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Phenotypic and Molecular Characterization of Haemolysins in relation to Antibiotic Resistance Profile among Uropathogenic *Escherichia coli* (UPEC)

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ABSTRACT

This study was conducted to detect the prevalence of haemolysis, its *hly* virulence genes and the antimicrobial resistance profile of *Escherichia coli* strains implicated in Urinary tract infections (UPEC) in Palakkad, India. From 280 urine samples 200 *E. coli* strains were isolated and identified. The antimicrobial resistances of *E. coli* isolates were characterized using Disk Diffusion method. Haemolysis was checked using 5% sheep Blood agar and finally PCR was carried out for strains showing haemolysis using *hly* D primer. Antimicrobial susceptibility testing revealed, 90.5%, 52.0%, 47.5%, 19.0%, 38.5%, 24.7%, 36% and 30% resistance rate to ampicillin, cefuroxime, ciprofloaxacin, ceftazidime, co-trimoxazole, gentamicin, nalidixic-acid, norfloxacin and nitrofurantion respectively. Haemolysis in sheep blood agar was observed in 55 (27.5%) of the isolates. *hlyD* genes were seen among 10 UPEC strains. The data suggest a high prevalence of antibiotic resistance in UPEC strains.

Keywords: Uropathogenic Escherichia coli (UPEC), antibiotic resistance, haemolysis, hlyD, Polymerase chain reaction (PCR).

1. INTRODUCTION

Uropathogenic *E.coli* (UPEC) which are a leading cause of both acute and chronic urinary tract infections often secrete a labile pore forming toxin known as alpha haemolysin (Hly A) [1]. Haemolysin owes its name because it cause lysis of erythrocytes. Water moves into the cell as a result of the increased intracellular osmotic pressure, causing the cell to swell and rupture. This is also a cytolytic protein toxin. The production of toxins helps bacteria to spread within the host tissue by disrupting cellular integrity. Bacteria gain access to nutrients from lysed host cells. Toxins help the pathogen to spread into deeper tissues after disrupting cell integrity and to gain access to nutrients inside the host cell; it will destroy immune effector cells and thus evade their potential antibacterial activity. The tissue damage will provoke a strong inflammatory response and thus eventually help the host terminating the infection [2].

The common assay, namely haemolysis is based on the property of the toxin to lyse red cells. Lyses of erythrocytes result in dissipation of trans-membrane ion gradients. Iron, is released from cells that can be used by the bacteria for propagation [3]. Most of the haemolytic *E.coli* produces haemolysins. Haemolysin is a toxin for a wide range of host cells which may result in inflammation, tissue injury, and impaired host defenses. It has been noted that monocytes and granulocytes are highly susceptible to haemolysin cytotoxicity, whereas lymphocytes are relatively resistant. Exposure of polymorphonuclear leukocytes (PMNLs) to haemolysin results in impaired chemotaxis and phagocytosis [4]. The chain of effects by which haemolysin results in degranulation and release of leukotrines of the phagocytic cells bringing about morphological alteration has been successfully demonstrated in zebra fish model [5]. Serum has been shown to partially prevent the action of the haemolysin on leukocytes [6].

Haemolysin belongs to the prototypical member of repeat in Toxin (RTX) protein family encoded by the CABD operon. There are two common types of this toxin, α-haemolysin and β-haemolysin. α-haemolysin is commonly produced by strains isolated from cases of human UTI and other extra intestinal infections. The prefix alpha denotes the exotoxin nature [7]. It is heat labile and will be coded either in the chromosome or the plasmid. The *hlyA* gene codes for the alpha-haemolysin protein (HlyA). It is secreted across both membranes of bacteria (outer and inner membrane) without lysis. HlyB and HlyD together with the outer membrane protein *Tol*C are required to transport HlyA from cytoplasm to the external medium. The HlyC protein is required for activation of HlyA to its haemolytic form by transferring a fatty acyl residue to it. *hlyC, hlyB* and *hlyD* genes code *for the* proteins HlyC, HlyB and HlyD [8]. β-haemolysin is a cell associated haemolysin with a similar range of haemolytic activity as α-haemolysin [9].Anti inflammatory action has been proposed for Hly toxin (1). Haemolysin stimulates superoxide anion and hydrogen peroxide release and oxygen consumption by renal tubular cells as well as histamine release from mast cells and basophils [10]. Natural Killer (NK) cells are destroyed by HlyA. Since NK cells promote secretion of TNF-α in response to infection, this action of HlyA suppresses the pro-inflammatory response to UPEC. Hly might also reduce the release of cytokines by various immune cells [11]. Hly producing uropathogenic *E.coli* is associated with exfoliation of the bladder epithelium. HlyA stimulates activity of serine proteases and caspases, which then mediate the degradation of paxillin (important to stabilise cell–cell contacts) and induce apoptosis [12].

UTIs are among the most common bacterial infections endured during clinical practice. The resolution of symptoms and sterilization of the urine is the aim of treating UTI. Several factors are considered when selecting the drugs. This include drug active at urinary pH values, drug excreted in the active form and the cost factor. Another factor to consider will be the effect of drug on the resident flora of vagina and gut and the prevalence of drug resistance within the community and its outcome upon public health [13]. The present scenario is showing rising antibiotic resistance among UPEC for commonly used antimicrobial agents like

fluoroquinolones, beta-lactams, co-trimoxazole and nitrofurantoin. Add to it the emergence of extended spectrum β lactamase (ESBL) which has complicated the treatment and its outcome of UTI.

The aim of this study was to look for the prevalence of haemolytic activity among UPEC, assessment of haemolysin gene (*hlyD*) of UPEC and the antibiotic resistance pattern shown by the strains isolated from patients with culture positive community acquired UTI, with significant bacteriuria from a tertiary hospital in Kerala.

2. MATERIALS AND METHODS

Clinical samples

Two hundred *E.coli* strains were isolated from 280 urine samples of patients suffering from acute cystitis. The urine samples were collected aseptically as mid stream voided specimen. The urine samples were examined macroscopically and microscopically by wet mount. The sample was spread on MacConkey agar and Blood agar by semi-quantitative method. A colony count of $\geq 10^3$CFU/ml of urine was considered as significant. The bacteria which had grown with significant counts were identified by their colony morphology, Gram's smear, motility and biochemical reactions. Diagnosis of *E.coli* cystitis was made based on clinical symptoms and culture having significant colony count. Samples associated with more than one bacterial species were excluded from the study.

Antimicrobial susceptibility testing

The antibiotic susceptibility testing was done by using the Kirby-Bauer disk diffusion method. The antibiotic disks which were used were ampicillin, ceftazidime, cefuroxime, nitrofurantoin, co-trimoxazole, gentamicin, norfloxacin, ciprofloxacin and nalidixic acid. The zone size around each antimicrobial disk was interpreted as sensitive, intermediate or resistant according to the CLSI criteria.

Phenotypic assay: Haemolysis

Production of α-haemolysin was tested on 5% sheep blood agar. *E. coli* strains were inoculated onto blood agar plates, incubated overnight at 37^0C and haemolysis was detected by the presence of a zone of complete lysis of the erythrocytes around the colony.

Virulence factor genotyping to detect the *hlyD* gene

Genomic DNA was isolated from the Bacterial culture by boiling; PCR reactions were used to screen each *E. coli* isolate for the presence of virulence gene *hlyD* using specific primers gene markers as previously described [14]. Appropriate positive and negative controls were included. Positive controls for *hlyD* gene were strains J96, 2H25, L31, 2HI6, S36 and P10. Negative controls were PM9, 11A and JJ055). The primers sequences used in this study are present in Table 1.

Table I The primer used for UPEC screening of hly gene

hlyD	*hlyD*	**F**	CTCCGGTACGTGAAAAGGAC	904bp
		R	GCCCTGATTACTGAAGCC**T**G	Ref:[14]

Statistical analysis

The significance of the results was established using the Chi square and Fisher's exact test and the level of significance was set at a P value<0.05

3. RESULTS

Antimicrobial susceptibility testing revealed, 90.5%,52.0%,47.5%,19.0%,38.5%,24.7%, 36% and 30% resistance rate to ampicillin, cefuroxime, ciprofloaxacin, ceftazidime, co-trimoxazole, gentamicin, nalidixicacid, norfloxacin and nitrofurantion respectively (Table II). About 54% were multidrug resistant to three or more classes of antibiotics. There was not any significant difference in the *p* values from the isolates of males and females.

Haemolysis in sheep blood agar was observed in 55 (27.5%) of the isolates. The frequency of distribution of the various classes of antibiotic -susceptible and antibiotic-resistance among the haemolysis positive and haemolysis negative strains are given in Table 3. Frequency of haemolysis seen among strains resistant to cephalosporins ranged from 27.3% for ceftazidime to 58.2% for cefuroxime. While among the haemolytic *E.coli* antibiotics susceptibility ranged from 41.8% for ceftazidime and 72.7% for cefuroxime. The resistance rate among haemolysis positive strains for nalidixic acid, ciprofloxacin, norfloxacin and trimethoprim-sulfamethoxazole (Co-trimoxazole) was found to be 41.7%, 41%, 35% and 35.3% respectively. Analysis revealed a significant difference between the

haemolysis positive and haemolysis negative strains that were resistant to ciprofloxacin and nalidixic acid ($p<=0.001$) and also significant difference between haemolysis positive and haemolysis negative strains that were co-trimoxazole resistant ($p<0.05$). Ampicillin resistance was found to be 28.2% in haemolysis positive strains and 71.8% in haemolysis negative strains. Gentamicin resistance was found to be 24.5% in haemolysis positive and 75.5% in haemolysis negative strains.

Table II Antibiotic susceptibility pattern among UPEC strains with and without haemolysis (R=resistant S=sensitive)

		haemolysis								
		Absent			Present			Total		
		Count	Column N %	Row N %	Count	Column N %	Row N %	Count	Column N %	Row N %
Ampicillin	R	130	89.7%	71.8%	51	92.7%	28.2%	181	90.5%	100.0%
	S	15	10.3%	78.9%	4	7.3%	21.1%	19	9.5%	100.0%
Cefuroxime	R	72	49.7%	69.2%	32	58.2%	30.8%	104	52.0%	100.0%
	S	73	50.3%	76.0%	23	41.8%	24.0%	96	48.0%	100.0%
Ciprofloxacin	R	56	38.6%	58.9%	39	70.9%	41.1%	95	47.5%	100.0%
	S	89	61.4%	84.8%	16	29.1%	15.2%	105	52.5%	100.0%
Ceftazidime	R	23	15.9%	60.5%	15	27.3%	39.5%	38	19.0%	100.0%
	S	122	84.1%	75.3%	40	72.7%	24.7%	162	81.0%	100.0%
Co-trimoxazole	R	62	42.8%	80.5%	15	27.3%	19.5%	77	38.5%	100.0%
	S	83	57.2%	67.5%	40	72.7%	32.5%	123	61.5%	100.0%
Gentamicin	R	37	25.7%	75.5%	12	22.2%	24.5%	49	24.7%	100.0%
	S	107	74.3%	71.8%	42	77.8%	28.2%	149	75.3%	100.0%
Nalidixic acid	R	42	29.0%	58.3%	12	22.2%	41.7%	72	36.0%	100.0%
	S	103	71.0%	80.5%	25	45.5%	19.5%	128	64.0%	100.0%
Norfloxacin	R	39	26.9%	65.0%	12	22.2%	35.0%	60	30.0%	100.0%
	S	106	73.1%	75.7%	34	61.8%	24.3%	140	70.0%	100.0%
Nitrofurantion	R	22	15.2%	64.7%	12	21.8%	35.3%	34	17.0%	100.0%
	S	123	84.8%	74.1%	43	78.2%	25.9%	166	83.0%	100.0%

We performed molecular analysis of *hlyD* in haemolysis positive strains. *hlyD* gene prevalence was checked for in haemolysis positive strains. The hlyD was isolated from 10 strains. The *hlyD* gene primer (*hlyD*, membrane fusion protein) was used in this study to look for the prevalence of *hly* operon (Figure 1). It was found to be 18.6%. Ampicillin resistance was 90% among these strains, the resistance pattern for other antibiotics were 50% to cefuroxime, 40% for ciprofloxacin and nalidixic acid, 30% for nitrofurantoin and cotrimoxazole, 20% for norfloxacin and gentamicin and 10% for ceftazidime. The lower percentage detection of *hly* gene prevalence could be due to the choice of primer *hly D*.

The general distribution of antibiotic resistant and antibiotic susceptible pattern among haemolysis positive and negative strains is illustrated in Figure 2. Among the haemolytic and non-haemolytic strains, Ampicillin showed highest resistance range and ceftazidime showed least resistance. The general distribution of antibiotic resistant and antibiotic susceptible pattern among *hly* positive and *hly* negative strains are illustrated in Figure 3. The prevalence of antibiotic resistance pattern was almost similar in *hly* positive and *hly* negative strains, with the exception of co-trimoxazole resistance being high among the *hly* negative strains (40%) compared to only (20%) resistance in *hly* positive strains. Nitrofurantoin resistance was found to be less in *hly* negative strains, less than 20% while among the *hly* positive isolates it was 30%.

To confirm the amplification products obtained by PCR, truly represented the expected sequences of *hly, one* each positive strain was individually amplified. The amplified product was sent for sequencing. The products of sequencing were subjected to BLAST

analysis and the sequences showed 99-100% similarity. Analyzed sequence was submitted to The Genbank and has got the following accession number KU933940.

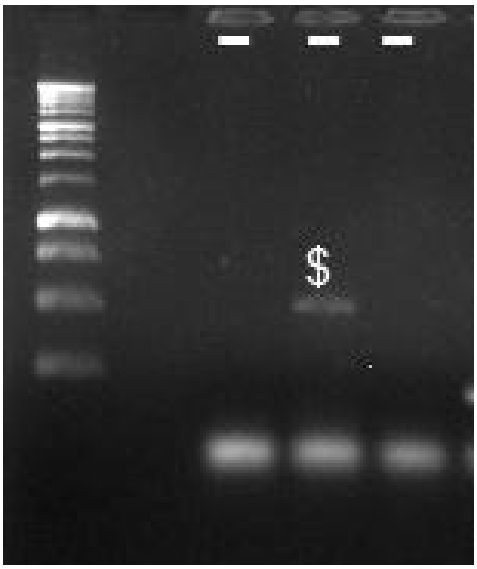

Figure1 PCR showing amplification of UPEC
$:hly gene Lane 2:*hly* gene from UPEC (Left side lane: 1kb Molecular ladder)

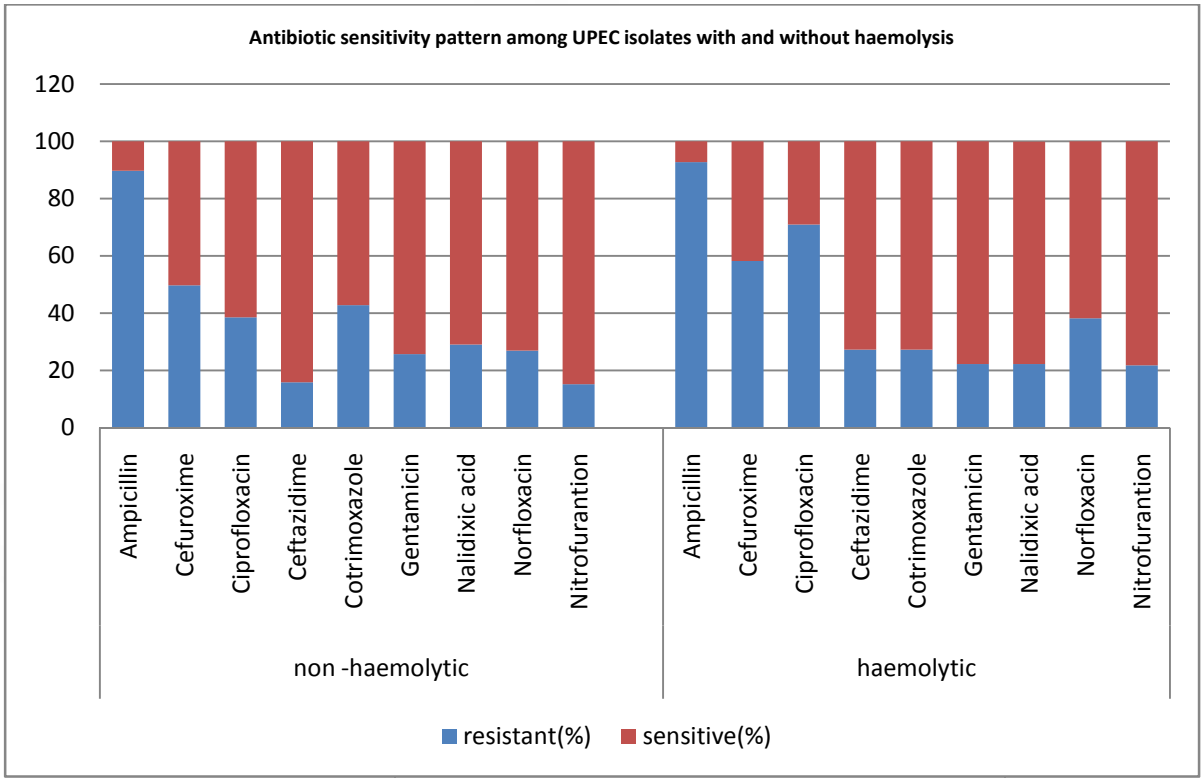

Figure 2 Antibiotic sensitivity pattern among UPEC isolates with and without haemolysis

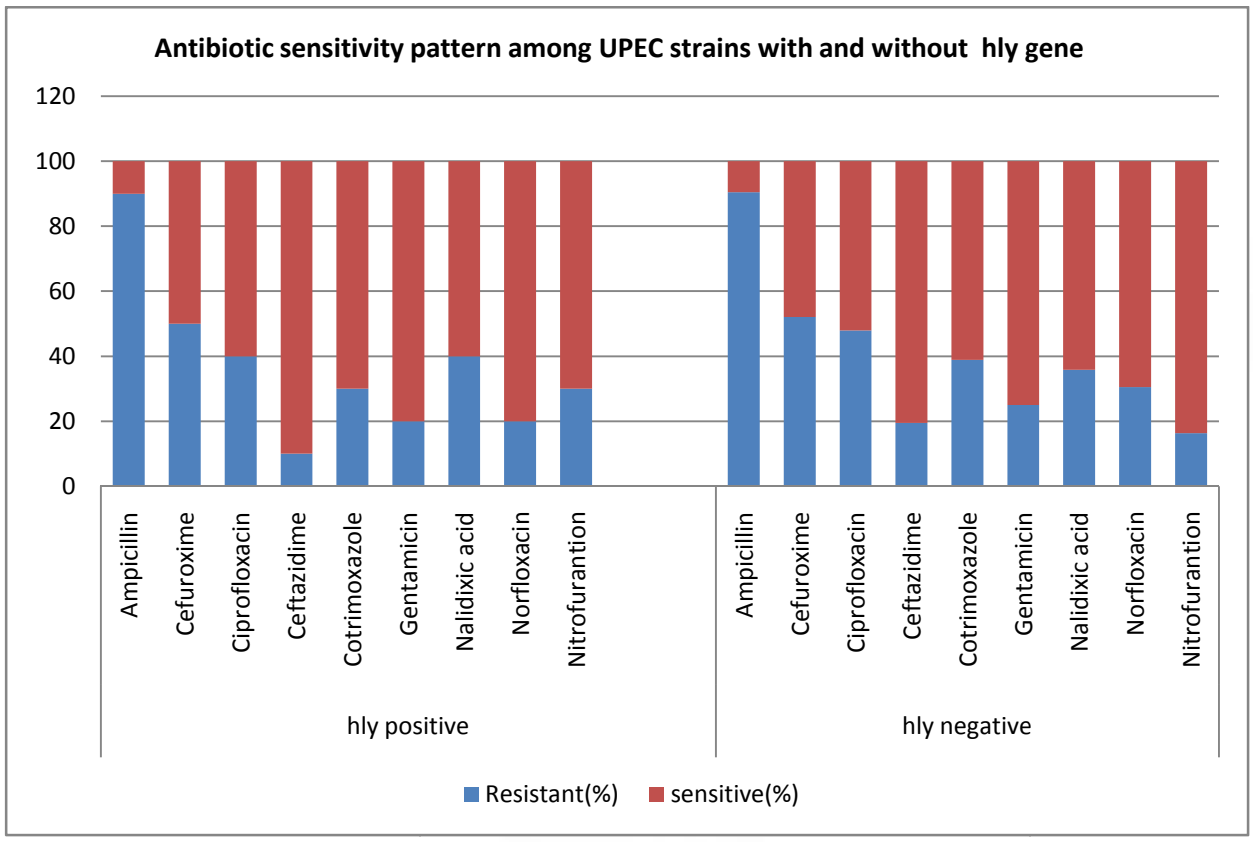

Figure 3 Antibiotic sensitivity pattern among UPEC isolates with and without *hly* gene

4. DISCUSSION

Treatment of UTI is important to prevent relapses. Antimicrobial drug resistance is further adding to the cost of treating these infections because they often require more complicated treatment regimens, and might result in treatment failures [15]. This study was conducted to determine the distribution of haemolysins in UPEC by haemolysis, check the prevalence of hlyD virulence genes, and the antimicrobial-resistance pattern in the related isolates. Resistance rates among common uropathogens to many commonly used antimicrobial agents have increased over the years and these resistance rates vary between countries. In this study, we investigated the antimicrobial susceptibility pattern of *E.coli* strains isolated from UTI cases in our region, for better prescription of empiric antimicrobial agents and to demonstrate the distribution of resistance patterns. Resistance to ampicillin was about 90% and this clearly demonstrates why this antibiotic shouldn't be used for empirical treatment of UTIs.

Co-trimoxazole is another commonly used first-line antimicrobial agent which had 38.5% resistance rate among the isolates. Fluoroquinolones are widely accepted as better empiric choice of UTI treatment. Low level resistance is being reported to nitrofurantoin. This drug exhibits low resistance rate in the major part of the world (0%–5.4%), despite of it's being used for many years [16]. Several factors may play roles in antimicrobial resistance, though the misuse of antibiotics in each geographic location directly affects the antibiotic-resistance pattern.

In 1921 Dudgeon, Wordley and Bowtree inferred that haemolytic strains were more common among UTI strains than the faecal flora [17]. The evidence for the trait as virulence factor epidemiological and experimental was established by Brooks *et al*, 1980 and O'Hanley *et al*, 1991 & Welch *et al*, 1981 respectively [18]. Haemolysin (*hly*) is produced by various pathogenic types of *E.coli* causing extraintestinal and intestinal infections, but its effect on virulence is not completely clarified. *hly* gene region being related with complicated urinary tract infections such as pyelonephritis has been reported by many researchers. *hly* plays a major role in evoking damage in the uroepithelium and in inducing hemorrhage in the bladder during the early stages of *E. coli*-mediated cystitis in the mouse [19].

Hly operon is often located adjacent to the P fimbrial genes on the same pathogenicity island on the chromosome of UPEC strains. The prevalence of *hlyD* gene was less in this study. Studies have shown varied frequencies from 1% - 44% for *hly* gene. Abe *et al* reported 44% [20], Maslow *et al* reported 60%, Wang *et al* 45% [21], Tiba *et al* 25% [22], Fattahi *et al* 26% [23], Miyazaki *et al* 19% [24], Farshad *et al* 15.6% [25], Emamghorashi *et al* 13.5% [26]. The difference observed in the occurrence of *hly* determinant and haemolysis could be because of the presence of a different allele of the primer expressing the phenotypic trait. Differences in geographical variation or differences in association with host characteristics could also be the reason for varied expression. In the Israeli study involving cystitis in women *hly* was 75% [27]. Ruiz *et al* studied the prevalence of virulence determinants in Spain and found *hly* genes in 52% [28]. In the study by Vaishnavi *et al* hly genes were detected in 5 of the 30 diarrheagenic *E.coli* isolates [29]. Chakraborty *et al* 2016 reported *hlyA* (23%) from Mangalore [30]. In the Iranian study of 2017 with 112 *E.coli,* 32 were *hly* positive [31]. *hly* was found to be in 9.3% out of 75 *E.coli* isolates in an Egyptian study [32]. In a study by Soto et al, biofilm-producing *E. coli* isolates isolated from the urinary tract and causing inflammation of prostate were often the highest haemolysin-containing isolates and biofilm production had significantly correlated with the expression of haemolysin [33]. In another 2015 study from Iran *hly* genes were present only in 10% of UPEC isolates [34]. Different results in various studies may be due to regional differences in targeted population and the increasing resistance to antibiotics. Limitations of this study included the checking for only a single primer, *hlyD* for polymerase chain reaction and the study population was confined to a specific geographic location. The fine tuned expression of HlyA protein helps to attenuate the host cell signaling and studies have shown it can induce host cell inflammation response [35].

5. CONCLUSION

Since antimicrobial drug resistance is high among the *Uropathogenic E.coli* strains, strict management of antibiotic policies and surveillance programs for resistant organisms together with infection control procedures need to be implemented and continuously audited. A regular evaluation of antimicrobial susceptibility patterns of various clinical isolates and proper surveillance of the virulence properties of the strains shall help to understand better about the strains that are prevalent within the community.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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