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## Comparative Analysis of Virulence Factors of *Escherichia coli* from Non-enteric Infections

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There is correlation between certain properties of *E. coli* strains and capacity of the strain to cause non-enteric infections. The aim of this study was to compare *Escherichia coli* strains isolated from non-enteric infection with those from faeces of normal healthy individuals, for their possession of haemolysin, mannose-resistant-haemagglutinin, colicin, protease, cell surface hydrophobicity and antibiotic susceptibility. Source wise distribution of the haemolytic strains were 60% from urine, 41.7% from blood, 33.3% from peritoneal fluid, 50% from pus and 26.7% from stool. Colicinogeny was found to be a common property of both clinical and faecal *E. coli* and only a few of the urine and blood isolates (52 and 41.7%, respectively) exhibited colicin V activity. Mannose Resistant Haemagglutinin (MRHA) test showed positive (MRHA<sup>+</sup>) reaction for 47.7% of the clinical *E. coli* isolates, while this value was only 26.7% for the controls. A significant association between haemolysin production and MRHA of human type O erythrocytes was found, as 75.86% of the Hly<sup>+</sup> *E. coli* strains were also MRHA<sup>+</sup>. The data obtained in this study suggested that haemolysin production, MRHA of human type O erythrocytes and hydrophobic cell surface might be important for *E. coli* strains to initiate and sustain infection at non-enteric sites.

**Key words:** *E. coli*, virulence, antibiotic resistance, haemolysin, mannose resistant, infection

## INTRODUCTION

*Escherichia coli* is a commensal organism inhabiting human and animal intestinal tract. It can cause variety of non-enteric infections when enters into unnatural sites (Sharma *et al.*, 2007; Frederick, 2011). Several virulence factors help *E. coli* isolates to survive under adverse conditions present at the non-enteric sites and contribute to the ability of *E. coli* to cause extra intestinal infections (Bamu *et al.*, 2011). Non-enteric or Extra-intestinal pathogenic *E. coli* (ExPEC) is a group of *E. coli* strains that induce extra-intestinal diseases (Russo and Johnson, 2000). ExPEC has a great impact on public health in terms of both morbidity and mortality, with an economic cost of several billion dollars annually (Russo and Johnson, 2003).

Considerable amount of information has been accumulated in recent years on *E. coli* virulence markers and their role in infections. Production of enterotoxins, hemolysins, colicins, haemagglutinins, proteases, colonization factors, cell surface hydrophobicity etc is some virulence-associated factors of *E. coli* (Kausar *et al.*, 2009). Secretory proteases are common virulence factors in many bacterial and nonbacterial pathogens including *E. coli* (Dezfulian *et al.*, 2003). Haemolysin is one of the most important virulence factors and according to some previous studies, approximately 50% of *E. coli* isolates causing extra-intestinal infections in humans are haemolytic (May *et al.*, 2000). Cell surface hydrophobicity has been identified to be important in cell adhesion and pathogenicity of *E. coli* (Najar *et al.*, 2007). Mannose Resistant Haemagglutination (MRHA) are adhesive factors, which are important in the establishment of pathogenic strains of *E. coli* to various host tissue (Drews *et al.*, 2005). Colicins are toxic proteins produced by *E. coli* and active against related bacteria and Phenotypic expression of colicin V (Col V) have been described as an indicator of complement resistance (Riley, 1993).

As *E. coli* infection involving the urinary tract, peritoneum, blood and meninges occur frequently in our country, a comprehensive study on the virulence factors of these bacterial strains is urgent. Development of resistance against antibiotics is making treatment of *E. coli* infections difficult (Mathur *et al.*, 2002). Practices of self-medication, drug abuse and indiscriminate misuse of antibiotics among the general people favored the emergence of drug resistant strains (Manikandan *et al.*, 2011). Considering the above facts in mind, knowledge on antibiotic susceptibility pattern of extra-intestinal

pathogenic *E. coli* is necessary to select correct antibiotic(s) for proper treatment of the infections caused by them (Sharma *et al.*, 2007).

The aim of this study was to compare *E. coli* strains isolated from urinary tract infection, septicemia, peritonitis and abscess with those from faeces of normal healthy individuals, for their possession of haemolysin, mannose-resistant-haemagglutinin, colicin V, protease, hydrophobic cell surface and antibiotic susceptibility. This would provide an opportunity to determine the importance of individual characteristics of *E. coli* strain in different clinical situations.

## 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 Cerebrospinal Fluid (CSF) were included in the study. Five *E. coli* strains isolated from stool of healthy individuals were also included. *Streptococcus pyogenes*, Nonpathogenic *E. coli* ATCC-35218, *E. coli* ATCC-25922, *E. coli* K-12 (Col), *E. coli* K-12 Col V<sup>+</sup>, *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.

**Sample collection:** Urine, blood, pus, peritoneal fluid, stool and Cerebrospinal Fluid (CSF) was collected through standard procedures. A midstream specimen of urine was collected in a sterile wide-mouthed bottle. Five milliliter of blood was drawn from the cephalic vein with a sterile disposable syringe. 0.2 mL pus was drawn from the abscess using a sterile disposable syringe. Immediately after abdomen was opened by the surgeon, 2 mL of peritoneal fluid was collected in a sterile disposable syringe. In case of stool sample, stool was touched with a sterile cotton swab which was returned immediately to a sterile cotton-plugged test tube. In case of cerebrospinal fluid, about 0.5 mL of CSF was collected in a sterile tube aseptically by lumbar puncture.

**Isolation of strains:** Immediately after collection, the samples of urine, blood, pus, CSF, peritoneal fluid and stool were directly inoculated onto MacConkey agar and blood agar plates by spread plate technique. Plates were incubated for 24 h at 37±0.5°C. Isolated colonies were streaked on nutrient agar plates for pure culture and for presumptive identification using biochemical tests (Adzitey *et al.*, 2012).

**Identification of strains:** The shape and type of Gram reaction are microscopically studied using 18 h culture from agar plate. The biochemical tests involved Klingler's Iron (KIA) Agar, Simmon's Citrate agar, Motility Indole Urease (MIU), Lysine Iron Agar (LIA), Urea broth, Peptone water, Methyl Red (MR), Voges Proskauer (VP), Nutrient Nitrate Broth (NB), Gelatin liquefaction, Eijkman test, carbohydrate fermentation test was done for lactose, sucrose, glucose and starch, Oxidase and Catalase tests (Apun *et al.*, 2008). Identification of isolates obtained in pure culture was based on Gram staining, biochemical characteristics and growth pattern on selective and differential media and according to the procedures recommended in the Bergey's Manual of Determinative Bacteriology (Holt, 2005; Edwards and Ewing, 1986).

**Detection of haemolytic strains:** The haemolytic activity was observed on washed blood agar plates according to Sharma *et al.* (2007) and Subashkumar *et al.* (2006). Sixty five *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:** The colicin production was determined by the 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<sup>+</sup> strains were used as control.

**Haemagglutinin test:** Slide haemagglutination of erythrocytes was performed as described by Kausar *et al.* (2009) and Peerayeh *et al.* (2008).

**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; Mansouri *et al.*, 2011).

**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 (Paniagua *et al.*, 1990; Shumi *et al.*, 2004). *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), Cefotaxime (Cep, 30 µg), Ceftriaxone (Cef, 30 µg), Trimethoprim-Sulfamethoxazole (Tms, 25 µg), Ampicillin (Amp, 25 µg), Chloramphenicol (Clr, 20 µg), Cefradine (Cef, 30 µg), Gentamicin (Gen, 30 µg), Penicillin (Pen, 10 µg), Nitrofurantoin (Nit, 100 µg), Ceftazidime (Caz, 30 µg), Polymyxin B (Pol, 300 IU) and Nalidixic acid (Nal, 30 µg). 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).

**Statistical analysis:** Analysis was performed by employing statistical package for social science (SPSS version 16) software and excel office program for the statistical analysis of this study. To compare mean values between groups t-test was done as a test of significance (Shahina *et al.*, 2011).

## RESULTS AND DISCUSSION

**Identification of *E. coli*:** The identification of the isolates of *E. coli* was confirmed by plating on them onto MacConkey and EMB agar. All of the isolates showed typical characteristics of *E. coli*. All the isolates were gram-negative, non-sporing and mostly motile. All the strains were oxidase negative, indole positive, urease negative, did not produce H<sub>2</sub>S in KIA media, catalase positive, methyl-red positive, Voges-Proskauer negative, reduced nitrate to nitrite, did not utilize citrate or liquefy gelatin.

**Haemolysin production:** *E. coli* strains were grown overnight on sheep and human blood agar plates at 37±0.5°C. The strain that gave clear zone of haemolysis larger than the overlying colony was considered as positive reaction. However, the strains that produced haemolysis on two consecutive days were labeled as haemolytic *E. coli*. The strain that produced haemolysis on sheep blood agar was also found haemolytic for human blood agar. The control (*Streptococcus pyogenes*) produced haemolysis on all the blood agar plates. It was found that 29 (44.6%) clinical isolates of *E. coli* were haemolytic. Among the clinical *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

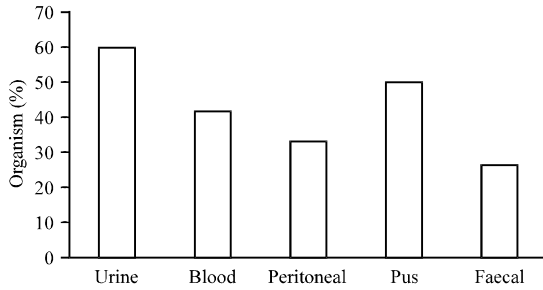


Fig. 1: Haemolysin production by *E. coli* isolates from different sources

haemolytic. While only four of the 15 (26.67%) faecal *E. coli* strains produced haemolysin. Haemolysin production by *E. coli* from different sources are shown in Fig. 1.

**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. Colicin production by *E. coli* isolates is shown in Fig. 2.

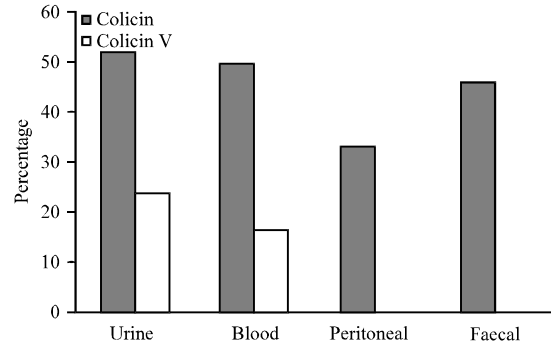


Fig. 2: Colicin production by *E. coli* isolates

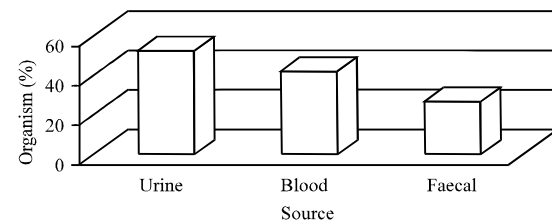


Fig. 3: Haemagglutinin (MRHA) production by *E. coli* isolates

**Mannose-resistant Haemagglutination (MRHA) test:** All the *E. coli* strains were screened for their possession of mannose-resistant-haemagglutinins by using human type O erythrocytes in the slide agglutination test. The strains that gave positive reactions on two successive days were labeled as MRHA positive *E. coli*. 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, whereas 26.7% *E. coli* strain of faecal origin were MRHA positive (Fig. 3). 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 (44%) strains produced both haemolysin and MRHA, 15 (30%) strains produced only haemolysin and 13 (26%) strains were MRHA positive but haemolysin negative (Fig. 4).

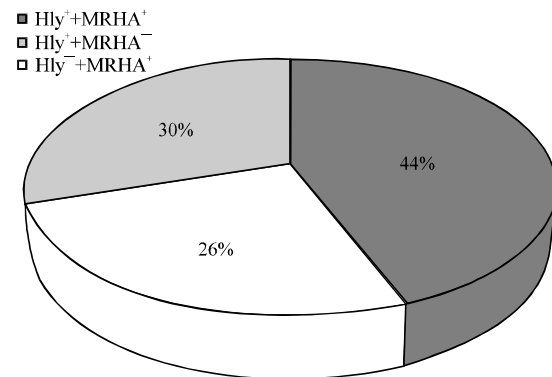


Fig. 4: Relationship between haemolysin production and MRHA of human type O erythrocytes by *E. coli* strains

**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  $\leq 2.0$  M concentration, whereas, 9 out of 15 (60%) of control strains had SAT value  $\leq 2.0$  M. Source-wise analysis showed that 18 (72%) urine isolates; 5 (41.7%) peritoneal isolates and 9 (75%) blood isolates had SAT value  $\leq 2.0$  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%) of 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. Comparison of cell surface hydrophobicity of *E. coli* isolates from different sources is shown in Fig. 5.

**Protease production:** All of the 65 *E. coli* strains isolated from different pathological samples were screened for protease production by cultivating in 2% milk-agar plates. At the end of 5 days of incubation at 37±0.5°C, 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. The control (*P. aeruginosa* NCTC-6750) always gave positive result on the same medium. Production of protease by *E. coli* isolates from different sources is shown in Fig. 6.

**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 Fig. 7.

**Antimicrobial resistance:** The clinical isolates of *E. coli* were tested for their susceptibility to 14 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. 85% strains was resistant to ampicillin, 73% strains were resistant to tetracycline, 77% strains were resistant to streptomycin, 69% strains were resistant to penicillin, while resistance to sulfamethoxazole-trimethoprim and chloramphenicol were 59.0 and 44.5%, respectively. The third-generation cephalosporin (ceftriaxone, ceftazidime and cefotaxime) and polymyxin B showed most effectiveness. Other drugs that appeared to be clinically useful were the first-generation cephalosporin (cephradine), nalidixic acid, gentamicin and nitrofurantoin. The percentage of resistance to the antibiotics is shown in Fig. 8.

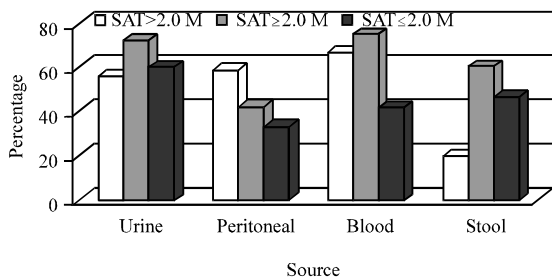


Fig. 5: Comparison of SAT value of *E. coli* isolates from different sources

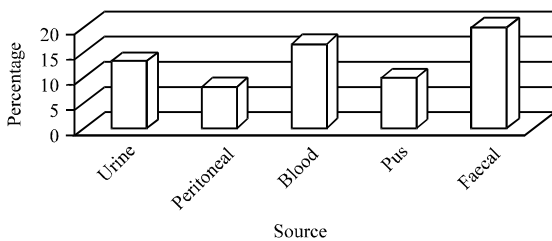


Fig. 6: Protease production by *E. coli* isolates

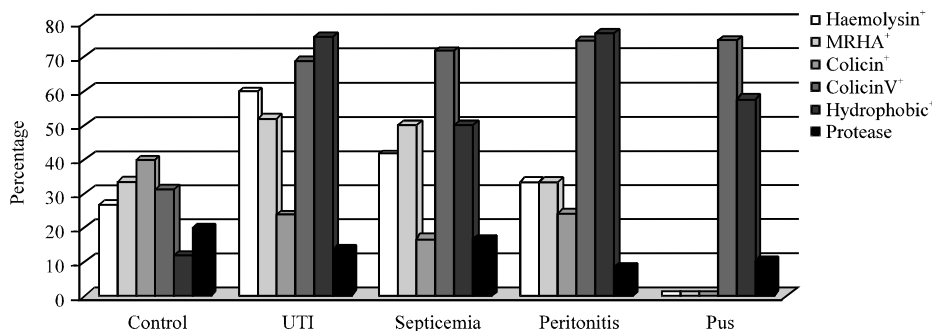


Fig. 7: Comparison of virulence factors of *E. coli* isolates from different sources

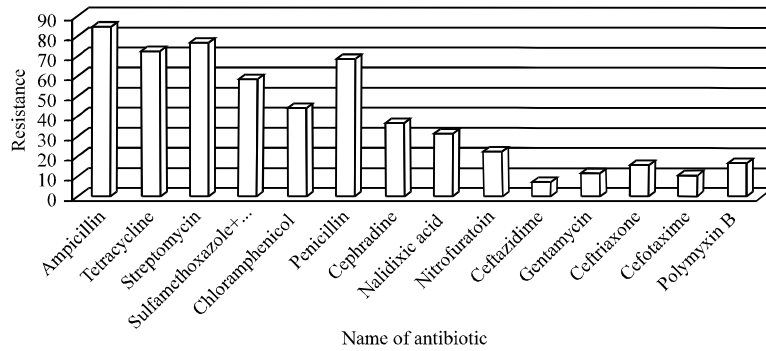


Fig. 8: Antibiotic resistance pattern of clinical isolates of *E. coli*

**DISCUSSION**

Discovering virulence factors is important to understand bacterial pathogenesis and interactions of them with the host. Understanding virulence properties may also aid to select novel targets in drug and vaccine development (Banu *et al.*, 2011). Capacity of *E. coli* to produce multiple virulence factors may contribute to its pathogenicity in extra-intestinal infections (Sharma *et al.*, 2007). These virulence factors make competent some members of the normal flora to cause an infection by overcoming the host defence mechanisms (Emody *et al.*, 2003). Production of haemolysin usually associated with pathogenicity of *E. coli*, especially responsible for more severe forms of infections (Johnson, 1991). 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 ( $p < 0.001$ ) indicating the importance of haemolysin in the pathogenesis of urinary tract infection (Najar *et al.*, 2007). Haemolytic *E. coli* strains may also at an advantage in producing septicemia as in this study 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  $p > 0.001$ , respectively). Few strains from pus and peritoneal fluid was also haemolytic indicating it may contribute to tissue injury (Sharma *et al.*, 2007).

Among the extra-intestinal pathogenic *E. coli* isolates, only the urinary and blood isolates produced colicin V. 6 (24%) urinary strains and 2 (16.7%) blood isolates showed colicin V activity. Production of colicin was found statistically not significant in urinary, blood and peritoneal isolates ( $p > 0.05$ ,  $p > 0.5$  and  $p < 0.5$ , respectively).

None of the *E. coli* strains isolated from peritoneal fluid gave MRHA positive reaction. Possession of mannose resistant haemagglutinin was found significant

for the urinary isolates ( $p < 0.001$ ) while, for the blood isolates the p value was  $p > 0.05$ . Similar results were found in the study of Soleimani and Nejad (1994).

Surface hydrophobicity is an important virulence factor of *E. coli* that causes extra-intestinal infections. *E. coli* strain with SAT value  $\leq 2.0$  M was described as hydrophobic while those with  $\geq 3.0$  M as less hydrophobic. Source wise analysis of the clinical isolates of *E. coli* strains indicated that the urinary and the peritoneal isolates were the most hydrophobic which is in accordance with results of Suman *et al.* (2001). Blood isolates have relatively high SAT values justifying the minor role of cell surface hydrophobicity in pathogenesis of septicemia. Another interesting finding was all the MRHA positive *E. coli* strains had SAT value  $\leq 1.0$  M.

In this study very few of the clinical or faecal isolates of *E. coli* produced extracellular protease and hence it can be presumed to be a minor virulence factor for *E. coli* infection. Some of the extra-intestinal *E. coli* isolates did not possess any of the virulence factors studied yet causing infections. May be these isolates induced infections in immuno-compromised hosts or they might possess properties different from those including in this study.

This study also revealed expression of multiple virulence factors by extra-intestinal *E. coli*. Most of the haemolysin and MRHA positive *E. coli* were also hydrophobic which is in accordance with Hughes *et al.* (1982). It is difficult to accurately predict virulence of an organism on the basis of the virulence phenotype, expression of multiple virulence factors contribute synergistically in overcoming normal host defence mechanisms (Sharma *et al.*, 2007).

Antibiotic susceptibility pattern was studied for all isolates of *E. coli*. Resistance was observed to commonly used antibiotics such as ampicillin, tetracycline, penicillin and streptomycin. The greater prevalence of resistance to common antibiotics has also been reported by other

workers (Chitnis *et al.*, 2003; Weiner *et al.*, 1999). The presence of multidrug resistance may be related to the dissemination of antibiotic resistance among hospital isolates of *E. coli*. Maximum number of isolates (85%) were resistant to ampicillin and the lowest (7.8%) to ceftazidime.

### CONCLUSION

From the data obtained in this study, it seemed probable that haemolysin production, MRHA of human type O erythrocytes and hydrophobic cell surface might be the important characteristics that enabled *E. coli* strains to cause extra-intestinal infections. Study on other characteristics, such as resistance to human serum, cytotoxin production, possession of aerobactin iron-acquisition system and adherence to uroepithelium might reveal the virulence determinants of the strains that were negative for all the characteristics studied. Further study involving larger number of *E. coli* strains from septicemia, peritonitis, abscess and meningitis is necessary before any factor could be implicated for the virulence of *E. coli* infection at those extra-intestinal sites and for better understanding of interaction of different virulence factors at molecular level.

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