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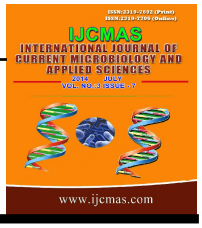

Original Research Article

Study of Prevalence of Virulence Factors in Extraintestinal Pathogenic *Escherichia coli* isolated from a tertiary care hospital

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A B S T R A C T

Escherichia coli cause extraintestinal infections in both community and hospitalized patients. These extraintestinal pathogenic *Escherichia coli* (ExPEC) strains exhibit certain virulence factors. The present study was undertaken to detect the virulence factors of extraintestinal pathogenic *E.coli*. A total of 150 clinical isolates of extraintestinal *E.coli* and 20 commensal *E.coli* isolated from apparently healthy individuals were screened for virulence factors like biofilm production, mannose resistant haemagglutination (MRHA), cell surface hydrophobicity, serum resistance and gelatinase production by standard methods. Chi square test was used to analyze the result. Of the 150 extraintestinal *E.coli* 91 (60.66%) showed cell surface hydrophobicity, 89 (59.33%) were biofilm producers, 68 (45.33%) were resistant to serum, 57 (38%) were MRHA positive. Hemolysin production and gelatinase activity was seen in 43 (28.66%) and 9 (6%) of extraintestinal *E.coli*. This study highlights the association of different virulence factors that enables *E. coli* strains to cause extra intestinal infections.

Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are *E.coli* strains with enhanced ability to cause infection outside the intestinal tract such as in the blood stream, cerebrospinal fluid, or urinary tract of mammalian and human hosts (Johnson and Russo., 2002). The barrier between commensalism and virulence results from a complex balance involving host condition, presence and expression of virulence factors. In general, pathogenecity has been correlated with the presence of genes encoding virulence factors (VFs) organized in large blocks called pathogenecity islands.

Such factors may be horizontally disseminated among distinct *E. coli* strains leading to infections of the urinary tract (UTI), meningitis and bacteremia (Hacker et al., 1997).

Extra intestinal pathogenic *E. coli* are distinct from most intestinal commensal *E. coli* as well as from diarrheagenic *E. coli* types (Russo and Johnson., 2000). The virulence of individual strains in a given infection is determined by the presence and actual expression of the virulence genes present in them and also by the environmental conditions in the host. The virulence factors of extraintestinal *E.coli* include ability to adhere to uroepithelial cells, haemagglutination, serum resistance, haemolysin, cell surface hydrophobicity, resistance to phagocytosis, cytotoxic necrotizing factor, K1 antigen, siderophore and gelatinase production and others.

The present study was undertaken to detect the virulence factors of extraintestinal pathogenic *Escherichia coli* and correlate them with commensal strains of *Escherichia coli*.

Materials and Methods

Biofilm formation

Biofilm production was demonstrated by tube adherence test (Christensen et al., 1982). Trypticase Soy Broth with glucose (10mL) was inoculated with loopful of test organisms from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted, washed with Phosphate Buffer Saline (PBS pH 7.3) and dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Experiments were performed in triplicate and repeated three times.

Mannose resistant hemagglutination (MRHA)

Haemagglutinating activity was determined by micro haemagglutination test using 96 well round bottom plates and fresh human group O positive erythrocytes (Duguid et al., *1979,* Smyth CJ et al.,1978,*). Escherichia coli* grown on Colonization Factor Antigen agar(CFA Agar) plates at 37° C for 18 hours were suspended in phosphate buffer saline (PBS) to yield a starting concentration of 10^9 CFU/ml. 100µl of the bacterial suspension was added to each well, followed by an equal volume of a 1% suspension of erythrocytes in PBS. Wells containing only the suspension of erythrocytes were taken as negative control. The microtitre plate was then incubated at 4°C for 1hour. The presence of a small pallet of erythrocytes at the bottom after incubation was considered as negative result, and that containing an even sheet of erythrocytes across the well was considered as positive. Tests were repeated with an equal volume of suspension of 1% erythrocytes and 1% D- mannose to find out MRHA. The presence of a small pallet of erythrocytes at the bottom of wells after incubation was considered as negative result, and that containing an even sheet of erythrocytes across the well was considered as positive.

Haemolysin production

Plate hemolysis test was done by using 5% sheep blood agar to detect alpha-haemolysin produced by *Escherichia coli* (Siegfried et al., 1994). Test organism was inoculated on sheep blood agar and incubated over night at 35^0C. Haemolysin production was detected by the presence of a zone of complete lysis of erythrocytes around the colony and clearing of the medium

Serum resistance

Fresh culture isolates were employed for studying their serum resistance. Cells harvested from the overnight cultures *Escherichia coli* grown at 37^0C on blood agar were suspended in Hank's balanced salt solution (HBSS). Equal quantities (0.05ml) of bacterial suspension and serum were incubated at 37^0 C for 180min. 10 µl of samples was inoculated on blood agar plates and the viable count was determined after incubating the plates at 37^0C for 18 hrs . Resistance of bacteria to serum bactericidal activity was determined by the percentage of bacteria survived after 180 minutes of incubation with serum in relation to the original count. Bacteria were termed serum sensitive, wherever viable count dropped to 1% of initial value, and resistant if >90% organisms survived after 180 minutes (Siegfried et al., 1994).

Cell surface hydrophobicity

This was determined by salt aggregation test (SAT) (Siegfried et al., 1994). One loopfull of bacterial suspension made in phosphate buffer was mixed with equal volume of ammonium sulfate solution of different molarities i.e., from 0.3125 M through 5.0 M on a glass slide and observed for 1min while rotating. The highest dilution of ammonium sulfate solution which showed visible clumping of bacteria was scored as salt aggregation value. Strains which showed aggregation in 0.02 M phosphate buffer alone (P^H 6.8) was considered auto aggregative. *Escherichia coli* strains with SAT value $\leq$ 1.25 M was considered cell surface Hydrophobic.

Gelatinase test

Gelatinase production was tested using gelatin agar (Collee et al., 1996). The plate was inoculated with test organism and incubated at 37 °C for 24 h. The plate was then flooded with mercuric chloride solution. Development of opacity in the medium and zone of clearing around colonies was considered positive for gelatinase.

Results and Discussion

Out of 150 clinical isolates of extraintestinal *Escherichia coli;* 57 were from urine, 55 from pus, 20 from sputum, 9 were from blood and endotracheal tubes+ catheter tips. All the isolates were studied for virulence factors such as cell surface hydrophobicity, biofilm formation, resistance to serum, MRHA, hemolysin production and gelatinase activity along with 20 commensal *Escherichia coli* isolated from healthy individuals as controls. The Chi-square test was used to find the significance of the study parameters.

Of the 150 extraintestinal *Escherichia coli* 91(60.66%) exhibited cell surface hydrophobicity, 89(59.33%) produced biofilm, 68(45.33%) were resistant to serum and 57(38%) were MRHA positive. Hemolysin production and gelatinase activity was observed in 43(28.66%) and 9(6%) of extraintestinal *Escherichia coli* respectively (Table 1).

There was a significant association between multiple virulence factors production in extraintestinal *Escherichia coli* (table 2).
Of the 150 extraintestinal *Escherichia coli 89* (59.33%) were biofilm producers and 61(40.67%) were non biofilm producers. Multiple virulence factor expression was seen more in biofilm producers compared to non biofilm producers (table3).

Escherichia coli is an important cause of urinary tract infections (UTIs), enteric

infections, and systemic infections in humans. The systemic infections include bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis, and infectious arthritis. *Escherichia coli* is also a leading cause of neonatal meningitis (Mandell et al., 2001).

Escherichia coli comprises of non-pathogenic commensal isolates that forms part of the normal flora of humans and various animals. However, several variants have been described that causes infection of the gastrointestinal system (intestinal pathogenic *E. coli*) while others cause infections outside the gastrointestinal system (extraintestinal pathogenic *E. coli* or ExPEC) (Kaper et al.,2004). The ability of *Escherichia coli to* cause extraintestinal infections depends largely on several virulence factors which help to survive under adverse conditions (Banu et al., 2011).

Hydrophobicity is a recently described novel virulence mechanism by *Escherichia coli which* promotes their adherence to various surfaces like mucosal epithelial cells. Crystalline surface layers present on both Gram negative and Gram positive organisms play a role in this (Sleytr et al., 1983). Of the 150 clinical isolates of extraintestinal *Escherichia coli* 91 (60.66%) showed cell surface hydrophobicity where as it was seen only in 4 (20%) of intestinal commensal isolates. Cell surface hydrophobicity was seen more isolates from urine followed by endotracheal tubes + catheter tips, pus, sputum and blood. This is correlates with results of previous studies (Raksha et al., 2003; Suman et al 2001 and Sharma et al., 2007).

Biofilms are microbial communities of organism's adherent to each other and/or a target surface. Biofilm formation protects bacteria from hydrodynamic flow conditions, host defence mechanisms and antibiotics. In the present study, biofilm production was observed in 89 (59.33%) of extraintestinal *Escherichia coli and* 4 (20%) of intestinal commensal isolates. Sleytr B and Messner P (1993) have shown the prevalence of biofilm production among *Escherichia coli* was approximately 17% for faecal strains, 43% for cystitis, 40% for pyelonephritis and 42% for bacteraemic *Escherichia coli* strains.

Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system. Bacterial resistance to killing by serum results from individual or combined effects of capsular polysaccharide and surface proteins (Taylor., 1983). The serum resistant gram negative bacteria possess a significant survival advantage in the blood during bacteriaemia. There is a strong correlation between serum resistance and the ability of a variety of Gram negative bacteria to invade and survive in human bloodstream. Serum resistance was seen in 68 (45.3%) of extraintestinal *Escherichia coli* and 6 (30%) intestinal commensals highlighting its pathogenic role in extraintestinal infection. In the present study, highest serum resistance was seen among isolates from blood followed by endotracheal tubes+ catheter tips, sputum, pus and urine. Raksha et al (2003), Sharma et al (2007) and Johnson (1991) have observed highest serum resistance among isolates from urine.

Mannose resistant hemagglutination (MRHA) activity mediated by P fimbriae, X, FIC, and Dr fimbriae (Johnson., 1991). MRHA plays a pivotal role in adhesion and establishment of pathogenic strains of *Escherichia coli* to various host tissues, and the genetic information for a number of

them is closely associated with other virulence factors (Drews., 2005). Out of 150 extraintestinal *Escherichia coli* 57 (38%) were MRHA positive whereas 2(10%) of 20 intestinal commensal *Escherichia coli* were positive for MRHA. MRHA positivity was highest in isolates from urine followed by pus, sputum, blood and endotracheal tube + catheter tips. This is consistent with the results of previous studies (Fakruddin et al., 2013). Haemolysin production is a property usually associated with *Escherichia coli* strains which infect extraintestinal sites in humans whereas it is rarely found in fecal isolates from healthy individual (Caprioli A., 1989). In the present study, hemolysin production was observed in 43 (28.66%) of extraintestinal *Escherichia coli* and 3(15%) of intestinal commensal *Escherichia coli* isolates respectively. Hemolysin production was highest among the isolates of endotracheal tubes + catheter tips followed by blood, urine, pus and sputum. Raksha et al (2003) Johnson (1991) and Fakruddin et al (2013) have observed highest hemolysin production among urinary isolates.

Table.1 Virulence markers of extraintestinal and intestinal commensal *E.coli* isolates

Comparison of virulence phenotypes in extra-intestinal *E.coli* isolates						
Virulence factors	Control (20)	Urine (57)	Pus (55)	Sputum (20)	Blood (9)	Endotracheal tubes + catheter tips(9)
Cell surface hydrophobicity	20%	80%	60%	56.14%	44.14%	66.66%
Biofilm	20%	50.87%	50.90%	80%	77.77%	100%
Serum resistance	30%	17.54%	54.54%	55%	100%	88.88%
MRHA	10%	54.38%	32.72%	25%	22.11%	11.11%
Haemolysin	10%	28.07%	21.21%	44.44%	20%	77.77%
Gelatinase Activity	5%	8.77%	3.6%	5.0%	11.11%	0%

Table.2 Virulence markers of Escherichia coli obtained from cases and controls

Sl. No	Virulence markers	Cases n=150	Control n=20	P value
01	MRHA	57(38%)	02(10%)	< 0.05(S)
02	Hemolysin	43(28.66%)	03(15%)	>0.05(NS)
03	Biofilm	89(59.33%)	04(20%)	< 0.05(S)
04	Cell surface hydrophobicity	91(60.66%)	04(20%)	< 0.05(S)
05	Serum resistance	68(45.33%)	06(30%)	>0.05(NS)
06	Gelatinase production	09(6%)	01(5%)	>0.05(NS)

Table.3 Virulence factors of biofilm and non biofilm producing extraintestinal *E.coli* isolates

Virulence factors	MRHA(57)	Serum resistance(68)	Hemolysin(43)	Gelatinase(9)
Biofilm producers(89)	48(53.93%)	52(58.42%)	30(33.70%)	05(5.61%)
Non Biofilm producer (61)	9(14.75%)	16(26.22%)	13(21.31%)	04(6.55%)

Gelatinase activity was observed in 9 (6%) of extraintestinal *Escherichia coli.* Gelatinase activity is not an important virulence factor and the similar observation was made by Nair et al (2013).

Expression of multiple virulence factors by extraintestinal *Escherichia coli* was observed in the present study. The present study also revealed expression of multiple virulence factors by extraintestinal *Escherichia coli*. Most of the biofilm producing isolates exhibited hydrophobicity, serum resistance, MRHA positive and hemolysin activity. This is consistent with the findings of Hughes et al(1982). Presence of multiple virulence factors increase the virulence of the organism and cannot be accurately predicted on the basis of its measurable virulence factor phenotype (Johnson., 1991). The virulence factors function additively or synergistically in overcoming normal host defenses. The strains with a more extensive complement of virulence factors are more effective pathogens, and the compromising host conditions decrease the need for multiple virulence factors in strains causing serious infections.

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