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BIOFILM FORMATION IN EXTRAINTESTINAL PATHOGENIC *Escherichia coli* STRAIN: RELATIONSHIP WITH ANTIMICROBIAL RESISTANCE AND PATIENTS CLINICAL OUTCOME

ARINDAM CHAKRABORTY*[1], PRABHA ADHIKARI[1] AND SHALINI SHENOY[2]

[1]*Department of Medicine. Kasturba Medical College .Mangalore. Manipal University. Karnataka. 575001. India.*
[2]*Department of Microbiology. Kasturba Medical College .Mangalore. Manipal University. Karnataka. 575001. India*

ABSTRACT

Extraintestinal pathogenic *Escherichia coli* infection is a serious health threat in the world. They show a high degree of resistance in various groups of antibiotics as biofilm production is one of the causes of antibiotic resistance. The aim of the present study is to detect in vitro biofilm formation by ExPEC isolates and resistance pattern of commonly used antibiotic, and patient's clinical outcome. A total of 300 isolates were screened for biofilm formation by the method described by O'Toole and kolter. Haemolysin production was tested on 5% sheep blood agar. Production of Extended spectrum-β-lactamases (ESBL) was detected by combination of disk diffusion method and in the same way AmpC was detected by AmpC disk test. Carbapenemase production was detected by modified Hodge test. Identification of Metallo- β- lactamases (MBL) activity was performed by MBL Etest. Of the 300 isolates 129 (43%) were biofilm producers. Twenty eight percent of the biofilm producing isolates showed clear zone of haemolysis in blood agar plate. Out of 129 isolates 72% were ESBL producers, ampC type β lactamase production were seen 31% isolates. Among the other group of antibiotics most number of resistance were seen in ciprofloxacin (73%). Most active antibiotic were amikacin and carbapenem class of drugs. Seventy and half percent patients were improved with proper antibiotic treatment were as relapses/ re-infection were seen in 14% patients and mortality seen in 11% patients. Biofilm producing isolates are associated with high level of drug resistance, mortality and relapses. They are producing ESBL, ampC along with carbapenemases which is a major problem worldwide. Early use of appropriate empirical antibiotic will probably reduce the mortality and morbidity of the patients.

KEYWORDS: Biofilm, Drug resistant, MBL, Outcome

ARINDAM CHAKRABORTY
Department of Medicine. Kasturba medical College. Mangalore.
Manipal University. Karnataka. 575001. India.

Corresponding author

INTRODUCTION

Extraintestinal pathogenic *E.coli* (ExPEC) strains are cause a large number of infection in humans and animals, such as urinary tract infection (UTI), meningitis, diverse intra abdominal infection, pneumonia, osteomyelitis and soft- tissue infection, besides bacteremia can accompany infection at any of the sites[1]. They generally exhibit several characteristics which contribute to their virulence including alpha hemolysin and biofilm formation, biofilm which is a hydrated matrix of polysaccharide and protein, forms a slimy layer. Antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronicity of infections such as those associated with implanted medical devices[2,3]. Recently, it has been observed that resistance of biofilms to antibiotics is greater than what is normally seen with planktonic cells[4,5]. The aim of the present study was to determine biological characteristics such as Biofilm formation, production of hemolysin, and antimicrobial susceptibility pattern of the ExPEC isolates and co-relate with clinical outcome of those patients after treatment with proper antibiotics.

MATERIALS AND METHODS

Clinical isolates

In this study 300 *Escherichia coli* non – repeated strains isolated from extraintestinal infections were studied over a period of 18 months (August 2010 to January 2012). The study population included hospitalized patients of all age groups who satisfied the following inclusion/ exclusion criteria. Inclusion criteria: Subjects whose extraintestinal clinical samples grew *E.coli* were included in the study. Exclusion criteria: Subjects who received antimicrobial drugs during the past one month, who had asymptomatic UTI, polymicrobial infection and those who were discharged without the treatment with antimicrobial drugs were excluded from the study. After obtaining the institution Ethics committee clearance, clinical outcome of patients were collected.

Specimens collected were clean catch midstream urine, blood, wound swab, pus, CSF, ascitic fluid and intravascular device 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[5]. Blood isolates were identified using biochemical system Vitek 2 (bioMerieux).

Biofilm production

The capacity to form biofilms was assayed in microtitre plates essentially as described by O'Toole & Kolter[6] with little modification. Briefly, cells were initially grown for 18 h in tripti- case soy broth (TSB) at 37^0 C. subsequently culture were diluted 1 in 100 with fresh TSB and 200 µl were inoculated into 96 well polystyrene microtitre plates and incubated for 18 h at 37^0 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 Bovine fixative and stained with crystal violet. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with an ELISA reader at a wave length of 630 nm. These OD values were considered as an index of bacteria adhering to the surface and forming biofilms. Interpretation: mean OD values <0.120 consider as a non biofilm producers and mean OD values >0.120 consider as a biofilm producers. Haemolysin production: Production of α- haemolysin was tested on 5% sheep blood agar. *E.coli* strains were inoculated onto blood agar plates, incubated overnight at 37^0 C and haemolysis was detected by the presence of a zone of complete lysis of the erythrocytes around the colony[7].

Antimicrobial susceptibility testing

Antimicrobial agents were tested by using modified Kirby-Bauer disk diffusion method in accordance with CLSI guidelines[8]. The

antibiotic disks. (HIMedia, Mumbai) usedwere:Ampicillin (10mcg), Piperacillin (10mcg), Piperacillin+Tazobactam (100/10mcg), Ceftriaxone (30mcg), Cefotaxime (30mcg), Ciprofloxacine (5mcg), Norfloxacin (10mcg), Amikacin (30mcg), Gentamicin (10mcg), Co-trimoxazole (1.25/23.75mcg), Cefoperazone+Sulbactam (75/30mcg), Imepenem (IPM) (10mcg), Meropenem (MRP) (10mcg), Etrapenem (ETP) (10mcg).

Screening for ESBL production

Isolates which were resistant to one or more third generation cephalosporin's were tested for ESBL production by the combination disk method using cefotaxime (30 µg) , cefotaxime/ clavulanic acid (10µg) ceftazidime (30µg) and ceftazidime / clavulanic acid (10µg). A ≥5mm 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

AmpC disk test

Isolates were tested for AmpC enzyme production by AmpC disk test[9]. Briefly A lawn culture of *E.coli* ATCC 25922 was prepared on Mueller - Hinton agar plate. Sterile discs (6 mm) moistened with sterile saline (20µl) and inoculated with several colonies of test organism .The inoculated disc was then placed besides a cefoxitin disc (almost touching) on the inoculated plate. The plates were incubated overnight at 35º C, aerobically. A positive test appears to 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 Mueller- Hinton agar were inoculated with suspensions of the tested strains and adjusted to turbidities equivalent to 0.5 McFarland standards. A set of discs (hymenia) of IPM, MRP and ETP (10µg each) was applied to the surface of the agar , plates were incubated overnight at 35^0 in air, and diameters of zones of inhibition (≥23 mm indicated sensitivity, 20 to 22 mm indicated intermediate resistance and ≤19 mm indicated resistance) were recorded . Carbapenemase production was further confirmed by modified Hodge test (MHT)[8].

Detection of metallo- β- lactamases producers

Identification of MBL activity was performed by MBL Etest (HI media) methods. For all tests known MBL producer isolate were used as a positive control.

MBL E tests

Several E test gradient formats for detecting MBLs have been developed and the strip containing a double sided seven – dilution range of IPM (4 to 256 µg/mL and IPM (1 to 64 µg/ m L) in combination with a fixed concentration of EDTA has been reported to be the most sensitive format [10]. Thus the IPM-EDTA Etest was performed according to the recommendations of the manufacturer (Himedia). Interpretation: When the ration of the value obtained for IPM: The value of IPM+EDTA is more than to 8 or if zone is observed on the side coated with IPM + EDTA & no zone is observed on the opposite the side coated with IPM interpreted the culture as MBL positive.

Statistical analysis

Data was expressed as percentage. Chi-square test was used to find an association between biofilm haemolysin and drug resistant patterns. P<0.05 was considered as significant. Analysis was performed using statistical package SPSS version 17.0 (SPSS, USA).

RESULTS

In total 300 patients with Extraintestinal *E.coli* infection were selected, out of these 159 (53%) were with UTI, 77 (26%) with sepsis, 40 (13.33%) with wound infection, 19 (6.33%) with pneumonia, 3 (1%) intravascular device infection and 2(0.66%) with meningitis.

Detection of biofilm

of the 300 ExPEC isolates 129 (43%) were biofilm producer, out of this maximum number

of isolates were from urine (51%) followed by blood(30%) and wound (13%). In blood agar plate 36 (28%) out of 129 isolates were shown clear zone of haemolysis (alpha haemolysis producer).

Drug resistance patterns of biofilms producing ExPEC

All the 129 *E.coli* isolates were analyzed for antimicrobial resistance by disk diffusion methods. Of these 93 (72%) isolates were confirmed ESBL producers by double disk diffusion assay. AmpC β lactamase production was seen in 40 (31%) isolates by AmpC disk test. All the 129 isolates were further screened for carbapenemase production by disk diffusion methods, isolates which were resistance to two or more carbapenem drugs were considered as carbapenemase producers. Modified Hodge test (MHT) was performed using both MER and ETP for further confirmation. In this study 10 (8%) isolates were carbapenemase producers. Carbapenemase producing isolates were farther evaluated phenotypically for the presence of a metallo β lactamase (MBL) using the metal chelating agent EDTA. 8 (7%) isolates were positive by MBL Etest. The resistant pattern of other group of antibiotics were as followed; gentamicin 52%, amikacin 24%, co-trimoxazole 31%, ciprofloxacin 73%.

Outcome

Out of 129 patients, who were infected with biofilm producers, 70.5% were improved with proper antibiotic treatment where as relapses seen in 14% patients and mortality seen in 11% patients. Table 1 showing the difference between biofilm producing and biofilm non - producing isolates.

Table 1
Comparison between biofilm and non biofilm producing isolates

	N=300	
	Biofilm producer N=129 (%)	**Biofilm non-producer N=171 (%)**
Haemolysin producers:	36(28)	40(23)
ESBL producers :	93(72)	119(70)
AmpC producers:	40(31)	55(32)
Carbapenemase producers	12(9)	17(10)
MBL producers :	8(6)	9(5)
Ampicillin resistance :	116 (90)	150(88)
Piperacillin + Tazobactam :	46 (36)	62(36)
Ciprofloxacine :	94(73)	106(62)
Co-trimoxazole :	41(32)	64(37)
Gentamicin :	67 (52)	75(44)
Amikacin :	31 (24)	43(25)
Patient outcome:		
Improved :	91 (70)	111(65)
Relapses / re-infection :	18 (14)	37(22)
Expired :	14 (11)	19(11)

DISCUSSION

Biofilm formation is one of the bacterial pathogenic determinants which allow the bacteria to persist a long time in vivo and interfere with bacterial eradication. Biofilm producing bacteria have several advantages, such as the acquisition of antibiotic tolerance, expression of several virulence factors and an increased resistance against phagocytosis and other host defense mechanisms. There are several mechanisms responsible for the resistance activity of the biofilm producers including failure of antibiotic penetration into biofilm as it act like a barrier [6 11]. Other well studied mechanism were efflux pumps since the efflux pumps can extrude antibiotics from the cell [12]. Induction of the pumps is one of the important alterations conferring resistance to biofilm cells; YhcQ is one of such putative multidrug resistance pump in *E.coli* which was reported to be involved in antibiotic resistance of biofilms [13 14]. In recent years the rapid spread of antibiotic resistance are mainly due to the antibiotic resistance marker genes on plasmids[15]. It has been reported that presence

of β- lactamase encoded plasmid reduced the amount of biofilm formed by *E.coli* strains. While the presence of either a gentamicin or tetracycline resistance gene did not effect in biofilm formation[16]. In our study, we found 72% of biofilm producing isolates were ESBL producers, where as only one third of the isolates were ampC producers. Only 8% isolates were carbapenemases producers out of this 7% were positive for MBL activity. For non β lactam antibiotics highest number of resistance were seen in ciprofloxacin (73%) and only 24% were resistance to Amikacin. There are several virulent factors are present in *E.coli*, out of this only haemolysin seems to have association with biofilm producers[17]. In our study we found only 28% isolates were haemolysin producers. Biofilm formation is the main cause of the persistence infection, despite appropriate antibiotic therapy[18]. More than 50% of all bacterial infections reported involve biofilm formation[19]. Several study have shown that UPEC strains were more frequent biofilm producer compare to other strains[7,20]. In our study we also found that half of biofilm producers were from urine isolates followed by blood isolates (30%). Acute UTI caused by UPEC can lead to recurrent infection were about 25% women with acute cystitis. Later develop recurrent UTI which is an important cause of morbidity. A study of women with recurrent UTI showed that 75% of strains causing relapse were biofilm formers[20]. However in our study population around 14% of the patients developed relapses because of biofilm producing isolates where as 21% patients developed relapses because of non biofilm producing isolates. In case of mortality 11% patients expired because of biofilm production isolated. However it is difficult to demonstrate attributable mortality solely to infection without proper study design and or autopsy provide evidence as those patients have some underlying conditions, where as 71% patients with biofilm producing isolates were improved with proper antibiotic treatment. In conclusion, our findings will help the clinician to choose the proper antibiotics for the biofilm producing isolates, which will reduced the mortality and morbidity of the patients.

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