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ANTIBIOTIC SUSCEPTIBILITY PROFILE AND ITS CORRELATION TO BIOFILM FORMATION AND MANNOSE RESISTANT HEMAGGLUTINATION IN EXTRAINTESTINAL PATHOGENIC *ESCHERICHIA COLI.*

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ABSTRACT

The present study was conducted to gain an insight into drug resistance and virulence factors in extraintestinal pathogenic *Escherichia coli.* This study was carried out on 150 *Escherichia coli* isolated from extraintestinal infections. Antimicrobial susceptibility testing was done by modified Kirby Bauer disc diffusion method. Extended spectrum β- lactamase (ESBL) production was confirmed by phenotypic confirmation tests. They were also screened for virulence factors, namely biofilm production, hemolysin, mannose resistant hemagglutination (MRHA), and gelatinase by standard methods. Majority of isolates included in this study were sensitive to imepenem (98%), amikacin (84.66%) and piperacillin-tazobactam (71.33%). In this study 76 isolates were ESBL producers (50.66%), but multi drug resistance was seen in many of isolates. Gelatinase activity was seen in 09 isolates (6%). Out of 150 isolates, 57 (38%) isolates were MRHA positive, 89 (59.33%) strains have formed biofilm and 43 (28.66%) isolates were hemolysin producers. The present study shows the expression of multiple virulence factors and multi drug resistance in extraintestinal pathogenic *Escherichia coli.* The present study also shows significant correlation between biofilm production, drug resistance and multiple virulence factors.

KEY WORDS: ***Escherichia coli,*** **biofilm, ESBL, MRHA, virulence**

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INTRODUCTION

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are implicated in a large number of infections in humans and animals, such as urinary tract infection (UTI), meningitis, diverse intra abdominal infection, pneumonia, osteomyelitis, and soft-tissue infection; besides, bacteriemia can accompany infection of any of these sites[1]. The virulence traits of extraintestinal pathogenic *Escherichia coli* are for the most part distinct from those of intestinal pathogenic *Escherichia coli* and other Gram negative bacilli that cause disease outside the bowel[2]. This difference reflects site-dependent differences in host environments and defense mechanisms. These specialized extraintestinal pathogenic *Escherichia coli* (ExPEC) strains acquire their unique pathogenicity from distinctive virulence factors that include adhesins, toxins, polysaccharide coating and invasions causing the host organism to overcome or subvert its defenses, colonize key anatomical sites, and disturbing host physiology by invading tissues causing disease[3]. In terms of morbidity and mortality, ExPEC has a great impact on public health, with an economic cost of several billion dollars annually. The treatment of *Escherichia coli* infections is increasingly becoming difficult because of the multidrug resistance exhibited by the *Escherichia coli*. Extended spectrum β-lactamase (ESBL) producing organisms pose a major problem for clinical therapeutics. The incidence of ESBL producing strains of *Escherichia coli* among clinical isolates has been steadily increasing over the past few years, resulting in limitation of therapeutic options [4,5]. The therapeutic choices for such infections are limited because of cross resistance. The carbapenems are the most active agents against such organisms. However, some strains are reported to produce New Delhi metallo- β-lactamase, NDM-1 a carbapenemase which make carbapenem antibiotics ineffective for the treatment[6]. The present study was undertaken to assess virulence factors and antimicrobial resistance in *Escherichia coli* isolates obtained from extraintestinal sites of infected patients with an emphasis on biofilm production and drug susceptibility.

MATERIALS AND METHODS

This study was carried out in the Department of Microbiology, Hassan Institute of Medical Sciences, Hassan., for a period of 18 months (October 2012 to August 2013). Totally 150 consecutive extraintestinal pathogenic *Escherichia coli* isolated from outpatients and inpatients of different age group were included in the study. Specimens collected were pus, exudates, clean catch midstream urine, endotracheal tubes, sputum, blood and body fluids using standard sterile procedures. All samples were processed immediately and isolates were identified and characterized on the basis of their colony characters, morphology and biochemical reactions as per the standard methods [7].

STUDIES DETERMINING VIRULENCE FACTORS

(i) Antimicrobial susceptibility testing

Antimicrobial susceptibility of all isolated *Escherichia coli* was determined by the modified Kirby Bauer disc diffusion method according to CLSI guidelines[8]. The following antibiotic discs from HiMedia were used in this study. Amikacin(30 µg),amoxicillin-clavulanic acid(20/10µg), ampicillin(10µg), cefepime(30µg) cefodoxime(10 µg), cefotaxime(30 µg), cefoxitin(30µg),ceftazidime(30µg), ceftazidime-clavulanic acid(30/10 µg),ciprofloxacin(5µg), cotrimoxazole(25µg), gentamicin(10µg), imepenem(10µg), norfloxacin(10µg),piperacillin-tazobactam((100/10 µg). Organisms were categorized as susceptible, intermediate or resistant to the antimicrobial agent on the basis of guidelines provided by the CLSI. *Escherichia coli* ATCC 25922 was used for the quality control.

(ii) Detection of ESBL

Isolates resistant to one or more third generation cephalosporins were tested for ESBL production by phenotypic confirmation test[8]. Ceftazidime (30µg) vs. ceftazidime/clavulanic acid (30/10 µg) and cefotaxime (30 µg) vs. cefotaxime/clavulanic acid (30/10 µg) discs (HiMedia) were placed on lawn culture of test organism on Muller Hinton agar plate (HiMedia) and incubated overnight at 35°C. *Escherichia coli* ATCC 25922 was used as the negative control and ESBL-producing organism *Klebsiella pneumoniae* ATCC 700603 was used as the positive control. Regardless of zone diameter, a ≥ 5mm increase in zone diameter of cephalosporin tested in combination with clavulanic acid vs. its zone diameter when tested alone was considered as phenotypic evidence of ESBL production.

(iii) Detection of biofilm formation

A qualitative assessment of biofilm production was done by modified Christensen method[9]. Trypticase soy broth with glucose (10mL) was inoculated with loopfull of test organisms from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and 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 an inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube.

(iv) Mannose resistant hemagglutination (MRHA)

Hemagglutinating activity was determined by micro hemagglutination test using 96 well round bottom plates and fresh human group O positive erythrocytes[10]. *Escherichia coli* were grown on colonization factor antigen agar (CFA Agar) plates at 37° C for 18 hours, then 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 utilized as negative control. The microtitre plate was then incubated at 4°C for 1hour. The presence of a small pellet of erythrocytes at the bottom after incubation was considered as a negative result, and that containing an even sheet of erythrocytes across the well was considered as positive. The 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 pellet of erythrocytes at the bottom of the wells after incubation was considered as a negative result, and that containing an even sheet of erythrocytes across the well was considered as positive.

(v) Hemolysin production

The plate hemolysis test was done for the detection of alpha hemolysin[11]. The bacteria were inoculated on 5% sheep blood agar and incubated overnight at 37°C. Hemolysin production was detected by the presence of a zone of complete lysis of erythrocytes around the colony and the clearing of the medium.

(vi) Gelatinase test

Gelatinase production was tested using gelatin agar[12]. 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 a zone of clearing around colonies was considered positive for gelatinase

RESULTS

Of the 150 clinical isolates of *Escherichia coli*, 57isolates were from urine, 55 from pus, 20 from sputum, 09 from blood and 09 from the tips of catheter and endotracheal tubes. The majority of isolates included in this study (Fig. I) were sensitive to imepenem (98%), amikacin (84.66%) and piperacillin-tazobactam (71.33%) whereas, the maximum resistance was observed for ampicillin (86.66%) followed by ceftazidime (83.33%), cefodoxime (82%), cefotaxime (82%), ciprofloxacin (73.33%),

gentamicin (57.33%) and cotrimoxazole (56%). ESBL production was seen in 76 (50.66%) isolates. Maximum number of ESBL producing *Escherichia coli* was isolated from the urine (47.36%) and pus (47.27%). Out of 150 strains, 89 (59.3%) were strong biofilm producers and remaining 61(40.66%) isolates were weak or non-biofilm producers; 57(38%) isolates were MRHA positive, 43(28.66%) isolates were hemolytic and 09(6%)gelatinase producers(Table I). Among biofilm producers, maximum numbers of strains were susceptible to imepenem and amikacin (Fig.II). When the biofilm producing strains were studied for the presence of multiple virulence factors, they showed a significant correlation between biofilm production and multiple virulence factors (Table II).

Figure I

Antibiotic resistance pattern in extraintestinal pathogenic Escherichia coli.(N=150)

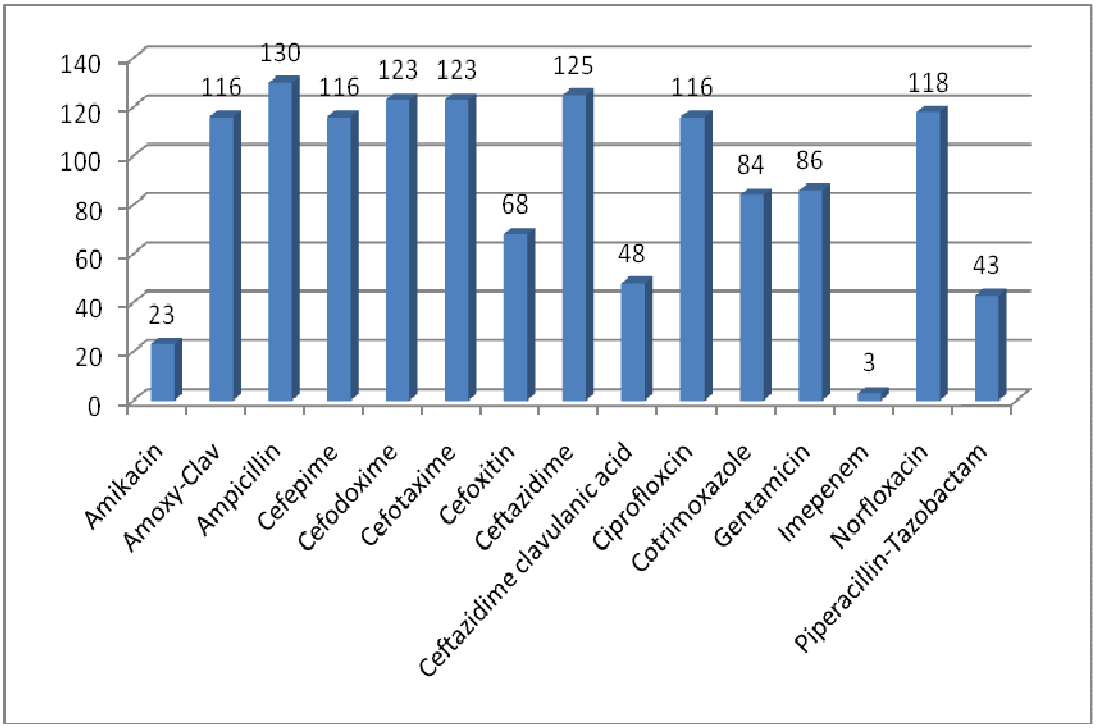

Figure II

Drug resistance pattern of biofilm producers and non biofilm producers.

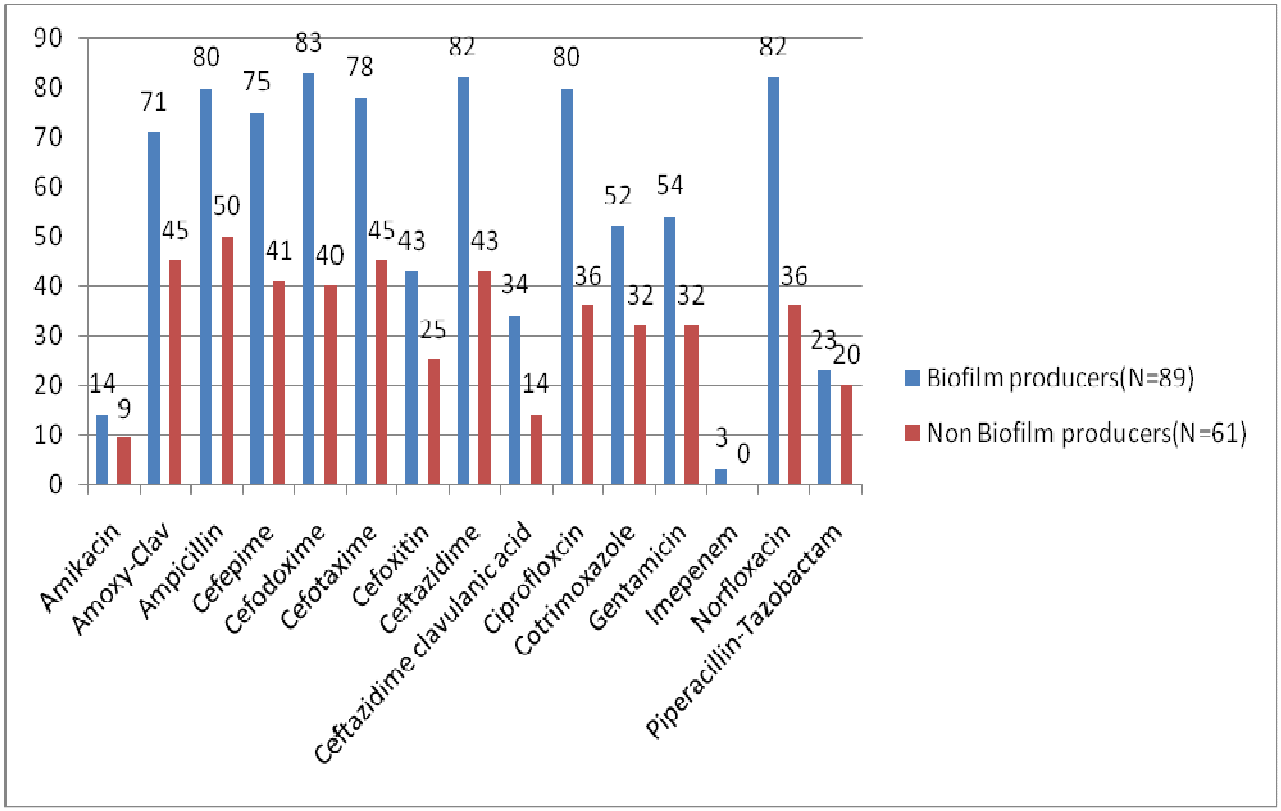

Table I

Presence of multiple virulence factors in extraintestinal pathogenic Escherichia coli.

Sl No	Virulence factors	No of isolates positive for virulence factor(N=150)	Percentage
01	ESBL production	76	50.66
02	Biofilm production	89	59.33
03	MRHA	57	38.00
04	Hemolysin	43	28.66
05	Gelatinase production	09	06.00

Table II

Occurrence of virulence factors in biofilm positive and biofilm negative strains of extraintestinal pathogenic Escherichia coli

Virulence factors	MRHA N=57	ESBL N=76	Hemolysin N=43	Gelatinase N=9
Biofilm producers(89)	48(53.93%)	49(55.05%)	30(33.70%)	05(5.61%)
Non Biofilm producer(61)	9(14.75%)	27(44.26%)	13(21.31%)	04(6.55%)

DISCUSSION

Discovering virulence factors are important to understand bacterial pathogenesis and interaction of them with the host. Capacity of *Escherichia coli* to produce multiple virulence factors and drug resistance to antibiotics may contribute to its pathogenicity in extraintestinal infections[5]. The extensive use of the antimicrobial substances led to the emergence of multidrug resistant strains, increasing the number of nosocomial infections and

complicating their clinical picture. Many species of Gram-negative organisms possess the ability to resist breakdown by β-lactam antibiotics through the production of β-lactamase enzymes. Over the past twenty years, new β-lactam antibiotics were developed with the ability to withstand the hydrolytic activity of these β-lactamases. Subsequently, new classes of "extended spectrum β-lactamases" have emerged. These enzymes are capable of breaking down beta-lactam antibiotics such as cefotaxime, ceftazidime and ceftriaxone as well as the monobactams such as aztreonam. ESBL production is encoded by plasmids which also carry genes for resistance to other classes of antibiotics such as, aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and sulfonamides. Although ESBLs have been identified in many Gram-negative species, they are predominantly found in Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae.* Different studies from India have quoted variable ESBL detection rates. The results of our study have shown that out of the 150 patients with the ExPEC infection, 76 (50.66%) were ESBL producers. ESBL production among *Escherichia coli* was 58% in Delhi[4], 32% in Bijapur, South India[13] and 51.4% in Mangalore, South India[5]. It was also observed that there was a high degree of resistance to the multiple classes of antibiotics among the ESBL producing organisms except for carbapenems which are the most active and reliable treatment options for infections caused by the ESBL producing isolates.

Biofilm formation by *Escherichia coli* is a known virulence factor. Antibiotic resistance in the biofilm mode of growth contributes to chronicity of infections such as those associated with implanted medical devices. The exopolysaccharide slime causes a diffusion barrier by restricting the rate of molecular transport to the interior of biofilm, chemically reacting with the molecules themselves. The exopolysaccharide is negatively charged and restricts the penetration of the positively charged molecules of antibiotics by chemical interactions or molecular binding. This also dilutes the concentration of the antibiotics before they reach the single bacterial cell in the biofilm[14]. Biofilm protects the bacterium from host defense mechanisms and antibiotic action. Apart from these, the efflux pumps can extrude antibiotics from the cell. Induction of the pump is one of the important alterations conferring resistance to biofilm cells[15]. The findings of the various studies showed that the biofilm producers were less susceptible to antibiotics[16,17]. In this study, 89 (59.33%) strains were in vitro biofilm producers. Among these strains 48(53.93%) were MRHA positive. Increased rate of biofilm production (53.93%) was observed in MRHA positive strains which clearly indicates that there is a strong correlation between biofilm production and MRHA, as fimbriae are probably one of the factor which contribute to biofilm production[18,19]. In this study, we found 55.05% of biofilm producing isolates were ESBL producers. Hemolysin production is a property largely associated with *Escherichia coli* strains which causes extraintestinal infections in human, whereas it is rarely found in fecal isolates from healthy persons[20]. In the present study, 28.66% of the strains were hemolysin producers. There was no significant correlation between hemolysin production and biofilm formation. Gelatinase activity was seen in only 09 (6%) isolates and it indicates, gelatinase is not an important virulence factor.

CONCLUSION

As the resistance pattern of microorganisms are changing continuously and causing public health problem, it is very essential to track changes in microbial population and rapidly detect the emergence of new resistance. With ongoing complications due to emerging antibiotic resistance, novel treatment strategies are necessary to control extraintestinal pathogenic *Escherichia coli* infections. Recognizing and treating risk posed by extraintestinal pathogenic *Escherichia coli* would greatly enhance

positive impact on human health.Along with drug resistance, *Escherichia coli* express other virulence factors which further complicate the outcome of the treatment. Therefore furthers studies are required at the molecular level to understand the interaction between different virulence factors expressed by *Escherichia coli*.

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