International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (1), pp. 001-006, January, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

-lactam antibiotic resistance in *Escherichia coli* commensal faecal flora of healthy population in Taif, Saudi Arabia

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Accepted 10 December, 2018

One hundred and twenty faecal sample of commensal *Escherichia coli* strains were collected from different healthy persons and tested for their susceptibility to 12 -lactam antibiotics by disc diffusion and minimum inhibitory concentration methods. Colonization with strains resistant to ampicillin (Amp), amoxicillin, carbenicillin and peperacillin was detected in 36.7% of the tested isolates. Resistance patterns to 3-6 -lactams was observed in 91.7% of the tested *E. coli* isolates. Transfer of Amp resistance marker by conjugation was usually associated with Strepomycin (Stm) and sulfonamise (Sul) in 100% of tested isoletes and with chloramphenicol (Clm) and tetracycline (Tet) in 63.3 and 45.6% of the isolates, respectively. This suggests that resistance markers to Amp, Stm, Sul, Tet and Clm existed as cossets on cojugative plasmids. Therefore, resistance to these antibiotics could rapidly disseminate and persist in the Saudi Society. All Amp resistant isolates produced one or two types of -lactamases with molecular weights of 28.9 and 28.8 KDa, which indicated TEM-1 and SHV-1. Both types of - lactamases are known to be plasmid-mediated in enteric bacterial species and are common in *E. coli* commensal faecal flora. Extended-spectrum -lactamases (ESBLs) were not detected in any of the tested strains and therefore, these types of -lactamases are uncommon in commensal *E. coli* in citizens of Taif. Measures should be taken to prevent the misuse of -lactams and the spread of antibiotic resistance in Saudi Society.

Key words: Antibiotic resistance, -lactams, -lactamases, commensal *Escherichia coli.*

INTRODUCTION

Commensal *Escherichia coli* is an inhabitant of the gastrointestinal tract of humans (Escobar-Páramo et al*.,* 2004). It may act as a reservoir of antibiotic resistance genes for human pathogens (Levy et al*.,* 1988; Shanahan et al*.,* 1994; Smith et al*.,* 2002; Bailey, et al., 2010). The normal *E. coli* flora, may also act as a major source for infections such as infections of urinary tract (Degener et al*.,* 1983; Pithei and Ellis, 1989; Rosen, et al., 2007). Misuse of antibiotics may lead to cumulative exposure of microorganisms to antimicrobial agents over the years and selection of resistant bacteria (Leistevuo et al*.,* 1996; Barza and Travers, 2002). The transmission of resistance genes between commensal bacteria harbored within the gut can occur both horizontally, through the movement of mobile genetic elements and vertically through proliferation and subsequent dissemination of resistant bacterial strains (Hoyle et al*.*, 2005).

-lactam antibiotics are the cornerstone of most of the severe bacterial infections (Holten and Onusko, 2000). They are generally characterized by their favorable safety and tolerability profiles as well as their broad spectrum of activity and hence, are typically used as first-line therapy in different types of infections (Lode, 2008).

The use of -lactam antibiotics could, however, be limited by the spreading of resistant bacteria in the society (Barberán et al., 2008). While many studies have evaluated resistance to -lactams in bacterial pathogens in Saudi Arabia (Kader and Kumar, 2005; Shibl, 1995; Gaillot et al*.,* 1997; El-Khizzi, and Bakheshwain, 2006), resistance in commensal bacterial flora has almost been

overlooked and little information is available. Therefore, in this investigation, resistance of commensal *E. coli* to -lactam antibiotics in some healthy individuals in Taif City was studied.

MATERIALS AND METHODS

Collection of samples

Faecal samples were collected from 120 healthy persons that had not been treated with antibiotics for at least 3 months preceding sampling and who were attending Edwani Hospital in Taif for general check up.

Isolation, identification and susceptibility of *E. coli* reisistant to ampicillin

Samples were diluted in saline and plated onto MacConkey agar plates. Five colonies apparently looking like *E. coli* were identified using API 20E kit (bioMèrieus, France) . One *E. coli* isolate from each faecal sample was further used in this study to evaluate the incidence of resistance to -lactams.

Disk diffusion assay

Disc diffusion method was done onto Muller-Hinton (Oxoid, UK) agar in accordance with National Committee for Clinical Laboratory Standards (NCCLS, 2003). Antibiotic discs used were, Ampicillin (Amp; 10 µg), amoxicillin (Amx; 10 µg), carbenicillin (Car; 100 µg), augmentin (Aug; 20/10 µg), piperacillin (Pip; 75 µg), cephalothin (Cpt, 30 µg), cephalexin (Cpx; 30 µg), cefoxitin (Cfx; 30 µg), ceftazidime (Cfz; 30 µg), ceftriaxone (Cfn; 30 µg), cefotaxime (Cfm; 30 µg), aztreonam (Azt; 30 µg).

Minimum inhibitory concentrations (MICs)

This was done by agar dilution method on Muller- Hinton medium (Oxoid, UK). The following -lactams were used at concentrations ranging between 0.5 - 128 µg/ml: Amp, Amx, Car, Aug, Pip, Cep, Cpx, Cfxi Cfz, Cfn, Cfm, and Azt. Bacterial inoculum of 10^4 CFU/ml was applied by a multipoint inoculator.

-lactamase production

Bacteria were tooth-picked onto Muller- Hinton agar (Oxoid, UK) plates and grown overnight at 37°C. The plates were overlaid with agarose containing toluene, soluble starch and a - lactam substrate, and were left for 10 min, before addition of lougol iodine solution. The plates were then left at room temperature for a few minutes. The appearance of a well defined halo zone indicated production of -lactamase (Abo-Kamar and Shohayeb, 1998). In case of testing inhibitors of -lactamase enzyme, both the inhibitor and -lactam substrate were added in the agarose overlay before the addition of iodine.

Conjugation studies

Commensal *E. coli* donor cells resistant to -lactams and recipient *E. coli* K12 ^{NxR} (UB 5202) cells, were grown to logarithmic phase, Mixed together and plated onto nutrient agar plates. Conjugation was allowed to take place for 48 h at 37^{o}C. The mating cells were subcultured onto nutrient agar plates containing 30 µg nalidixic acid plus tetracycline (25 µg/ml), chloramphenicol (20 µg/ml), streptomycin (50 µg/ml) or sulfamethoxazole (200 µg/ml) (Shohayeb and Sonbol, 1994).

Release of periplasmic –lactamases by osmotic shock

The method of Georgopapadakou and McCaffrey (1994) was used for release of periplasmic contents by osmotic shock. *E. coli* cells were grown overnight and resuspended in 50 M Tris-HCl (pH 7.5) containing 20% sucrose and 1 mM EDTA. The suspension was incubated at room temperature for 10 min, harvested and resuspended in cold deionized water and the mixture was stirred vigorously for 10 min at 4°C. After harvesting the cells the supernatant containing crude –lactamases was subjected to electrophoresis.

Detection of –lactamases by SDS-PAGE

Periplasmic crude extract of –lactamases was subjected to gel electrophoresis according to the method of Laemmli (1970). After electrophoresis the gels were either stained with Coomassie brilliant blue or –lactamases in unstained gels, were renatured by incubation in 1% Triton X100 for 4 h at 37°C. Gels were overlaid with 1% agarose containing 0.2% soluble starch and 1% penicillin in phosphate buffer. Lugol iodine was poured onto the surface of the gel. The appearance of a zone of discoloration indicated the presence of a –lactamase (Massida et al*.,* 1991).

RESULTS

The susceptibility of the tested commensal cells to -lactams was tested by disc diffusion and minimum inhibitory concentration methods. Results of both methods were comparable (Table 1). Resistant to Amp, Amx, Car and Pip was observed in 36.7% of isolates and resistance to Aug was detected in 30.0% of the isolates. Intermediate resistance to Cpt and Cpx was observed in 23.3% of isolates respectively and all isolates, were sensitive to 2nd and 3rd class cephalosporins and aztreonam (Table 1).

The MICs of - lactams are shown in Table 2. Isolates resistant to Amp, Amx, Car and Pip had MICs as high as 128 g/ml or more (Table 2). Both Cpt and CPx had MICs of an intermediate level of 16 g/ml. The Mic for 2nd a, 3rd generation cephalosporins and Azt did not exceed 2 g/ml (Table 2).

A part from four isolates which were resistant to only cephalosporins, all other isolates were resistant to 3 to 6 of the tested -lactams (Table 3). -lactams that were common in all resistance patterns were Amp, Car, Pip (Table 3).

The first generation antibiotics, streptomycin (Stm), tetracycline (Tet), sulfamethoxazole (Sul) and chloramphenicol (Clm) were tested for their co-transfer with Amp in conjugation studies. Amp transfer was usually associated with Stm and Sul in 100% of the

Table 1. Comparison between susceptibility of isolates to -lactams by disc diffusion and minimum inhibitory concentration (MIC) methods.

Antibiotic*	Susceptibility (%)				
	Sensitive		Intermediate	Resistant	
	Disc	MIC	Disc	Disc	MIC
Amp	63.3	63.3	-	36.7	36.7
Amx	63.3	63.3	-	36.7	36.7
Car	63.3	63.3	-	36.7	36.7
Aug	70.0	70.0	-	30.0	-
Pip	63.3	63.3	10	26.7	36.7
Cpt	86.7	86.7	13.3	-	-
Cpx	76.7	76.7	23.3	-	-
Cfx	100	100.	-	-	-
Cfz	100	100	-	-	-
Cfn	100	100	-	-	-
Cfm	100	100	-	-	-
Azt	100	100	-	-	-

*Amp, Ampicillin; Amx, amoxicillin; Car, carbenicillin; Aug, augmentin; Pip, piperacillin; Cpt, cephalothin ; Cpx, cephalexin; Cfx, cefoxitin; Cfz, ceftazidime; Cfn, ceftriaxone; Cfm, cefotamixme; Azt, aztreonam.

Table 2. Minimum inhibitory concentration of twelve different -lactams against *E. coli* isolates.

Antibiotic	Number of isolates for which the minimum inhibitory concentration was (g/ml)									
	0.5	1	2	4	8	16	32	64	128	>128
Amp	12	28	36	-	-	-	-	-	16	28
Amx	8	4	16	32	16	-	-	-	8	36
Car	16	28	32	-	-	-	-	-	16	28
Aug	24	12	20		28	36	-	-	-	-
Pip	-	8	8	52	8	-	-	-	36	8
Cpt	32	52	20	-	-	16	-	-	-	-
Cpx	40	44	8	-	-	28	-	-	-	-
Cfx	76	40	4	-	-	-	-	-	-	-
Cfz	72	48	-	-	-	-	-	-	-	-
Cfn	60	52	8	-	-	-	-	-	-	-
Cfm	64	56	-	-	-	-	-	-	-	-
Azt	72	48	-	-	-	-	-	-	-	-

Table 3. Patterns of resistance to the tested -lactams.

No. of -lactams	Pattern	No of isolates
2	Cpt, Cpx	4
3	Amp, Car, Pip	8
4	Amp, Car, Pip, Aug	12
4	Amp, Car, Pip, Cpx,	12
6	Amp, Car, Pip, Aug, Cpt, Cpx	12

tested isolates (Table 4). Clm and Tet were transferable at frequencies of 63.6 and 54.6%, respectively (Table 4).

Isolates were tested for their ability to produce -lactamases by iodometric method. All isolates resistant to Amp produced -lactamases (Figure 1) . The differences in the diameter of discolouration zone around colonies (Figure 1) suggest variation in the amount of enzyme produced.

Table 4. Incidence of transfer of resistance determinants of first generation antibiotics with ampicillin by conjugation.

Antibiotic	No of strains	% of cotransfer
Streptomycin	44	100
Tetracycline	24	54.5
Sulfamethoxazole	44	100
Chloramphenicol	28	63.6

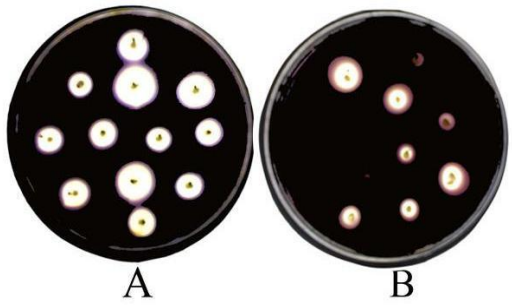

Figure 1. -lactamase production of strains resistant to -lactams by plate iodometric method. Amp and Cpx were used as substrates in plates A and B respectively.

Agar plate iodometric method was used to test the ability of –lactamases to hydrolyse Penicillin (Pen), Amp, cloxacillin (Clx) and Cpx as subtrates (Table 5). Both Pen and Amp were equally susceptible for hydrolysis by –lactamases of all the tested isolates except for 4 isolate which did not hydrolyse Amp. None of the isolates was able to hydrolyse cloxacillin (Table 5).

-lctamases were released from *E. coli* isolates by osmotic shock and subjected to electrophoreis in order to identify their molecular weights. As shown in Figure 2, two types of -lactamases were detected. The two -lactamase had molecular weights of 28.9 and 28.8 KDa.

DISCUSSION

-lactam antibiotics are a broad class of antibiotics commonly used in treatment of infections. In this study the susceptibility of commensal *E. coli* strains was tested to different classes of -lactams (Holten and Onusko, 2000) which included penicillins (Amp, Amx, Pip) , carbapenems (Car), 1st generation cephalosporins (Cpt and Cpx), 2nd generation cephalosporins (Cfx), third generation cephalosporins (Cfm, Cfn , Cfz, Cfn), monobactams (Azt) and a - lactamase inhibitor (clavulanic acid) which was used in a mixture with amoxicillin (Aug).

Colonization with Amp-resistant commensal fecal flora was found in 40% of *E. coli* commensal isolates which could cause a rapid disseminate of antibiotic resistance in the society. Resistance patterns to 3- 6 -lactams were observed in 91.7% of the tested *E. coli* isolates that were resistant to Amp.

The relationship between maintenance of resistance within a population and the use of selective antibiotic is complex (Summers, 2002). Resistant bacteria persist for a long time after the selecting agent has been withdrawn (Enne et al*.,* 2001). One explanation is that resistance markers to antibiotics may be carried on cassettes, and therefore simply one antibiotic would contribute to maintain resistance to others. Conjugation studies revealed that ampicillin resistance determinants transfer was 100% associated with transfer of resistance to streptomycin and sulfamethoxazole and more than 50% in the case of chloramphenicol and tetracycline. Therefore, it seems that conjugative plasmids detected in this study carry cassettes of genes for resistance to ampicillin together with non- -lactam antimicrobials.

There are several mechanisms of resistance to -lactams. The rate of antibiotic entry into the cell is a detrimental variable for susceptibility to -lactams (Bush et al*.,* 1985; Vu and Nikaido, 1985; Nitzan, et al., 2002). -Lactams penetrate the outer membrane of gram-negative bacteria through specific porin channels (Nikaido et al*.,* 1983; Yoshimura and Nikaido, 1985; Nitzan et al., 2002). Mutations that prevent production of the outer membrane porin proteins OmpC and OmpF causes - lactam resistance (Harder et al*.,* 1981, Jaffe et al*.,* 1983; Zhanel, et al., 1995). Modification in penicillin-binding proteins to forms that do not bind to -lactams is a second mechanism of resistance. The most frequent mechanism of resistance is the production of -lactamase
- enzymes that hydrolyze the amide bond at the -lactam ring (Mason and Kietzmann, 1999; Hujer et al*.,* 2009). Large amounts of -lactamase in the periplasmic space either bind or slowly hydrolyze the antibiotic before it can reach its targets (Then and Angehrn, 1982; Vu and Nikaido, 1985; Marre and Aleksi , 1990; Lai, 2009).

In this study, all Amp resistant isolates produced one or two types of -lactamases. Molecular weights of the detected -lactamses indicated that they were TEM-1 and SHV-1. Both types of -lactamses were produced individually or together by the tested isolates. In the past, SHV-1 -lactamase was most commonly found in *Klebsiella pneumoniae*, however it predominates now in both *E. coli* and *K. pneumoniae* (Shaokat et al*.,* 1987;

Table 5. Susceptibility -lactams to hydrolyse by -lactamases produced by different resistant isolates and their inhibition by clavulanic acid and EDTA as determined by iod0metric mtethod.

Isolates (%)	Hydrolysis of				Inhibition by	
	Pen	Amp	Clx	Ceph*	Clv**	EDTA
50	+	+	-	-	±	-
41.6	+	+	-	+	±	-
8.4	+	-	-	+	±	-

*Digestion required longer time; ** partial hydrolysis.

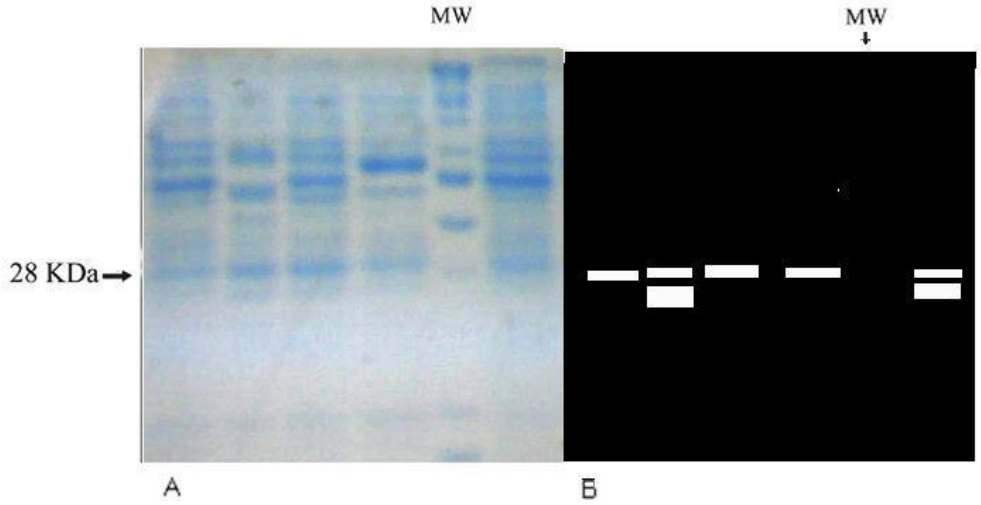

Figure 2. Electrophoresis of crude extracts of periplasmic space proteins of isolates by SDS-PAGE, followed by gel renaturation and localization of -lactamases by iodometric technique. MW, position of molecular weight markers; A, SDS-PAGE; B, tracing of bands which appeared after iodometric technique in gel A.

Hujer et al*.,* 2009). SHV- 1 shares 68% of its amino acids with TEM-1 and has a similar overall structure. Both types of - lactamses are known to be plasmid-mediated in enteric bacterial species (Jacoby, 2000), which explains the ability of -lactams resistant isolates to transfer their resistance determinants to *E. coli* K12 recipient cells.

Strains of *Enterobacteriaceae* resistant to extended spectrum -lactams (ESBLs) have become a concern in medical bacteriology as regards both antimicrobial treatment and infection control. ESBLs has disseminated globally and have been reported in Asia and Europe. ESBLs have been detected even among fecal *E. coli* isolates in non-hospitalized children and adults (Ho et al*.,* 2008). Resistance is usually due to the production of extended spectrum -lactamases (Drieux et al., 2008; Ho et al*.,* 2008). ESBLs were not detected in *E. coli* commensals in this study. This indicates that resistance of commensals to ESBLs, at the present time, is not a serious problem. However it should be mentioned that ESBLs have been detected in pathogenic enteric bacteria isolated from different types of infections in the Kingdom of Saudi Arabia (Kader and Kumar, 2005; Shibl, 1995; El-Khizzi and Bakheshwain, 2006).

In summary, this study demonstrates that - lactamases of types TEM-1 and SHV- 1 are common in *E. coli* commensal fecal flora in the citizens of Taif and ESBLs are still not common. Measures should be taken to make sure that resistance to ESBLs would not become a problem in the Saudi society in the future.

ACKNOWLEDGMENTS

The author gratefully thanks God and asks him to accept this work, and acknowledges the support and helpful advices of Prof. Mohammed Shohayeb and Taif University for providing all required facilities.

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