

Characterization of *Escherichia coli* Phylogenetic Groups Associated with Extraintestinal Infections in South Indian Population

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Abstract

Background: *Escherichia coli* strains mainly fall into four phylogenetic groups (A, B1, B2, and D) and that virulent extra-intestinal strains mainly belong to groups B2 and D. **Aim:** The aim was to determine the association between phylogenetic groups of *E. coli* causing extraintestinal infections (ExPEC) regarding the site of infection, expression of virulence factors, antimicrobial resistance patterns, and clinical outcome. This descriptive study was carried out in a multi-specialty Tertiary Care Hospital. **Materials and Methods:** A total of 300 *E. coli* causing ExPEC were studied. Triplex polymerase chain reaction was used to classify the phylogenetic groups; hemolysin production was assessed on sheep blood agar and biofilm production in a microtiter plate assay. Production of extended spectrum of beta-lactamase (ESBLs) was detected by combination disk method; AmpC was detected by AmpC disk test, Carbapenemase production was detected by modified Hodge test and metallo-β-lactamase by metallo-beta-lactamases (MBL) E-test. **Results:** Of 300 isolates, 61/300 (20%) belonged to phylogroup A, 27/300 (9%) to phylogroup B1, 104/300 (35%) were B2 and 108/300 (36%) belonged to group D, respectively. Phylogroups B2 and D were the most predominant groups in urinary tract infection and sepsis. Prognoses were better in infections with group A and B1 isolates, and relapses and death were common in infections with B2 and D. Expression of biofilm was greatest in B1 and hemolysin in group B2. Group A and B1 showed higher resistance to ciprofloxacin and were most frequent β-lactamase (ESBL, AmpC, Carbapenemase and MBL) producers. **Conclusions:** Phylogenetic group B2 and D were predominant in ExPEC and exhibited least antimicrobial resistance among the groups. Resistance to multiple antibiotics was most prevalent in group A and B1. Regular monitoring of antimicrobial susceptibility in commensal strains is essential as they might transfer the property of antimicrobial resistance to pathogenic strains.

Keywords: Drug resistance, *Escherichia coli*, Extraintestinal infections, Polymerase chain reaction, Phylogenetic group, Virulence

Introduction

Escherichia coli strains are implicated in a large number of extraintestinal infections (ExPEC) in humans such as urinary tract infection (UTI), bacteremia, pneumonia, soft-tissue

infection, and neonatal meningitis.^[1] Phylogenetic analysis has shown that *E. coli* strains fall into four main groups (A, B1, B2, and D). It has been found that pathogenic *E. coli* strains causing extraintestinal infections mainly belong to group B2 and a lesser extent to group D whereas commensal strains belong to group A and B1.^[2] Results of various studies have also indicated that the phylogroups A and B1 (commensal strains) exhibit an increased drug resistance pattern but possess few virulence genes whereas the phylogroups B2 and D (pathogenic strains) possess several pathogenicity associated islands and express multiple virulence factors such as adherence factors including biofilm production and high surface hydrophobicity, toxin (hemolysin and CNF)

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and siderophore production but are more susceptible to antibiotics.^[3]

In recent years, much attention has been given to the analysis of the phylogenetic affiliation of pathogenic *E. coli* strains, so as to understand the sources of such ExPEC and also to limit the spread of multidrug resistance among such strains.^[4] Although several studies have been reported from outside India, not much information is available regarding the phylogenetic lineage of ExPEC causing extraintestinal infections from within our country with only very few studies being reported regarding phylogenetic grouping. The aim of the present study was to assess the association between phylogenetic groups of *E. coli* and site of infection, phenotypic characters, antibiotic resistance, and clinical outcome, respectively.

Materials and Methods

Participants and clinical isolates

The study was conducted during the period from August 2010 to January 2012, from hospitalized patients of two Tertiary Care Hospitals, after obtaining permission by way of informed consent from patients and ethical clearance from the Kasturba Medical College, Mangalore institutional ethical committee. Sample size was determined with 55% confidence and 90% power based on previous studies. Three hundred non-repeat strains of *E. coli* were isolated from specimen such as urine, blood, wound swab, pus, cerebrospinal fluid, ascitic fluid, and intravascular devices, collected using standard sterile procedures from the study population. The study population included hospitalized patients of all age groups whose extraintestinal clinical samples grew *E. coli* and excluded those subjects who had received antimicrobial drugs during the past 1 month, who had asymptomatic UTI, polymicrobial infections and those who were discharged without treatment with antimicrobial drugs. Clinical data from the patient's records were collected in a proforma. All patients were followed up for the period of 1 year to monitor clinical outcome. 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.^[5] ExPEC isolates from blood were identified using automated biochemical system Vitek 2 (BioMerieux, France).

Biofilm production

The capacity to form biofilms was assayed in microtiter plates with minor modification as described by O'Toole and Kolter.^[6] Briefly, cells were initially grown for 18 h in Trypticase soy broth (TSB) at 37°C, subsequently cultures were diluted 1:100 with fresh TSB and 200 µl were inoculated into 96 well polystyrene microtiter plates and incubated for 24 h at 37°C. After incubation, content of each well was gently removed by tapping the plates. The wells were washed four times with 200 µl of phosphate buffer saline (PBS, pH 7.2) to remove free-floating planktonic bacteria. Biofilms formed by adherent

organisms in plate were fixed with Bovin's fixative and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical densities (OD) of stained adherent bacteria were determined with an ELISA reader at wavelength of 630 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms; strains with mean OD values of <0.120 were considered as non-biofilm producers and those with mean OD values >0.120 were considered to be biofilm producers. *Pseudomonas aeruginosa* ATCC 27853 was used as the control organism.

Hemolysin production

Production of α-hemolysin was tested on 5% sheep blood agar. *E. coli* strains were inoculated onto blood agar plates, incubated overnight at 37°C and hemolysis was detected by the presence of a zone of complete lysis of the erythrocytes around the colony.^[7]

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done by the modified Kirby-Bauer disk diffusion method in accordance with CLSI guidelines.^[8] The antibiotic disks (HiMedia, Mumbai) used were ampicillin (10 µg), piperacillin (10 µg), piperacillin + tazobactam (100/10 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), amikacin (30 µg), gentamicin (10 µg), co-trimoxazole (1.25/23.75 µg), cefoperazone + sulbactam (75/30 µg), imipenem (IPM; 10 µg), meropenem (MRP; 10 µg), and ertapenem (ETP; 10 µg).

Screening for extended spectrum of beta-lactamase production

Isolates which were resistant to one or more third-generation cephalosporins were tested for Extended spectrum of beta-lactamase (ESBL) production by the combination disk method using, ceftazidime (30 µg) and ceftazidime/clavulanic acid (10 µg). A ≥5 mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disc, when compared to the cephalosporin disc alone was interpreted as phenotypic evidence of ESBL production.^[8]

Detection of AmpC production

Isolates were tested for AmpC enzyme production by AmpC disk test.^[9] Briefly, a suspension of ATCC *E. coli* 25922 standardized to 0.5 McFarland was inoculated on the surface of Mueller-Hinton Agar (MHA) plate. A 30 µg cefoxitin disk was placed on the inoculated surface of the agar. A sterile plain disc containing Tris-ethylenediaminetetraacetic acid (EDTA) inoculated with several colonies of the test isolate was placed beside the cefoxitin disc almost touching it, with the inoculate in contact with the agar surface. The plates were incubated overnight at 35°C, aerobically. A positive test was indicated as flattening or indentation of cefoxitin inhibitory zone in the vicinity of the test disc. A negative test had an undistorted zone.

Detection of carbapenemase production

Plates of MHA were inoculated with suspensions of test strains and adjusted to a turbidity equivalent to 0.5 McFarland standard. A set of discs (HiMedia) of IPM, MRP, and ETP (10 µg each) were applied to the surface of the agar, plates were incubated overnight at 35°C aerobically, and diameters of zone of inhibition (≥ 23 mm indicating sensitivity, 20–22 mm indicating intermediate resistance and ≤ 19 mm indicating resistance) were recorded. Carbapenemase production was further confirmed by modified Hodge test.^[8]

Detection of metallo- β -lactamase producers

Identification of metallo- β -lactamases (MBL) activity was performed by two methods: A carbapenem–EDTA combined disk method and MBL *E*-test (HiMedia).^[10] A known MBL producing isolate (PCR positive with MBL genes) was used as a positive control for all tests.

Determination of *Escherichia coli* phylogenetic groups

Isolates were assigned to one of the four main phylogenetic groups of *E. coli* (A, B1, B2, and D) by using the triplex PCR as described by Clermont *et al.*^[11] Briefly, template DNA was obtained by boiling-lysis method. The genes *chuA*, *yjaA* and *TSPE4.C2* were amplified using appropriate oligonucleotide primers.^[11] The PCR was performed in a final reaction volume of 50 µl containing 750 mM Tris-HCl, 200 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.2 mM each of dNTP's, 0.4 µM of each primers, 1 U of Taq DNA polymerase and 5 µl template DNA. An Eppendorf thermocycler was used for amplification. The program for amplification included a step of initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension step at 72°C for 7 min. The PCR products were loaded in 2% w/v agarose gel prepared in Tris-borate–EDTA buffer at 120V for 1 h and detected by ethidium bromide staining after electrophoresis.

Statistical analysis

Chi-square test was used to find an association between the phylogroups, virulence factor genes and patient's clinical outcome. Analysis was performed using statistical package SPSS version 17.0 (USA).

Results

A total of 300 *E. coli* isolates from extraintestinal sources were collected. One hundred forty-three isolates were from the medical unit, 44 from surgical, 43 from urology, 20 from oncology, 20 from gastroenterology, 13 from obstetrics and gynecology, 12 from orthopedics and 5 from pediatrics units. The primary site of infection included 159/300 (53%) cases of UTI, 77/300 (25.6%) with bacteremia, 40/300 (13.3%) with wound infection, 19/300 (6.3%) with pneumonia, 3/300 (1%) intravascular device infection, and 2/300 (0.6%) with meningitis.

Phylogenetic analysis of isolates carried out by triplex PCR [Figure 1] indicated that 61/300 (20%) isolates belonged to Group A, 27/300 (9%) were group B1, 104/300 (35%) were group B2, and 108/300 (36%) belonged to group D. The distribution for each phylogenetic group according to gender, age group, site of infection, and outcome is summarized in Table 1. The phylogroups B2 and D occurred with greater frequency (statistically significant, $P < 0.05$) than A and B1 among all isolates of ExPEC without any sex disparity. Furthermore, phylogroups B2 and D were seen to occur at a far greater frequency in all age groups, when compared to A and B1. The largest number of ExPEC infections were observed in the > 60 years age group (130/300 strains; 43.3% of all infections, statistically significant, $P = 0.04$) indicating that this group was more susceptible than others to infection with ExPEC. UTI was the clinical entity wherein highest number of ExPEC were isolated (159/300 strains; 53% statistically significant, $P = 0.03$), again with phylogroups B2 and D being isolated most frequently (38.4 and 33.9% respectively, $P < 0.01$).

Regarding clinical outcome, prognosis of infections with strains belonging to A and B1 phylogroups was far better than with infections due to strains from B2 and D. Seventy-eight percent of cases due to ExPEC strains belonging to A and 62.9% of cases due to infection with strains belonging to B1 were successfully treated and such ExPEC were eradicated completely with appropriate antibiotic therapy. On the other hand, higher morbidity (relapses and recurrent infections) and mortality was observed in infections due to ExPEC strains belonging to the virulent phylogroups B2 and D (37% and 26%, respectively; Table 1).

The study of phenotypic characteristics such as expression of virulence factors and antimicrobial resistance of the isolates belonging to each phylogenetic group showed that expression of biofilm production was greatest with groups B1 and B2; group B2 isolates were observed to be most frequently associated with hemolysin production (37.5%, statistically significant, $P < 0.05$) and 17.3%

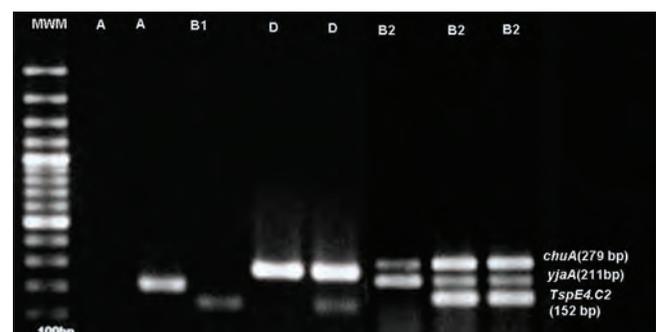


Figure 1: Triplex polymerase chain reaction results of phylogenetic grouping of extraintestinal infections isolates (phylogenetic group A [*chuA*–, *TspE4.C2*–]; Group B1 [*chuA*–, *TspE4.C2*+]; Group B2 [*chuA*+, *yjaA*+]; and Group D [*chuA*+, *yjaA*–])

Table 1: Comparison of different phylogenetic groups of ExPEC in relation to demographical features and clinical outcome

Feature	Phylogenetic groups (n=300)				Total (%)
	A	B1	B2	D	
Gender					
Male	35	14	56	58	163 (54.3)
Female	26	13	48	50	137 (45.6)
Age					
>1	-	1	2	1	4 (1.3)
1-18	-	-	5	3	8 (2.6)
19-44	15	9	20	27	71 (23.6)
45-59	18	10	32	27	87 (29)
>60	28	7	45	50	130 (43.3)#
Infection					
UTI	27	17	61	54	159 (53)*
Sepsis	16	7	27	27	77 (25.6)
Wound	8	2	13	17	40 (13.3)
Pneumonia	9	-	3	7	19 (6.3)
Meningitis	-	1	-	1	2 (0.6)
Outcome					
Improved	48	17	62	75	202 (67.3)
Re-infection	4	6	28	17	55 (18.3)
Expired	8	2	12	11	33 (11)
Lost to follow-up	1	2	2	5	10 (3.3)
Total* n (%)	61 (20.3)	27 (9)	104 (34.6) [®]	108 (36) [®]	300 (100)

*Numbers represent total number of each phylogenetic group in each category; that is, a, b, c and d. Among 300 isolates *Statistically significant, $P=0.04$, #Statistically significant $P=0.03$, Among the phylogroup [®]Statistically significant, $P<0.05$. UTI: Urinary tract infection

were non-lactose fermenters [Table 2]. The expression of various enzymes associated with antimicrobial resistance and resistance patterns exhibited by isolates of the four phylogenetic groups against representative antimicrobials from different classes are shown in Table 2 and Figure 2. Results of our study showed that group B2 isolates produced the least amount of beta lactamases when compared with group A, B1, and D. Resistance to ciprofloxacin, norfloxacin, co-trimoxazole, nalidixic acid and cefoperazone/sulbactam was especially prevalent among group B1 isolates and group A isolates showed comparatively higher resistance to ciprofloxacin and norfloxacin when compared with groups B2 and D. However, in case of ampicillin and cefotaxime, resistance rates were higher with group B2 and D. The presence of multi-drug resistance (resistance to three or more antibiotic groups) strains were significantly higher in group B1 isolates (statistically significant, $P = 0.04$). Among the four groups, A and B1 group isolates were most frequent β -lactamase (ESBL, AmpC, Carbapenemases, MBL) producers when compared to group B2 and D [Table 2].

Discussion

Escherichia coli is emerging as an important cause of extraintestinal infections in our hospitals. These strains are seen to exhibit several virulence properties as well as a high rate of antibiotic resistance which is of major concern for management of cases. Extraintestinal pathogenic *E. coli* which routinely cause infections have been shown to belong to phylogroups B2

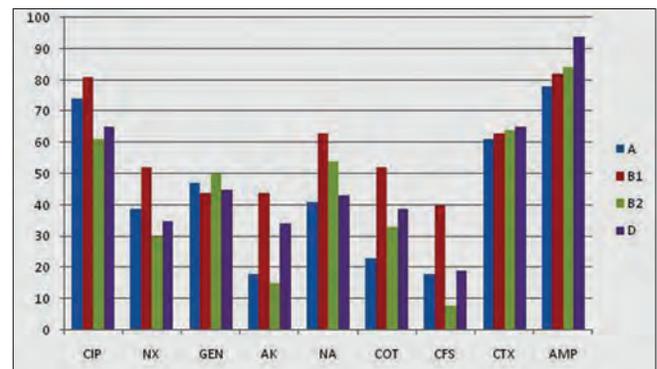


Figure 2: Antimicrobial resistance (in percentage) to representatives from different classes of antibiotics among 300 *Escherichia coli* isolated from various extraintestinal sources (CIP: Ciprofloxacin, NX: Norfloxacin, GEN: Gentamicin, AK: Amikacin, NA: Nalidixic acid, COT: Co-trimoxazole, CFS: Cefoperazone/sulbactam, CTX: Cefotaxime, AMP: Ampicillin)

and D.^[2,3] Results of our study indicated that approximately 70.6% of the *E. coli* isolates from our patients also belonged to phylogenetic group B2 and D which is in agreement with previous findings.^[3,4] The least frequently isolated phylogenetic group in our study was group B1, which is also in accordance with similar studies done elsewhere.^[4]

Our study on the prevalence of different phylogroups in various clinical entities yielded the following facts: In cases of sepsis among our study population, we found that all four phylogroups occurred with approximately equal frequency whereas in other studies, groups B2 and D have been reported to be more common.^[4,12] However, in cases of UTI, B2,

Table 2: Expression of phenotypic properties associated with virulence and antimicrobial resistance among ExPEC strains

Phenotypic properties	Phylogenetic group (n=300) (%)				Total*
	A (n=61)	B1 (n=27)	B2 (n=104)	D (n=108)	
Biofilm production	29 (47.5)	14 (51.8)	51 (49.1)	35 (32.4)	129 (43)
Hemolysis on sheep blood agar	12 (19.6)	4 (14.8)	39 (37.5)	21 (19.4)	76 (25.3)
Lactose fermentation	58 (95.1)	26 (96.3)	86 (82.7)	91 (84.3)	261 (87)
ESBL production	42 (68.8)	20 (74.1)	72 (69.3)	78 (72.2)	212 (70.6)
AmpC producers	20 (32.7)	12 (44.4)	23 (22.1)	40 (37.1)	95 (31.6)
Carbapenemase producers	5 (8.2)	10 (37.1)	4 (3.8)	10 (9.3)	29 (9.6)
MBL producers	3 (4.9)	7 (25.9)	3 (2.8)	4 (3.70)	17 (5.6)

*Percentage of ExPEC strains exhibiting a particular phenotypic character. ESBL: Extended spectrum of beta-lactamase, MBL: Metallo-beta-lactamases

and D phylogroups were more common. The most common phylogroup which was responsible for relapses and/or deaths was group B2, which probably indicates the capability of strains of this group to cause persistent and severe infections.

Previous studies have indicated that phylogroups B2 and D possess more virulence properties such as biofilm formation and hemolysin secretion when compared with phylogroups A and B1 isolates.^[13,14] In our study, we found group B2 consisted of a greater number of non-lactose fermenting isolates, and also more strains produced hemolysin which is considered to be a potent virulence factor essential for survival in extraintestinal sites.^[3,13] Interestingly, it was group B1 isolates which expressed a higher capacity to form biofilms, a virulence factor which has been shown to be responsible for the initial step of adherence to various cell/tissue surfaces, during the process of infection.^[14]

Regarding antimicrobial resistance exhibited by isolates from different phylogroups, our study results indicated that phylogroup B2 isolates were the most susceptible (statistically significant) to antibiotics than isolates belonging to the other three groups. Similar results have been obtained by other investigators, indicating that although being more virulent, the isolates of phylogroup B2 were more susceptible to antibiotics.^[12] However, in our study, we found gentamicin resistance was more common in group B2 isolates. Several studies have reported a significant association of ciprofloxacin-resistant extraintestinal *E. coli* with phylogeny group A and ciprofloxacin susceptibility with group B2, which is similar to our study findings.^[15,16]

Our study of the ability of isolates to produce antimicrobial degrading enzymes such as ESBLs, AmpC, carbapenemase, and MBLs indicated that beta lactamase producing isolates occurred with greater frequency in group A and B1 isolates when compared with B2 and D, a finding which is in accordance with other studies.^[15,17] This finding indicates that strains belonging to phylogroups A and B1 were carrying more resistance properties than the strains belonging to phylogroups B2 and D. By this finding, it may be assumed that in our population a large number of people act as a reservoir for such resistant strains. This may be due to inappropriate antibiotic use in both animals and humans.

Conclusion

Results of our study indicate that pathogenic strains belonging to phylogenetic groups B2 and D express more virulent properties but were seen to be more susceptible to antibiotics than phylogroups A and B1 isolates. This finding indicates a need for regular monitoring of antimicrobial susceptibility in commensal strains as these strains might transfer the property of antimicrobial resistance to pathogenic strains. There is also an urgent need to undertake measures which would prevent the development of multidrug resistance among commensal strains such as reducing inappropriate antibiotic treatment, the overuse of antimicrobials in the agricultural industry which might contribute to increased numbers of multidrug resistant strains. Clearly, antimicrobial resistance may lead to decreased therapeutic outcomes in the near future.

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