African Journal of Biotechnology Vol. 8 (21), pp. 5995-5999, 2 November, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Altered membrane permeability in multidrug resistant *Escherichia coli* isolated from extra-intestinal infections

Bindu Arora* and Trupti Jagdale

Department of Microbiology, Abeda Inamdar Senior College, Azam Campus, Camp, Pune- 411 001, India.

Accepted 29 September, 2009

The study was conducted with the objective of examining the outer membrane proteins and their involvement during the transport of β - lactams in multidrug resistant *Escherichia coli* isolated from extra-intestinal infections. Also, the response of gram negative bacterial biomembrane alteration was studied using extended spectrum β-Lactamase (ESBL) positive strains of *E.coli* in the presence and absence of stress. Outer membrane protein analysis revealed that multidrug resistance was mediated by loss of porins in the ESBL isolates of *E. coli*. Parallel to this, the level of outer membrane proteins decreased dramatically with increased antibiotic stress in the isolates. In non-ESBL isolates of *E. coli*, porins seems to have no influence in co-modulation of resistance.

Key words: Outer membrane proteins, porins, *Escherichia coli*, multidrug resistance, extra-intestinal, extended spectrum β-Lactamase (ESBL).

INTRODUCTION

Membrane permeability is the first step involved in resistance of bacteria to an antibiotic. The outer membrane proteins that constitute porins play major roles in the definition of intrinsic resistance in gram-negative bacteria that is altered under antibiotic exposure (Viveiros et al., 2007). Outer membrane proteins (OMPs) of gram-negative bacteria have diverse functions. They are directly involved in the interaction with various environments encountered by pathogenic organisms. Thus, OMPs represent important virulence factors and play essential roles in bacterial adaptation to host niches, which are usually hostile to invading pathogens (Martínez-Martínez, 2008). Porins are outer membrane proteins that form water-filled channels that permit the diffusion of small hydrophilic solutes like β-lactam antibiotics across the outer membrane (Nikaido et al., 1983; Varadhachary and Maloney, 1990; Martinez-Martinez et al., 2002). Two major porins that facilitate diffusion of antimicrobials have been described in *Escherichia coli* namely OmpF and OmpC (Cowan et al., 1992; Basle et al., 2006). The decrease of OmpC and OmpF appears to result from their being degraded by proteases as long as the activity of genes that code for these proteins are also significantly elevated during prolonged antibiotic stress (Viveiros et al., 2007). Since β-lactam antibiotics penetrate the outer membrane of gram-negative bacteria, resistance could also be caused by loss or deficiency of specific porins that reduce the outer membrane permeability to β-lactam antibiotic (Ananthan and Subba, 2005). This might be an important factor in mediating β-lactam resistance in multidrug resistant *E. coli* in ESBL and non-ESBL isolates. It has been noted that the response to prolonged exposure to increasing levels of antibiotic cause major changes in the permeability of the bacterium due to over-expression of efflux pumps and down-regulation of porins (Bradford, 2001; Nikaido, 2003).

Cephalosporins enter the cell through OmpC and OmpF porin in the strains of *E.coli*. Proteomic studies indicate that OmpC is an important outer membrane protein for tetracycline resistance in *E. coli* (Zhang et al., 2008). OmpC forms trimeric porin allowing ions and other hydrophilic solutes to cross the outer membrane (Neidhart and Curtiss, 1996). The solutes tend to be less than 500 daltons. OmpC is tightly but noncovalently

*Corresponding author. E-mail: bindu_arora@yahoo.co.uk. Tel: +919766317202.

Table 1. Phenotypes of *E. coli* strains isolated from extra-intestinal infections (N= 80).

Phenotype	Phenotype detected with		
	Cefotaxime[1]	Ceftazidime[2]	Both antibiotics[3]
Presumptive ESBL producers	29 (36.25%)	32 (40%)	26 (32.5%)
Confirmed ESBL producers	21 (26.25%)	24 (30%)	18 (22.5%)

[1]Phenotype detected with cefotaxime but not with ceftazidime.
[2]Phenotype detected with ceftazidime but not with cefotaxime.
[3]Phenotype detected with ceftazidime and also with cefotaxime.

associated with the peptidoglycan layer (Lambert, 1988). Also, OmpF allows the passage of solutes such as sugars, ions, and amino acids which are less than 600 daltons, with a weak preference for cationic molecules (Cowan et al., 1992). This suggests that these two porins are crucial for entry into survival mode (Darcan et al., 2003).

In the present study the presence of porin mediated resistance to β-lactam antibiotics in ESBL producing and non-producing strains in multidrug resistant *E. coli* recovered from persons suffering from extra-intestinal infections was examined.

MATERIALS AND METHODS

A total of 80 isolates of *E.coli* from extra-intestinal infections were obtained between July 2008 and September 2008 from hospital of Pune (India). Isolates collected were from the urine, pus and sputum and 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 (Crichton, 1996). Reference strain of *E. coli* 113-D was obtained from National Collection of Industrial Micro-organisms (NCIM), Pune.

Screening for ESBL production was done according to criteria recommended by National Committee for Clinical Laboratory Standards (NCCLS). Two discs, ceftazidime (30 µg) and cefotaxime (30 µg), were used for in vitro sensitivity testing by Kirby-Bauer disc diffusion method (1966). For phenotypic confirmatory testing, 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 incubated overnight at 37 °C for 20 h. An increase in zone of inhibition greater than 5 mm of an antimicrobial agent tested in combination with clavulanic acid vs. its zone size when tested alone, indicated ESBL production (NCCLS, 2002, CLSI 2006).

The sensitivity of the isolates to third generation cephalosporins (3GC) viz., ampicillin (10 µg), co-trimoxazole (30 µg), cefotaxime (30 µg), ciprofloxacin (30 µg), ceftriaxone (30 µg), ceftazidime(30 µg), imipenem (30 µg). (Hi-Media, India) was determined by the disc diffusion method. The results were interpreted as per National Committee for Clinical Laboratory Standards recommendations (NCCLS, 2002, CLSI, 2006).

Konnings and Kaback (1973) procedure was used for preparation of OMP's with few modifications. The OMP profile was analysed by polyacrylamide gel elecrophoresis (30% acrylamide/0.8% bis-acrylamide, 20% SDS). Gels were visualized by staining with coomassie blue (Sigma).

RESULTS AND DISCUSSION

The ESBL phenotypic screening by double disc diffusion synergy test showed that the maximum number of ESBL producing *E. coli* 17 (94.4%) were isolated from urine. Out of 80 clinical isolates, 26 (32.5%) were positive for ESBL by screening test, 18 (22.5%) were positive by confirmatory test for ESBL, whereas all the 54 isolates that were negative by screening were also negative by confirmatory test. These results were consistent with previous studies (Joumana and Araj, 2003; Hong et al., 2004; Sharma et al., 2007) (Table 1).

Antibiotic susceptibility pattern was studied for all isolates of *E. coli*. 100% resistance was observed to ciprofloxacin, co-trimoxazole followed by 58.7% to ampicillin in the isolates. The prevalence of resistance to common antibiotics has also been reported earlier (Wiener et al., 1999; Chitnis et al., 2003; Ajith, 2006). All isolates were sensitive to ceftazidime and imipenem. These results were consistent with the previous studies on drug resistance in *E. coli* (Naoki and Shinzaburo, 1999; Rupal et al., 2007). It has been reported earlier that ESBLs are capable of hydrolyzing broad spectrum cephalosporins and monobactams but inactive against cephamycins and imipenem (Chaudhary and Aggarwal, 2004). In this study, the levels of sensitivities to various antimicrobial drugs commonly used for treatment were compared (Table 2). The isolates showed reduced susceptibility to cefotaxime and ceftriaxone as compared to other cephalosporins. However, cefotaxime was found to be more active than ceftriaxone. In addition, the susceptibility pattern was not similar for ESBL and non-ESBL isolates (Table 2). In another study using *Neisseria meningitidis* , it was found that on the basis of the minimum inhibitory concentrations (MICs), ceftriaxone was found to be more active than cefotaxime (cefotaxime MIC_{90}, 0.007 µg/ml; ceftriaxone MIC_{90}, 0.0015 µg/ml) (Arreaza et.al., 2000).

OMP profile showed the absence of 35 kDa porin protein in ESBL- positive isolate of *E. coli* (ampicillin R, cefotaxime I) (Figure 1) in the presence as well as absence of stress . Loss of porins) contributes to antimicrobial resistance, particularly when additional mechanisms of resistance were expressed. The study shows that the membrane proteins got affected by β-lactam transport

Table 2. Antimicrobial susceptibility pattern of ESBL and non ESBL clinical isolates of *E. coli*.

S/N	Antibiotic	ESBL positive isolates of *E. coli* (n= 18)			Non ESBL isolates of *E. coli* (n= 62)		
		Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
1	Ampicillin	17 (94.4%)	1 (5.5%)	-	30 (48.3%)	32 (51.6%)	-
2	Co-trimoxazole	18 (100%	-	-	62 (100%)	-	-
3	Ciprofloxacin	18 (100%)	-	-	62 (100%)	-	-
4	Ceftriaxone	-	3 (16.4%)	15 (83.3%)	-	30 (48.4%)	32 (51.6%)
5	Cefotaxime	-	1(5.6%)	17 (94.4%)	-	29 (46.8%)	33 (53.2%)
6	Ceftazidime	-	-	18 (100%)	-	-	62 (100%)
7	Imipenem	-	-	18 (100%	-	-	62 (100%)

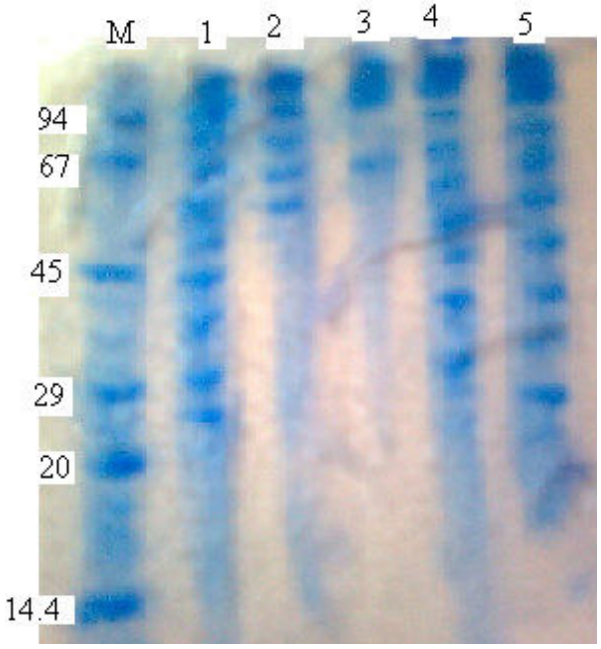

Figure 1. The OMP profile was studied for well characterized strains. Lane M: Marker protein. Lane 1: Reference strain (*E.coli* 113-D). Lane 2: ESBL positive (ampicillin R, cefotaxime I) without cefotaxime stress. Lane 3: ESBL positive (ampicillin R ,cefotaxime I) with cefotaxime stress. Lane 4 : ESBL- negative, (ampicillin I ,cefotaxime S) without cefotaxime stress. Lane 5: ESBL negative (ampicillin I ,cefotaxime S) with cefotaxime stress.

revealing the mediation of direct antibiotic protein interaction. Many studies on the structure and regulation of porins in *E. coli* are available, but there is little information concerning clinical isolates of this species. It has been reported earlier that in *Klebsiella pneumoniae*, two major porins, OmpK35 and OmpK36, were produced, but many extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* isolates do not express OmpK35 (Domenech-Sanchez et al., 1999). Loss of both OmpK35 and OmpK36 in ESBL-producing *K. pneumoniae* causes resistance to cefoxitin, increased resistance to expanded-spectrum cephalosporins, and decreased susceptibility to carbapenems, particularly ertapenem (Perez et al., 2007). Porin loss also decreases the susceptibility to other non-β-lactam compounds, such as fluoroquinolones of ESBL-producing organisms (Jacoby and Han, 1996; Livermore, 1998).

All isolates which showed multidrug resistance, do not show loss of outer membrane proteins (Figure 1, Lane 4 and 5). In non-ESBL isolates (cefotaxime S), the porins were found intact confirming the susceptibility of cefotaxime towards these clinical strains. From the OMP profile, there is a clear indication that the resistance to cefotaxime was due to both porin and ESBL mediated resistance. ESBLs are not active against imipenems, and 94.4% strains expressing ESBLs are susceptible to cefotaxime. However, ESBL producing strains might be resistant to ampicillin and cefotaxime due to the loss of outer membrane porin protein (Figure 1, lane 2 and 3). Loss of porins in *E. coli* was found to augment resistance provided by ESBLs to include resistance to cefotaxime, oxyimino- β-lactams. Reduced permeability of *E. coli* isolates due to porin loss was found to increase the MIC of cefotaxime. This is consistent with the findings of Rasheed et al., (2000) and Laura, (2006).

Recently, cefotaximase (CTX-M) enzymes have been recognized in a number of focal outbreaks from many parts of the world, for instance in Japan (Toho-2) 45, India (CTX-M-15) 46 and UK 47, suggesting their wide dispersal that preferentially hydrolyzes cefotaxime. CTXM- 15 has also been found in Indian *E. coli* and *K. pneumoniae* strains (Medeiros, 1997). Deficiency in expression of an outer membrane protein (OmpK35) was also observed. These observations led us to postulate that the extremely low level of OmpK35 expression and the co-existence of Tumor endothelial marker 1 (TEM-1) and Suid herpes virus1 (SHV-1) resulted in an increased MIC of cefotaxime and the false designation of the isolates as ESBL producers (Tsu-Lan et al., 2001). Alteration of Omps, especially the loss of OmpK35 when OmpK36 is present, can result in an increase in the MIC of cefotaxime to 4 mg/L. Possibly the combination of decreased outer membrane permeability and the hydro-

lytic effect of TEM-1 and SHV-1 ß-lactamases increased the MIC of cefotaxime slightly (Domenech-Sanchez, 1999).

In the ESBL isolates of *E. coli*, there was an elevated level of ampicillin resistance which resulted from a combination of increased enzyme production and OMP changes. The relatively low rates of cefotaxime coresistance provided indirect evidence that 36-kDa OMP loss may play an important role in the expression of cefotaxime resistance in ESBL strains. In *K. pneumoniae,* cefoxitin resistance due to reduced permeability of porins to β-lactam antibiotics has been reported (Ananthan and Subha, 2005). In the present study, the loss of porins in ESBL isolates shows the direct involvement of outer membrane components in developing resistance towards β-lactams.

In conclusion, knowledge of the resistance mechanisms in these clinical isolates will provide data on multidrug resistant pathogens which would be helpful in making recommendations on the best use of antibiotics and to formulate therapeutic strategies to control infections. More prudent use of antibiotics and control of the spread of these resistant organisms are necessary. Understanding the role of bacterial OMPs in transport of β-lactams will facilitate the design of antimicrobial drugs and vaccines. However, the porin-mediated mechanism is important in the virulence and drug-resistance of pathogenic gram-negative bacteria and is of interest as a therapeutic target.

ACKNOWLEDGEMENTS

BA is grateful to Dr. D. R. Majumder, Head of Microbiology Department and Dr E. M Khan, Principal, Abeda Inamdar College, Pune for laboratory assistance and also Col Satish Kumar, AFMC hospital, Pune for providing clinical isolates.

REFERENCES

Ajith K (2006). Multi Drug Resistance: Another Fatal ICU Concern. Pulmon. 8(3): 84-88.

Ananthan S, Subha A (2005). Cefoxitin resistance mediated by loss of a porin in clinical strains of *Klebsiella pneumonia* and *Escherichia coli*. Indian J. Med. Microbiol. 23(1): 20-23.

Arreaza L, Fuente L, Vázquez JA (2000). Antibiotic Susceptibility Patterns of *Neisseria meningitidis* Isolates from patients and asymptomatic carriers. Antimicrob. Agents Chemother. 44(6): 1705-1707.

Basle A, Rummel G, Storici P, Rosenbusch JP, Schirmer T (2006). Crystal structure of osmoporin OmpC from *E. coli* at 2.0Å. J. Mol. Biol. 362(5): 933-42.

Bauer AW, Kirby WM, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Bradford PA (2001). Extended spectrum β-lactamases in 21st century: Characterisation, epidemiology and detection of this important resistance threat. Clin. Microbiol. Rev. 14: 933-951.

Chaudhary U, Aggarwal R (2004). Extended spectrum ß-lactamases-An emerging threat to clinical therapeutics. Indian J. Med. Microbiol. 22(2): 75-80.

Chitnis SV, Chitris V, Sharma N, Chitnis DS (2003). Current status of drug resistance among gram negative bacilli isolated from admitted cases in a tertiary care centre. J. Assoc. Physicians Indian. 51: 8-31.

Clinical and Laboratory Standards Institute (2006). Methods for Dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th ed., M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA: 20-76.

Cowan SW, Schirmer T, Rummel G, Steiert M, Ghosh R, Pauptit RA, Jansonius JN, Rosenbusch JP (1992). Crystal structures explain functional properties of two *E.coli* porins. Nature, 358(6389): 727-33.

Crichton PB (1996). Enterobacteriaceae: *Escherichia, Klebsiella, Proteus* and other genera. In: Collee JG, Fraser AG, Marmion BP & Simmons A (eds), Mackie and McCartney Practical Medical Microbiology, 14 th ed., Churchill Livingston, New York, pp. 361-364.

Darcan C, Ozkanca R, Flint KP (2003). Survival of nonspecific porin-deficient mutants of *Escherichia coli* in black sea water. Lett. Appl. Microbiol. 37(5): 380-385.

Domenech-Sanchez A, Hernandez-Alles S, Martinez-Martinez L, Benedi JC, Arberti S (1999). Identification and characterization of a new porin gene of *Klebsiella pneumonia* in role in ß - lactam antibiotic resistance. J. Bacteriol. 181: 2726-2732.

Hong F, Christina L, Barbro OL, Göran H, Emma L, Åsa R and Struwe J (2004). Molecular epidemiological analysis of *Escherichia coli* isolates producing Extended spectrum ß - lactamases for identification of nosocomial outbreaks in Stockholm, Sweden. J. Clin. Microbiol. 42(12): 5917-5920.

Jacoby GA, Han P (1996). Detection of extended spectrum ß-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. J. Clin. Microbiol. 34: 908-911.

Joumana S, Araj GF (2003). Recent developments in β - lactamases and Extended spectrum β - lactamases. British Medical J. 327(7425): 1209-1213.

Konning WN, Kaback HR (1973). Anaerobic transport in *E.coli* membrane vesicles. Proc. Natl. Sci., 70: 3376-3381.

Lambert PA (1988). Enterobacteriaceae: composition, structure and function of the cell envelope. Soc. Appl. Bacteriol. Symp. 17: 21S-34S.

Laura JVP (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin. Microbiol. Rev. 19(2): 382-402.

Livermore DM (1998). ß-lactamase mediated resistance and opportunities for its control. J. Antimicrob. Chemother. 41: 25- 41.

Martinez-Martinez L, Hernandez-Alberti S, Tomas J, Bebedi V, Jacoby GA (2002). In vivo selection of porin deficient mutant of *Klebsiella pneumonia* with increased resistance of cefoxitin and expanded spectrum cephalosporins. Antimicrob. Agents Chemother. 46: 3679-3682.

Martínez-Martínez l (2008). Extended spectrum ß-lactamases and the permeability barrier. Clinical Microbiology and Infection. 14: s1: 82-89.

Medeiros AA (1997). Evolution and dissemination of ß-lactamases accelerated by generations of ß-lactam antibiotics. Clin. Infect. Dis. 24: S19-45.

Naoki M, Shinzaburo M (1999). Role of permeability in the activities of β-Lactams against gram-negative bacteria which produce a group 3 β-Lactamase. Antimicrob. Agents Chemother. 43: 8-10.

National Committee for Clinical Laboratory Standards (2002). Screening and confirmatory tests for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, and *Escherichia coli*, 5th ed., vol. 22. Document M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA: 27-75.

Neidhart FC, Curtiss R (1996). *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press Washington, D.C: pp. 75-100.

Nikaido H, Rosenberg, Foulds J (1983). Porin channels in *Escherichia coli*: studies with ß-lactams in intact cells. J. Bacteriol. 153(1): 232-240.

Nikaido H (2003). Molecular basis of bacterial outer membrane permeability Revisited. Mol. Biol. Rev. 67(4): 593-656.

Perez F, Endimiani A, Hujer KM, Bonomo RA (2007). The continuing challenge of ESBLs. Curr. Opin. Pharmacol. 7(5): 459-469.

Rasheed JK, Anderson JG, Yigit H, Queenan AM, Donebecg-Sanchez A, Swenson MJ, Biddle WJ, Ferraro JM, Jacoby AG, Tenover CF (2000). Characterisation of the Extended spectrum ß-lactamase reference strain, *Klebsiella pneumoniae* K6 (ATCC 700603) which produces the Novel Enzyme SHV-18. Antimicrob. Agents Chemother. 44: 2382-8.

Rupal MM, Daniel PE, Amy MS, Hector AC, Edward BS, Allesa JE, Kimberty AM (2007). Ertapenem susceptibility of Extended spectrum ß-lactamase producing organisms. Ann. Clin. Microbiol. Antimicrob. 6: 6-19.

Sharma S, Bhat GK, Shenoy S (2007). Virulence factors and drug resistance in *Escherichia coli* isolated from extraintestinal infections. Indian J. Med. Microbiol. 25(4): 369-373.

Tsu-Lan W, Siu LK, Lin-Hui Su, Lauderdale TL, Lin FM, Leu HS, Lin TY, Ho M (2001). Outer membrane protein change combined with co-existing TEM-1 and SHV-1 ß-lactamases lead to false identification of ESBL -producing *Klebsiella pneumoniae*. J. Antimicrob. Chemother. 47: 755-761.

Varadhachary A, Maloney PC (1990). A rapid method for reconstitution of bacterial membrane proteins. Mol. Microbiol. 4(8): 1407-1411.

Viveiros M, Dupont M, Rodrigues L, Couto I, Davin-Regli A (2007). Antibiotic stress, genetic response and altered permeability of *E.coli*. PLoS ONE, 2(4): e365.

Wiener J, Quinn JP, Bradford PA, Georing RV, Nathan C, Bush K (1999). Multiple antibiotic resistant *Klebsiella* and *Escherichia coli* in a nursing home. JAMA. 281: 517-523.

Zhang DF, Jiang B, Xiang ZM, Wang SY (2008). Functional characterisation of altered outer membrane proteins for tetracycline resistance in *Escherichia coli*. Int. J. Antimicrob. Agents NIL. PMID: 18620846.