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Research article

Virulence factors and antibiotic sensitivity pattern in *E. coli* isolated from extra intestinal infections in a tertiary care hospital

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Abstract

Identification of virulence determinants among the clinically isolated microorganisms assumes a greater significance in the patient management perspective.

Aim: To detect the virulence factors produced by Escherichia. coli isolated from various clinical samples, collected from patients with extra intestinal infections and to detect the antibiotic sensitivity pattern of the isolates.

Method: 130 isolates of E. coli obtained from various clinical samples, collected from patients with extra intestinal infections were screened for virulence factors such as haemolysin, cell surface hydrophobicity, serum resistance, mannose resistant haemagglutination and Gelatinase. 50 E. coli isolates obtained from stool samples of healthy subjects were included as controls for comparison with the study samples. Antibiotic sensitivity pattern of the isolates were detected by Kirby Bauer disc diffusion method.

Results: Among 130 isolates, 26 (20%) produced haemolysin, 36 (27.69%) were hydrophobic, 107 (82.31%) were serum resistant, 37(28.46%) were positive for mannose resistant haemagglutination and 2 (1.54%) produced Gelatinase, whereas among controls only 2 (4%) were haemolytic, 3 (6%) were hydrophobic, 4 (8%) were serum resistant, 2 (4%) were positive for mannose resistant haemagglutination and none of the control produced Gelatinase. More than one virulence factor in each sample was observed in 64 (49.3%) of isolates. 100% of isolates were resistant to Ampicillin and 100% were sensitive to Cefaperazonesulbactam and Imipenem.

Conclusion: Virulence of an organism results from the cumulative impact of one or several virulence factors which serve to distinguish potential pathogens from harmless intestinal strains.

Keywords: Virulence factor, Escherichia coli, Antibiotic, Haemagglutination, Gelatinase.

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1. Introduction

ExPEC– Extra intestinal pathogenic *E. coli* are *E. coli* strains that possess currently recognized extra intestinal virulence factors or have been demonstrated to possess enhanced virulence in an appropriate animal model [1].

ExPEC is common in all age groups and may occur at almost any extra intestinal site. The most common infections include urinary tract infections ranging from uncomplicated to febrile to invasive, pyelonephritis, neonatal and post neurosurgical meningitis and septicaemia. This group is epidemiologically and phylogenetically distinct from commensal and intestinal strains of *E. coli* [2].

A broad range of virulence factors have been described that confer the ability to overcome host defenses, invade host tissues, and cause extra intestinal disease [3]. These virulence factors may be present in different combinations from strain to strain and include serum resistance, haemagglutination as well as the production of adhesions, aerobatic, toxins, iron acquisition factors and colicin V.

Aim

1. To isolate *E .coli* from various clinical samples received from symptomatic patients with extra intestinal infections.
2. To detect the virulence factors of *E. coli*isolated and to compare it with *E .coli* isolates obtained from stool samples of healthy subjects.
3. To determine the Antimicrobial susceptibility pattern of the isolates using Kirby Bauer disc diffusion method.

2. Materials and methods

This study was carried out over a period of one year at the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai. Ethical clearance was obtained from the Institutional Ethical Committee, Government General Hospital and Madras Medical College, Chennai. India.

A total of 130 isolates of *E. coli* obtained from extra intestinal infections were included in the study. Specimens collected were Urine, Blood, Sputum, Pus, Tracheal swab, Body fluids, Bronchoalveolar lavage, and Devices (Shunt tube, catheter tips etc). A total of 50 *E. coli* isolates obtained from stool samples of healthy subjects were included as controls for comparison with the study samples. The proportional data of this study was tested using Pearson's chi – square analysis test. The criterion for statistical significance was $P \leq 0.05$.

Sample processing for *Escherichia coli* isolation

Clinical samples from symptomatic patients of all age groups with extra intestinal infections were collected and transported according to the specific specimen collection and transport techniques [4]. Samples received in the laboratory were processed immediately using standard procedures [5, 6]. Stool samples from healthy individuals were also collected, transported and processed as per standard procedures. The isolates were identified based on colony morphology on Mac Conkey's agar, blood agar, Gram staining and by standard biochemical tests [6]. Only typical *E. coli* strains isolated from the above mentioned samples were included in the study further for detection of virulence factors.

Detection of virulence factors

Haemolysin production

Plate haemolysis test was done for the detection of alpha haemolysin production by *E. coli.* The cytolytic protein toxin secreted by most haemolytic *E. coli* isolates is known as alpha haemolysin [7]. Haemolysin was detected by determining a zone of lysis around each colony on (5%) sheep blood agar plates after overnight incubation [8].

Gelatinase test

Gelatinase production was tested using Gelatine agar. The plate was incubated with test organism and incubated at 37 °C for 24 hours. The plate was then flooded with (1%) Tannic acid solution. Development of relative opacity around gelatine liquefying colonies quick to develop but fading as the medium also becomes opaque were considered positive for gelatinase [9].

Serum resistance test

Serum resistance was studied using fresh culture of the isolates [8, 10]. Overnight cultures of *E. coli* grown at 37°C on blood agar, were harvested and the cells were suspended in Hank's Balanced Salt Solution (HBSS). Bacterial suspension 0.05ml was incubated with serum 0.05ml at 37 °C for 180 minutes .Ten micro litres of samples were withdrawn and spread on blood agar plates which were then incubated at 37° C for 18 hours and the viable count was determined. Resistance of bacteria to serum bactericidal activity was expressed as the percentage of bacteria surviving after 180 minutes 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 minutes [11].

Cell surface hydrophobicity

The cell surface hydrophobicity of *E. coli* was determined by salt aggregation test (SAT) [8, 10]. One loopful 10µl of bacterial suspension made in phosphate buffer was mixed with equal volume of Ammonium sulphate solution of different molarity, i.e., from 0.3125 M through 5.0M, 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 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 [11].

Haemagglutination

Red cells separated from fresh citrated guinea pig blood are washed twice in physiological saline and made up to a 3% (v/v) suspension in fresh saline. A nutrient broth culture of the test organism is centrifuged to deposit the bacilli. After removal of the culture supernatant, the bacillary deposit is re suspended in the small amount of fluid remaining. A drop of the very dense bacillary deposit is mixed with an equal drop of the red cell suspension in a depression on a white tile at room temperature, and the tile is then rocked gently for 5 minutes.

The haemagglutination produced by the fimbriate organisms is seen with the naked eye and usually develops as a coarse clumping within a few seconds. Weakly active cultures produce a fine granularity within 2–3min. Very poor haemagglutinating cultures may show positive reactions only if mixing is continued for up to 30 min. If a very dense bacterial suspension is not used, weak reactions may be missed.

Inhibition of fimbrial haemagglutination with mannose

The incorporation of a small drop of a 2% solution of D–mannose in the haemagglutination mixture (final mannose

concentration 0.5%) specifically inhibits type I fimbrialhaemagglutination [9].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was done using Kirby Bauer disc diffusion method. The antibiotic discs used were Ampicillin, Amikacin, Cefotaxime, Ciprofloxacin, Gentamicin, Ceftazidime, Cefoperazone – sulbactam, Cotrimoxazole, and Imipenem.

Mueller Hinton agar plate was inoculated with 0.5 McFarland standard inoculums to obtain a lawn culture. Using a sterile forceps, discs were placed over the agar surface, incubated at 37 °C for overnight. The results were interpreted as per Clinical Laboratory Standards Institute (CLSI) standards [12]. The *E. coli* strain ATCC 25922 was included as a quality control in all tests.

3. Result

The study undertaken at the Institute of Microbiology, Madras Medical College, Chennai, showed the following results.

Out of 953 total samples received from patients with extra intestinal infections, 130 (13.64%) of *E. coli* were isolated from different clinical samples.

Table No 1: *E. coli* isolates from different clinical samples (n =130)

Samples	No	%
Urine	84	64.62
Pus	32	24.62
Sputum	07	05.38
Blood	05	03.85
Tracheal swab	01	0.77
Shunt tube	01	0.77

Maximum number of *E. coli* 84 (64.62%) was isolated from urine samples.

Table No 2: Comparison of virulence factors among cases and controls

Virulence factor	Cases (n=130)		Control (n=50)	
	No	%	No	%
Hemolysin positive	26	20	2	4
Cell surface hydrophobicity positive	36	27.69	3	6
Serum resistance positive	107	82.31	4	8
Gelatinase Positive	2	1.54	-	-
Mannose resistant haemagglutination Positive	37	28.46	2	4

Hemolysin production

(20%) 26 of cases produced Hemolysin compared to (4%) 2 of controls. $P < 0.001$ – highly significant

Cell surface hydrophobicity

(27.69%) 36 of cases were positive for cell surface hydrophobicity while compared to (6%) 3 of controls. $P < 0.001$ – highly significant

Serum resistance

(82.31%) 107 of cases were serum resistance when compared to (8%) 4 of controls. $P < 0.001$ – highly significant

Gelatinase production

2 cases out of 130 produced Gelatinase and no control produced Gelatinase.

Gelatinase production was observed in two isolates from urine samples.

Haemagglutination pattern

(28.46%) 37 of cases showed Mannose resistant Haemagglutination pattern when compared to 4% of controls. P–highly significant and (36%) 47 cases showed Mannose sensitive Haemagglutination pattern

Table No 3: Virulence factors in *E. coli* from extra intestinal infection

Nature of specimen with number of isolates	Hemolysin positive		Cell surface hydrophobicity positive		Serum resistance positive		Gelatinase Positive		Mannose resistant haemagglutination Positive	
	No	%	No	%	No	%	No	%	No	%
Urine (84)	18	21.43	25	29.76	73	86.90	2	2.38	23	27.38
Pus (32)	5	15.62	7	21.87	25	78.13	-	-	9	28.13
Sputum (7)	1	14.29	2	28.57	4	57.14	-	-	2	28.57
Blood (5)	2	40	1	20	4	80	-	-	2	40
Tracheal swab(1)	-	-	1	-	-	-	-	-	1	-
Shunt tube (1)	-	-	-	-	1	-	-	-	-	-

Hemolysin production was observed in 2 isolates out of 5 from blood samples and in 18 (21.43%) out of 84 urine samples. 25 (29.76%) *E. coli* isolates from urine samples were observed to be positive for cell surface hydrophobicity. 73 (86.90%) *E. coli* isolates from urine samples were observed to be positive for serum resistance, followed by 4 (80%) from blood samples and 25 (78.13%) *E. coli* isolates from pus samples were

observed to be positive for serum resistance. Gelatinase production was observed in two isolates from urine samples. Mannose resistant haemagglutination was observed in 37 (28.46 %) of total isolates.
Serum resistance was positive in 107 (82.31%) of total isolates and was observed to be the predominant virulence factor.

Table No 4: Number of virulence factors versus number of samples (n=130)

Number of virulence factors-present	Number of samples	%
1	66	50.7
2	50	38.46
3	12	9.23
4	2	1.54
5	-	-

More than one virulence factor in each sample was observed in 64 (49.3 %) of isolates.

Table No 5: Occurrence of *E. coli* with virulence factors

Virulence factors	Number of cases	% of cases
Single factor		
Serum Resistance (SR)	49	37.69
Haemolysin (H)	6	4.62
Cell surface hydrophobicity (CSH)	6	4.62
Mannose Resistant Haemagglutination(MRHA)	4	3.08
Gelatinase (G)	1	0.77
Total	66	50.7
Two factors		
SR + MRHA	20	15.39
SR + CSH	15	11.54
SR + H	10	7.7
CSH + MRHA	3	2.31
CSH + H	1	0.77
H + MRHA	1	0.77
Total	50	38.46
Three factors		
SR + CSH + MRHA	5	3.85
SR + CSH + H	4	3.08
SR + MRHA + H	2	1.54
SR + MRHA + G	1	0.77
Total	12	9.23
Four factors		
SR + CSH + H + MRHA	2	1.54
Total	2	1.54

The predominant virulence factor in *E. coli* isolates, with single virulence factor was Serum resistance (49 out of 66), with two virulence factors - was Serum resistance + Mannose resistant haemagglutination (20 out of 50), with three virulence factors- was Serum resistance + Mannose resistant haemagglutination + Cell surface hydrophobicity (5 out of 12) . All four virulence factors except Gelatinase were present in 2 isolates.

Table No 6: Antimicrobial susceptibility pattern (n =130)

Antibiotic	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	110	84.62	20	15.38
Ampicillin	-	-	130	100
Cefoperazonesulbactam	130	100	-	-
Ceftazidime	10	7.70	120	92.30
Cefotaxime	23	17.69	107	82.31
Ciprofloxacin	20	15.38	110	84.62
Cotrimoxazole	32	24.62	98	75.38
Gentamicin	49	37.69	81	62.31
Imipenem	130	100	-	-

100% of isolates were resistant to Ampicillin. 100% of isolates were sensitive to Cefoperazone sulbactam and Imipenem,

3. Discussion

In the study undertaken, 130 strains of E. coli were isolated from 953 samples received from patients with extra intestinal infections. Maximum number of *E. coli* was isolated from urine samples - 84 (64.62%) (Table-1). 50 E. coli isolated from stool samples of healthy individuals were included as controls. These were investigated for the possession of virulence factors.
Haemolysin production was detected in (20%) 26 of cases whereas only (4%) 8 of controls produced haemolysin, which was highly significant (Table-2). Among cases, haemolysin production from strains of E. coli isolated from blood was 2 out of 5. The higher rate of haemolysin producing strains isolated from blood may indicate its importance in the invasive strains [11]. Hemolysin production was observed in 18 (21.43%) out of 84 urine samples (Table -3). It has been suggested that colonization with haemolytic strains of E. coli is more likely to develop into urinary tract infections.
Cell Surface hydrophobicity was positive in (27.69%) 36 of cases while only (6%) 3 of controls showed positive, which was highly significant. It was more common among the E. coli strains isolated from urine 25 (29.76%) (Table -3).This is consistent with the result of previous study [8].The high hydrophobicity of the bacterial cell surface promotes their adherence to various surfaces like mucosal epithelial cells.
107 (82.31%) of cases were found to be serum resistant while only (8%) 4 of controls were serum resistant, which was highly significant. Maximum isolates from urine 73 (86.90 %) were serum resistant.A previous study showed serum resistance in 32.7% of E. coli isolated from urine [8]. The serum resistant gram negative bacteria possess a significant survival advantage in the blood during bacteraemia.

Gelatinase production was noted in 2 strains isolated from urine whereas no *E.coli* strains isolated from control produced Gelatinase.

37 (28.46%) of cases showed mannose resistant haemagglutination, when compared to only (4%) 2 of controls, which was highly significant. Haemagglutination by mannose resistant fimbriae is not inhibited by D–mannose or mannosides and their receptors contain various carbohydrates. The best studied of these are P fimbriae. Agglutination of erythrocytes is an indirect evidence of the presence of fimbriae and it provides a simple indirect method of virulence testing [13].

The most common virulence factor identified was serum resistance, in 107 (82.31 %) isolates. The present study revealed expression of multiple virulence factors by extra intestinal *E. coli*. Multiple virulence factors were present in (49.3%) 64 of the isolates (Table-4). The predominant virulence factor in *E. coli* isolates, with single virulence factor was Serum resistance (49 out of 66) , with two virulence factors - was Serum resistance + Mannose resistant haemagglutination (20 out of 50), with three virulence factors - was Serum resistance + Mannose resistant haemagglutination + Cell surface hydrophobicity (5 out of 12). All four virulence factors except Gelatinase was present in 2 isolate (Table -5).
Antimicrobial susceptibility pattern was studied for all isolates of *E. coli*. Resistance was observed to commonly used antibiotics such as ampicillin, ciprofloxacin, co-trimoxazole, cefotaxime, ceftazidime, gentamicin and amikacin. Maximum number of isolate (100%) were resistant to ampicillin, followed by 92.30% to ceftazidime, 84.62% to ciprofloxacin, 82.31% to cefotaxime, 75.38% to cotrimoxazole, 62.31% to Gentamicin and lowest to Amikacin (15.38%). The greater prevalence of resistance to common antibiotics has also been reported by other workers [14, 15]. The presence of multidrug resistance may be related to the dissemination of antibiotic resistance among hospital isolates of *E. coli*. All the isolates were sensitive to Cefoperazone sulbactam and Imipenem (Table 6).

Conclusion

The present study shows the capacity of E. coli to adapt and survive in different tissues by producing virulent factors. By virtue of their numerous virulence traits ExPEC clearly possess a unique ability to cause disease outside the host intestinal tract. The practical goal of investigations into the virulence properties of any pathogen is the development of specific anti virulence factor interventions (such as vaccine) to prevent infection [16].

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