlation; data is not shown).

DISCUSSION

ExPEC represent a diverse group of *E. coli* isolates known to cause disease in human, animals and several food-borne infections worldwide. In Pakistan, studies have been done to characterize these isolates, mainly focusing on the UTIs. The current investigation was done to characterize the ExPEC population isolated from patients presenting with infections other than gastroenteritis.

In this study, 63% of the isolates were recovered from UTIs followed by 30% of isolates associated with SSIs

and 7% were recovered from lower respiratory tract infections ($p < 0.001$). In some other investigations the isolation rate was close to our findings. Micenkova are reported 37 and 27% of the isolates from SSIs and lower respiratory tract infections, respectively (Micenkova *et al*., 2006). Wide majority of ExPEC isolates were recovered from females as compared to males and this finding was also reported (Tabasi *et al*., 2015). However, these findings were not in accordance with some other investigations, where the percentage of ExPEC isolated from males was slightly higher than females. Age distribution revealed the highest percentage of ExPEC in age group 41-60 years in our study which was 115 (31.3%) ($p< 0.001$). These results are similar to another study where higher isolates were observed in age-group 40-70 (Kukanur *et al*., 2015).

Toxins are important virulence factors of *E. coli* involved in numerous diseases. *HlyA* is the most common pore-forming *E. coli* secreted toxin able to lyse erythrocytes and other nucleated cells in order to make access for the extra-intestinal pathogens to infect the host and survive by utilizing the host nutrients. However at low concentrations, it causes the induction of targeted cells apoptosis and damage to epithelial and endothelial cells (Bien *et el.*, 2012). In this study the expression of *hlyA* was investigated for all isolates representing a higher (82.9%) incidence rate of hemolytic isolates ($p<0.001$) (Johnson and Stell, 2000). Only 10% isolates were confirmed as hemolytic in another study reported by Grover *et al.* (2013). Whilst these hemolytic results were comparable with the findings of Kukanur *et al.* (2013) yet there is a difference in their association with the specimen type (Kukanur *et al*., 2015; Grover *et al.,* 2013). In a study, hemolysin production was found to be highest among ExPEC isolated from catheter tips followed by isolates isolated from blood, urine, pus and sputum (Shetty *et al*., 2014). An interesting finding in our study is that we observed hemolytic activity by isolates that were negative for *hlyA* gene which probably means that hemolysis could be due to the release of silent hemolysin (*sheA*) that is a pore-forming toxin and a cytolysin A, causing the release of hemoglobin from erythrocytes (Oscarsson *et al*., 2002).

Studies have been carried out worldwide to determine virulence genotyping by molecular based methods. Several virulence factors are characterized in this study including *hlyA, cnf*1, *sat*, *vat* and *cdt*. High frequency of these toxin genes was detected in patients with UTIs. Previous studies showed nearly similar findings supporting our investigations (Micenkova *et al*., 2016). The *cnf1* gene was observed in approximately 12% isolates and majority was isolated from UTIs cases, while Bashir *et al.,* documented the prevalence of *cnf1* in up to 20% of UTIs cases. These results are different from the reports of Micenkova *et al*. (2016) where only 1.9% isolates were positive for *cnf1* obtained from skin and soft tissues. The prevalence of *sat* in our study was found to be 64.3% while *vat* in about 12.5% of the ExPEC isolates ($p<0.001$). Majority of them were obtained from pus and urine and our findings for *sat* gene are almost in agreement with the findings of Sarayulu *et al*. (2012) where *sat* gene was observed in 75% of isolates but showed *vat* gene in 36% isolates, showing higher prevalence rate (Saraylu *et al.*, 2015). In a study done by Johnson and Stell (2000) only 8% of the uropathogenic isolates were *cdtB* positive regarding *cdtB* as possible ExPEC virulence factor. In contrast, none of the isolates were positive for *cdtB* gene in our study (Johnson and Stell, 2000). This result was in agreement with the investigations done in Iran (Mirzarazi *et al.*, 2015). In Pakistan, no work has been reported on ExPEC SPATE and CDT toxins.

The current investigation provided occurrence of 16 different combinations of genes (virotypes) in ExPEC isolates containing 1 or more virulence genes. A combination of 4 genes (*hlyA/cnf1/sat/vat*) was exhibited by 4 isolates, while 4 different combinations of 3 virulence genes were observed and the highest frequency of *hly*A/*cnf*1/*sat* virotype was observed (7.1%). While 6 different combinations were observed for 2 virulence genes of which *hly*A/*sat* virotype was determined to be the most prevalent combination in about 6.3% isolates. Apart from this, 4 virotypes were found to contain single virulence gene, of which the most prevalent was *sat,* 154 (42%) isolates. Overall, there was a significant difference noted in the distribution of toxin genes among the studied isolates of ExPEC ($p<0.0001$). These findings are in accordance with the results reported previously (Smith *et al*., 2007; Bien *et al*., 2012). Likewise, only 5.2% of the isolates possessed *sat*/*vat* virotype and only 1.1% isolates contained *cnf1*/*vat* virotype (Johnson and Stell, 2000).

In this study, 41% of the *E. coli* isolates were resistant to at least three different classes of antibiotics (MDR) and 91 different drug resistant patterns were observed by Kirby Bauer disc diffusion method. This elucidates the emergence of a highly variable resistance profile in ExPEC. Nearly all isolates (99.2%) were highly resistant to cefotaxime, followed by ampicillin (97.5%), aztreonem (89.6%), ciprofloxacin and levofloxacin (83.4%). The current results are comparable with other previous findings where resistance to ampicillin was 97 and 92%, respectively (Bashir *et al.,* 2012; Abdi and Ghalehnoo, 2015) Until late 1990s, ExPEC showed a relatively high susceptibility to penicillins, cephalosporins, amino-glycosides and fluoro-quinolones but the surveillance studies reports in 2000s have shown the emergence of resistant ExPEC in North and South America and also in Europe (Johnson and Russo, 2002; Micenkova *et al*., 2016, Foxman, 2010).

Table 1: Optimized PCR conditions and primers used for bacterial confirmation and molecular characterization of toxin genes in ExPEC isolates

Gene target	Primer oligonucleotide (5 to 3) sequence	Product Size (bp)	Optimized amplification conditions			Original reference
			Denaturation	Annealing	Extension	
E. coli, hemolysin A (*hlyA*)	F: AACAGGTATTCGGCACAGCA	263	94°C for 30s	57°C for 60s (30 cycles)	72°C for 60s	Present study
	R: AGAACTGACATTGCCACCAGA					
E. coli, cytotoxic necrotizing factor 1(*cnf1*)	F:CTGACTTGCCGTGGTTTAGTCGG	1295	94°C for 30s	59°C for 60s (30 cycles)	72°C for 60s	(kuhar *et al*., 1998)
	R:TACACTATTGACATGCTGCCCGGA					
E. coli, secreted autotransporter toxin (*sat*)	F: CTACAGCTTGATCACCTATGGC	410	94°C for 60s	59°C for 60s (30 cycles)	72°C for 60s	(Saraylu *et al*, 2012)*
	R: CTCCCTGGTATTTCTTTGTGG					
E. coli, vacuolating autotransporter toxin, (*vat*)	F: TTCACGGTACTGTTGTTCGC	217	94°C for 60s	54°C for 60s (30 cycles)	72°C for 60s	(Saraylu *et al*, 2012)*
	R: CAGATAACTCCAGCGTCACG					
E. coli, cytolethal distending toxin B (*cdtB*)	F:AAATCACCAAGAATCATCCAGTTA	430	94°C for 30s	63°C for 30s (30 cycles)	68°C for 3min	(Sorsa. J., 2007)
	R:AAATCTCCTGCAATCATCCAGTTTA					
E. coli, Universal Stress Protein (*uspA*)	F: CCGATACGCTGCCAATCAGT	884	94°C for 30s	56°C for 45s (30 cycles)	72°C for 30s	(Chen and Griffiths, 1998)*
	R: ACGCAGACCGTAGGCCAGAT					
E. coli, Beta D glucouronidase (*uidA*)	F: TCACCGTGGTGACGCATGTCGC	486	94°C for 30s	55°C for 30s (30 cycles)	72°C for 30s	(Bashir et at., 2012)*
	R:CACCACGATGCCATGTTCATCTGC					

*= with slight modifications, F= forward primer, R= reverse primer, *hlyA*= hemolysin A, *cnf1*= cytotoxic necrotizing factor 1, *sat*= secreted autotransporter toxin, *vat*= vacuolating autotransporter toxin, *cdtB*= cytolethal distending toxin B, *uspA*= universal stress protein A, *uidA*= Beta-D-glucouronidase

Table 2: Frequency distribution of ExPEC clinical isolates based on patient demographics, clinical presentation, phenotypic and molecular data (n=367).

	Frequency (n)	Percent (%)	*p* value
Gender			
Male	148	40.3	P<0.0001
Female	219	59.7	
Age Groups Overall			
0-10	24	6.5	p< 0.001
11-20	76	20.7	
21-40	95	25.9	
41-60	115	31.3	
> 60	57	15.5	
Specimen Type			
Urine	230	62.7	p < 0.001
Pus	89	24.3	
Sputum	26	7.1	
Wound	22	6	
Hemolytic Activity			
Strong	42	11.4	p < 0.001
Moderate	159	43.3	
Weak	103	28.1	
No Hemolysis	63	17.2	
Toxin Genes			
Sat	236	64.3	

Table 3: Frequency distribution of toxin genes (virotypes) among ExPEC isolates (*n*=367).

Virotypes (Toxin genes)	Frequency, n (%)
hlyA	10 (2.7)
Vat	7 (1.9)
Sat	154 (42)
cnf1	1 (0.3)
hlyA/vat	1 (0.3)
cnf1/sat	2 (0.6)
hlyA/cnf1	3 (0.8)
cnf1/vat	4 (1.1)
sat/vat	19 (5.2)
hlyA/sat	23 (6.3)
hlyA/cnf1/sat	26 (7.1)
hlyA/sat/vat	5 (1.4)
cnf1/sat/vat	3 (0.8)
hlyA/cnf1/vat	1 (0.3)
hlyA/cnf1/vat/sat	4 (1.1)
No gene	104 (28.3)

Table 4: Distribution of antibiotic resistance phenotypes among ExPEC isolates (n=367).

Antibiotic	Resistance (%)	Antibiotic	Resistance (%)
Ampicillin	97.5	Gentamicin	40.3
Amoxicillin-clavulanic	60.8	Tobramycin	50.1
Ampicillin-Sulbactam	14.4	Amikacin	33.8
Piperacillin-Tazobactam	51	Doxycycline	46
Cefipime	78.2	Ciprofloxacin	83.4
Cefotaxime	99.2	Levofloxacin	83.4
Ceftazidime	73.3	Nalidixic acid	82.6
Aztreonem	89.6	Seftran-sulphamethoxazole	89.1
Meropenem	30.5	Chloramphenicol	25.3
Imipenem	30.5	Fosfomycin	12.3

The high rate of antibiotic resistance in present study may reflect over irrational usage of antimicrobial agents, poor infection control practices or other factors such as possession of resistance genes and mutations contributing towards MDR phenotypes. Therefore, strong surveillance programs and infection control measures are indispensable steps need to be taken to control the dissemination of antibiotic resistance in hospital and community.

The association among virulence genes and resistance phenotypes varied in this study. Overall, we observed negative correlation between virulence genes and antibiotic resistance. Our findings suggest that the antibiotic resistance is not augmented by the presence of toxin genes and resistance might be strain-specific or due to various antibiotics used in different geographical regions.

CONCLUSION

The current study revealed a high prevalence of *sat* and *hlyA* toxin genes among ExPEC isolate which thereby suggests a role of these genes in the pathogenesis. In present study ExPEC isolates pathogenicity was commonly associated with UTIs and SSIs and provides base line epidemiological data in hospitalized patients. Further large scale studies are needed for better understanding of the molecular mechanisms responsible for the pathogenesis of such isolates beside correlation of virulence factors and resistance phenotypes. Also, there is need to fully characterize the ExPEC isolates both phenotypically as well as genotypically to find rapid diagnostic strategies and design new drugs.

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