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Original Article

VIRULENCE FACTORS AND DRUG RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM EXTRAINTESTINAL INFECTIONS

S Sharma, *GK Bhat, S Shenoy

Abstract

Purpose: To determine the virulence factors produced by *Escherichia coli* isolated from extraintestinal infections, to study the drug resistance pattern in *E. coli* with special reference to extended spectrum β-lactamase (ESBL) and to evaluate screening methods for ESBL. **Methods:** A total of 152 isolates of *E. coli* from various extraintestinal infections were screened for virulence factors such as haemolysin, surface hydrophobicity, serum resistance and protease. All the isolates were also studied for antibiotic susceptibility pattern using modified Kirby Bauer disk diffusion method. ESBL production was screened by standard disk diffusion method and confirmed using phenotypic confirmatory method. **Results:** Among 152 isolates, 36 (23.7%) were haemolytic, 42 (27.6%) were hydrophobic, 132 (86.8%) were serum resistant and only four were positive for protease. Multiple virulence factor were observed in 67 (44%) of isolates. Seventy-nine (51.4%) isolates produced ESBL. ESBL producing isolates showed multidrug resistance. There was a significant association ($P < 0.001$) between multiple virulence factors and ESBL production by extraintestinal *E. coli*. **Conclusions:** The present study shows the expression of virulence factors and multidrug resistance in *E. coli* isolated from various extraintestinal infections. The study also shows that appropriate methods of detecting drug resistance and ESBL production are required for the judicious use of antibiotics in managing these infections.

Key words: *Drug resistance, Escherichia coli, haemolysin, serum resistance, surface hydrophobicity*

Escherichia coli is one of the commensals in the human intestinal tract. As a commensal, it contributes to the maintenance of health of a person. However, *E. coli*, when enters into unnatural sites, can cause variety of infectious diseases such as urinary tract infections, wound infections, bacteraemia, meningitis and other soft tissue infections.[1] The ability of *E. coli* to cause extraintestinal infections depends largely on several virulence factors, which help in the survival of *E. coli* under adverse conditions present in those sites. 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.[2]

The treatment of *E. coli* infections is increasingly becoming difficult because of the multidrug resistance exhibited by the organism. Extended spectrum β-lactamase (ESBL) producing organisms pose a major problem for clinical therapeutics. The incidence of ESBL producing strains of *E. coli* among clinical isolates has been steadily increasing over the past few years resulting in limitation of therapeutic options.[3]

The knowledge of drug resistance pattern in a geographical area and the formulation of an appropriate hospital antibiotic policy will go a long way in the control of these infections. Therefore, it is necessary to know the antibiotic susceptibility pattern of pathogenic *E. coli* to select the correct antibiotic(s) for proper treatment of infections caused by it. The objectives of the present study were to demonstrate the virulence factors and drug resistance in *E. coli* isolated from extraintestinal infections and to evaluate screening procedures for ESBL.

Materials and Methods

Clinical isolates

A total of 152 isolates of *E. coli* from extraintestinal infections obtained from January 2004 to June 2005 were included in the study. The study population included hospitalised patients of all age groups at Government Wenlock Hospital, Government Lady Goschen Hospital, KMC Hospital Attavar and KMC Hospital Ambedkar Circle, Mangalore.

Specimens collected were pus, exudates, clean catch midstream urine, urine from indwelling catheter, blood and CSF using standard sterile procedures. The samples were processed immediately using standard procedures. The isolates were identified based on colony morphology on blood agar, MacConkey's agar, Gram staining and by standard biochemical tests.[4]

Detection of virulence factors

Cell surface hydrophobicity: The cell surface hydrophobicity of *E. coli* was determined by salt aggregation test (SAT).[5,6] One loopful (10 μL) of bacterial suspension made in phosphate buffer was mixed with equal volume

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of ammonium sulphate solution of different molarity, i.e., from 0.3125 M through 5.0 M, on a glass slide and observed for 1 min while rotating. The highest dilution of ammonium sulphate solution giving visible clumping of bacteria was scored as the salt aggregation test (SAT) value. Strains showing aggregation in 0.002 M phosphate buffer alone (pH 6.8) were considered auto aggregative. *E. coli* strains that had SAT value ≤1.25 M were considered hydrophobic.[5,6]

Haemolysin production: Plate haemolysis test was done for the detection of α-haemolysin produced by *E. coli*.[5] The bacteria were inoculated onto 5% sheep blood agar and incubated overnight at 35 ºC. Haemolysin production was detected by the presence of a zone of complete lysis of the erythrocytes around the colony and clearing of the medium.

Serum resistance: Serum resistance was studied using fresh culture of the isolates.[5,6] Overnight cultures of *E. coli*, grown at 37 ºC on blood agar, were harvested and the cells were suspended in Hank's balanced salt solution (HBSS). Bacterial suspension (0.05 mL) was incubated with serum (0.05 mL) at 37 ºC for 180 min. Ten microlitres of samples were withdrawn and spread on blood agar plates which were then incubated at 37 ºC for 18 h and the viable count was determined. Resistance of bacteria to serum bactericidal activity was expressed as the percentage of bacteria surviving after 180 min of incubation with serum, in relation to the original count. Bacteria were termed serum sensitive, if viable count dropped to 1% of initial value and resistant, if >90% of organisms survived after 180 min.

Gelatinase test: Gelatinase production was tested using gelatin agar.[7] The plate was inoculated with test organism and incubated at 37 ºC for 24 h. The plate was then flooded with mercuric chloride solution. Development of opacity in the medium and zone of clearing around colonies were considered positive for gelatinase.

Test for extended spectrum β*-lactamase production*

Screening by standard disk diffusion method: Screening for ESBL production was done according to criteria recommended by NCCLS.[8] Two discs, ceftazidime (30 μg) and cefotaxime (30 μg), were used for *in vitro* sensitivity testing by Kirby-Bauer disk diffusion method. Zone diameters were read using NCCLS criteria. An inhibition zone of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime indicated a probable ESBL producing strain requiring phenotypic confirmatory testing.

Phenotypic confirmatory methods: Disk diffusion method was used to confirm ESBL production by *E. coli* strains.[9] Ceftazidime (30 μg) vs. ceftazidime/clavulanic acid (30/10 μg) and cefotaxime (30 μg) vs. cefotaxime/clavulanic acid (30/10 μg) were placed onto Mueller Hinton agar plate lawned with the test organisms and incubated overnight at 35 ºC. Regardless of zone diameters, a ≥ 5 mm increase in a zone diameter of an antimicrobial agent tested in combination with clavulanic acid vs. its zone size when tested alone, indicated ESBL production.

Antibiotic susceptibility testing: The antibiotic susceptibility testing was done using modified Kirby-Bauer disk diffusion method. The antibiotic disks (Hi Media, Mumbai) used were ampicillin (10 μg), amikacin (30 μg), co-trimoxazole (25 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), gentamicin (30 μg), netlimicin (30 μg).

After 18 hours of incubation at 37 ºC, the diameter of the zone of inhibition was measured using a millimetre scale around each antimicrobial disk on the undersurface of the plate. The zone size around each antimicrobial disk was interpreted as sensitive, intermediate or resistant according to NCCLS criteria.[10]

Results

A total of 152 strains of *E. coli* were isolated from specimens collected from extraintestinal infections. Out of these, 62 (40.8%) were from pus, 60 (39.5%) from urine, 14 (9.2%) from blood, 8 (5.3%) from ascites, 7 (4.6%) from sputum and 1 (0.7%) from CSF (Table 1).

Virulence factors such as haemolysin, surface hydrophobicity, serum resistance and protease were studied for all the isolates. The most common virulence factor identified was serum resistance in 132 (86.8%) isolates. Haemolysin was produced by 36 (23.7%) isolates, 42 strains (27.6%) were hydrophobic and only 4 (6.9%) produced protease. Multiple virulence factors were observed among 67 (44.0%) isolates of *E. coli*.

Table 1: Virulence factors in extraintestinal *E. coli*

Nature of specimen	Number of isolates	Number (%) of *E. coli*			
		Haemolysin positive	Hydrophobic	Serum resistant	Protease positive
Pus	62	10 (16.1)	16 (25.8)	48 (77.4)	0
Urine	60	15 (25.0)	20 (33.4)	52 (86.7)	4 (6.9)
Blood	14	9 (64.2)	3 (21.4)	12 (85.7)	0
Ascites	8	2 (25.0)	2 (25.0)	5 (62.5)	0
Sputum	7	0	0	4 (57.1)	0
CSF	1	0	1	1	0

The analysis of drug resistance pattern showed that among 152 isolates of *E. coli*, maximum numbers (76.9%) were resistant to ampicillin and lowest to netillin (42.8%). ESBL production was detected in 79 (51.4%) of isolates. The maximum number of ESBL producing *E. coli* (66.1%) was isolated from pus.

Out of the 75 isolates resistant to cefotaxime, 70 (93.4%) were ESBL producers whereas 9 (11.7%) isolates sensitive to cefotaxime were positive for ESBL production. On the other hand, of the 85 isolates, which were positive for ESBL by screening test, 79 (92.9%) were positive by confirmatory test for ESBL, whereas all the 67 isolates negative by screening were also negative by confirmatory test.

The ESBL producing isolates were also studied for the presence of co-resistance to other antibiotics. Among the 79 ESBL producers, all were resistant to ampicillin followed by 89.8% to ciprofloxacin, 64.5% to cotrimoxazole, 88.6% to cefotaxime, 65.8% to gentamicin, 46.8 to amikacin and 37.9% to netillin.

All the ESBL producing isolates were studied for the presence of multiple virulence factors (Table 2). Application of Gaussian's formula showed that, there was significant association ($P < 0.001$) between multiple virulence factors and ESBL production in extraintestinal *E. coli*. Haemolysin production was predominantly correlated with MDR/ESBL production. As the number of virulence factors increased, the rate of ESBL production in *E. coli* decreased.

Table 2: Presence of multiple virulence factors in ESBL producing isolates of *E. coli*

Multiple virulence factors	ESBL+	ESBL−
+	21	46
−	58	27
Total	**79**	**73**

Discussion

Virulence factors enable *E. coli* to colonise selectively the mucosal uro-epithelium, evoke an inflammatory reaction and eventually proceed from lower urinary tract to renal cavities and tissue invasion. The capacity of *E. coli* to produce many virulence factors contributes to its pathogenicity. *E. coli* is able to cause a variety of infections such as urinary tract infection, soft tissue infections, bacteraemia and neonatal meningitis. These virulence factors enable some members of the normal flora to elicit an infection by overcoming the host defence mechanisms.[11]

Haemolysin production is associated with pathogenicity of *E. coli*, especially the more severe forms of infection.[12] In the present study, a few strains of *E. coli* (23.7%) produced haemolysin. Haemolysin production was more common among the isolates from blood. Haemolysin production as a virulence factor by urinary isolates of *E. coli* has been shown by previous workers.[6,12] It has been suggested that colonization with haemolytic strains of *E. coli* is more likely to develop into urinary tract infections. Haemolysis, though not essential for establishment of acute pyelonephritis, may contribute to tissue injury, survival in renal parenchyma and entry into blood stream. The higher rate of haemolysin producing strains isolated from blood may indicate its importance in the invasive strains.

Surface hydrophobicity is another important virulence factor of *E. coli* that causes extraintestinal infections. In the present study, 27.6% of the strains were hydrophobic and compared to other specimens, more isolates from urine (33.4%) were hydrophobic. This is consistent with the results of previous studies.[6,13] The high hydrophobicity of the bacterial cell surface promotes their adherence to various surfaces like mucosal epithelial cells.

Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system. In the present study, 86.8% of the isolates were resistant to serum bactericidal activity, majority of which were isolated from urine (86.7%) and blood (77.4%). A previous study showed serum resistance in 32.7% of *E. coli* isolated from urine.[6] In another study, 68% of the urinary isolates were resistant to serum bactericidal activity which is comparable to our results.[5] The rate of serum resistant strains of *E. coli* isolated from blood in the present study is consistent with a previous study.[12]

Isolates from patients with pyelonephritis, cystitis and bacteraemia are typically serum resistant whereas patients with asymptomatic bacteriuria have serum sensitive strains.[12] The serum resistant gram negative bacteria possess a significant survival advantage in the blood during bacteraemia. 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. A previous study has shown that serum resistance is important in the pathogenesis of symptomatic UTI, regardless of the severity.[12]

The present study also revealed expression of multiple virulence factors by extraintestinal *E. coli*. Most of the haemolysin producing isolates were also hydrophobic and serum resistant. This is consistent with the findings of a previous study.[14] A combination of all three virulence factors such as haemolysin, surface hydrophobicity and serum resistance was present in 11.2% of the isolates. A previous study indicated that although virulence of an organism cannot be accurately predicted on the basis of its measurable virulence factor phenotype, the presence of multiple virulence factors does increase the virulence of organisms.[12] The virulence factors function additively or synergistically in overcoming normal host defences. 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.

Antibiotic susceptibility pattern was studied for all isolates of *E. coli*. Resistance was observed to commonly used antibiotics such as ampicillin, ciprofloxacin, co-trimoxazole, cefotaxime, gentamicin, amikacin and netillin. The greater prevalence of resistance to common antibiotics has also been reported by other workers.[15,16] The presence of multidrug resistance may be related to the dissemination of antibiotic resistance among hospital isolates of *E. coli*. Among aminoglycosides, netillin was found to have an edge over gentamicin and amikacin. Similar observations have been made by a previous group of workers.[15] Maximum number of isolates (76.9%) were resistant to ampicillin and the lowest (42.8%) to netillin. These results are consistent with the previous studies on drug resistance in *E. coli*. [17,18]

We observed a high rate of ESBL production by *E. coli* which may be due to the selective pressure imposed by extensive use of antimicrobials. The indiscriminate use of cephalosporins is responsible for the high rate of selection of ESBL producing microorganisms. These results are consistent with previous studies from India.[9,19] For predicting ESBL production, it is important to mention that for screening test, negative results are a better guide than positive results. Therefore, confirmation of all positive results by screening should be done to prevent unnecessary avoidance of conventional β-lactams. These results are consistent with the other studies on ESBL detection.[9,20]

In the present study, we found that a majority (68.7%) of ESBL negative strains of *E. coli* produced multiple virulence factors whereas most of the ESBL producers (68.2%) did not produce multiple virulence factors. These results support the hypothesis that although virulence factors and antibiotic resistance may confer increased fitness for extraintestinal infections in humans, they may do so via mutually exclusive pathways and in distinct populations.[21] A rise in the number of virulence factors was associated with a decrease in the rate of ESBL production. A robust virulence factor repertoire may be essential for a pathogen to overcome intact host defences, whereas it may be unnecessary in a compromised host, where antibiotic resistance may provide a substantial advantage to the survival of the pathogen. Some strains sensitive to cefotaxime were positive for ESBL. The false susceptibility observed could be due to inoculum effect.[22] Since ESBL production is usually plasmid mediated, it is possible for one specimen to contain both ESBL producing and non-producing cells.

The present study has shown the capacity of *E. coli* to adapt and survive in different tissues by producing virulent factors and developing drug resistance. The expression of virulence factor(s) may depend on the need and it varies in different kinds of infections. Drug resistance is on the rise among *E. coli* strains that cause human infections. Proper selection of antibiotics for treatment depends on the results of antibiotic sensitivity test. Therefore, the correct detection of drug resistant bacteria is important. Judicious use of antibiotics and good antibiotic policy are needed to limit the emergence and spread of antibiotic resistance in bacteria.

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