PCR detection of plasmid mediated TEM, SHV and AmpC β-lactamases in community and nosocomial urinary isolates of *Escherichia coli*

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Abstract

Fifty clinical isolates of Escherichia coli from two groups of subjects (hospitalized and outpatients) were studied for their susceptibility to twelve β-lactam antibiotics. All isolates were resistant to ampicillin, amoxicillin, oxacillin and cefradine. Carbenicillin resistance was found in all but one outpatient isolate. Resistance to cephalothin, cephalexin and cefazoline ranged from 76% to 96% among the test bacteria. On the other hand, the majority of the isolates were sensitive to cefoxitime, ceftizoxime and ceftriaxone (84-100%). β-lactamases from all bacteria were inhibited by clavulanic acid and none harbored extended spectrum β-lactamases as shown by the double disc diffusion method. Gene amplification by polymerase chain reaction (PCR) using specific primers for AmpC, TEM and SHV enzymes showed that 92% of all organisms carried bla_{TEM} alone, or along with SHV or ampC genes. Presence of all three genes was shown in 8% of the hospitalized patients and 20% of the outpatients. Conjugative transfer of β-lactam resistance markers into susceptible host recipients occurred for 72% of the isolates in each group. Overall, the antibiotic susceptibility profile, the distribution of the β-lactamase gene type and the rate of conjugative transfer of the resistance markers were similar in both subject groups.

Keywords: *Escherichia coli; TEM; SHV; AmpC; β-lactamases and PCR Typing.*

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INTRODUCTION

Escherichia coli is one of the main causes of urinary tract infections both in the community and nosocomial infections. The widespread use of β–lactam antibiotics in humans and veterinary medicine is thought to be associated with the selection of antibiotic resistance in pathogenic and nonpathogenic isolates of *E. coli* (Livermore, 1998). Resistance to β–lactam antibiotics is mostly mediated by β–lactamases which inactivate the antibiotic by hydrolyzing the β–lactam ring. Over two hundred β-lactamases are recognized which are classified into four main groups and eight subgroups according to their functional and structural characteristics (Bush and Jacoby, 1997; Bush *et al*., 1995). Many Gram-negative bacteria have been shown to possess naturally occurring chromosomally mediated β-lactamases (Bradford, 2001). The first plasmid mediated β–lactamase (TEM-1) was described in *E. coli* in 1960 and within a few years it was found in many different genera of Gram-negative bacteria (Bradford, 2001; Medeiros, 1984). Up to 90% of ampicillin resistance in *E. coli* is due to TEM production (Livermore, 1995). Another common plasmid mediated β-lactamase in *E. coli* is SHV-type enzymes (Bradford, 2001). With the use of numerous new β-lactam antibiotics over the last twenty years, extended spectrum β-lactamases (ESBLs) have emerged which are mostly derivatives of TEM-1 and SHV-1 and are capable of hydrolyzing a wide range of β-lactams including the most recently developed cephalosporins (Bradford, 2001; Bush *et*

al., 1995; Coudron *et al*., 1997; Tenover *et al*., 1999; Tzouvelekis *et al*., 2000). The AmpC family of β-lactamases occur both chromosomally and plasmid-mediated in *E. coli* and plasmid encoded AmpC β-lactamases are found to be responsible for global outbreaks (Ben Redjeb *et al*., 1988; Coudron *et al*., 2000; Philippon *et al*., 2002). Rapid detection and discrimination between diffrent β-lactamases are important not only to study the epidemiology but also choosing the correct antibiotics for treatment of these infections. The objective of this study was to detect and compare plasmid mediated β-lactam resistance in the urinary isolates of *E. coli* from the community and nosocomial infections. Fifty urinary isolates from hospitalized and outpatients were studied for their susceptibility to β-lactam antibiotics including the third generation cephalosporins. Transfer of plasmid mediated β-lactam resistance to susceptible bacterial hosts was carried out by conjugation and molecular identification of the β-lactamase types TEM, SHV and AmpC was carried out by PCR using type specific primers.

MATERIALS AND METHODS

Bacterial strains: Fifty clinical isolates of *E. coli* were randomly selected from a collection of urinary isolates of *Enterobacteriaceae* from the bacteriology laboratory of Vali-e-Asr hospital (Hosseini-Mazinani *et al*., 2003). Twenty five isolates were from hospitalized patients and twenty five were obtained from outpatients at the same time. The organisms were grown on nutrient agar plates and biochemical identifications were carried out by the API 20E system (Biomerieux, France). *Escherichia coli* strains used for conjugation studies were, *E. coli* K12 (nal^r), *E. coli* HB101 (Sm^r) and *E. coli* XL1Blue (Tet^r). *E. coli* ATCC 25922 was used as a control for antibiotic susceptibility tests. *E. coli* MK148 carrying the ampC gene and *E. coli* harboring pTEM-1 (Hosseini-Mazinani *et al*., 1996) and finally *K. pneumoniae* 57-1 (SHV gene, donated by Dr. Radu, Malaysia) were used as positive controls for DNA amplification by PCR with ampC, TEM and SHV specific primers respectively.

Antibiotic susceptibility testing: The antibiotic susceptibility of bacteria was initially carried out by the disc diffusion method using Mueller Hinton agar plates according to the NCCLS recommendations (NCCLS, 2000a). The antibiotics tested were ampicillin, amoxicillin, carbenicillin, cefalexin, cephalothin, cefazoline, cephradin, oxacillin, ceftazidime, ceftriaxone, ceftizoxime (Padtan Teb, Tehran) and coamoxyclave (Difco). Minimum inhibitory concentrations (MICs) were determined by the microdilution broth method using the NCCLS standard procedure (NCCLS, 2000b). The antibiotics used were ampicillin (Jaber-ibne Hayan Co., Tehran), ceftazidime (Glaxo Ltd), cefotaxime and ceftriaxone (Lorestan Co., Tehran), cefepime (Bristol-Myers, USA) and imipenem (Sigma, USA).

Screening for ESBL producing strains: The double disc synergy test was used to screen for ESBL producing strains (Bradford, 2001; Livermore and Brown, 2001). Cefotaxime (30 μg), ceftriaxone (30 μg) and ceftizoxime (30 μg) were placed on Mueller Hinton agar plates adjacent to an amoxicillin-clavulanic acid disc (20 μg of amoxicillin and 10 μg of clavulanic acid). The ESBL production is inferred when the cephalosporin inhibition zone is expanded by the clavulanate.

Conjugation experiments: Bacterial matings were carried out using overnight grown donor (clinical) and recipient strains in Lauria Bertani (LB) broth at 37°C followed by adding 1 ml of each pair into 2 ml of LB and reincubation for 6-24 hrs at 37°C (Winokur *et al*., 2001). Samples were taken at two hr intervals up to 6 hrs and at 24 hrs and were plated on LB agar plates containing the appropriate antibiotics. Ampicillin with nalidixic acid, streptomycin or tetracycline (each used at 100 mg/l) were used for selection of the transconjugants. Presence of β-lactamases in the clinical isolates as well as the transconjugants was shown by the iodometric paper strip method (Livermore and Brown, 2001).

PCR amplification of bla_{TEM}, SHV and ampC: DNA extraction was carried out using a rapid alkaline lysis method (Winokur *et al*., 2001). The oligonuclotide primers used for the PCR assays were; 5´-ATAAAATTCTTGAAGACGAAA3´ and 5´-GTCAGTTACCAATGCTTAATC-3´ for TEM (Sutcliffe 1978), 5´-TGGTTATGCGTTATATTCGCC-3´ and 5´-GGTTAGCGTTGCCAGTGCT-3´ for SHV (Kim *et al*., 1998) and finally 5´-ATGCAACAACGACAATCCATC-3´ and 5´-GTTGGGGTAGTTGCGATTGG-3´ for AmpC type β-lactamases (Bret *et al*., 1998). TEM and SHV primers were synthesized at the National Center for Genetic Engineering and Biotechnology (Iran) and ampC primer was synthesized at Faza Pajooh Company (Tehran, Iran). Reactions were carried out in a Techne DNA thermocycler in 25 μl mixtures containing Taq DNA polymerase (Fermentes, Sinagen) in 1 x buffer consisting of 10 mM Tris-HCl (pH 8.3), 1.5 mM

$MgCl_2$, each deoxyribonucleoside triphosphate at a concentration of 200 μM and each oligonucleotide primer at a concentration of 2 μM. Following a 4 min incubation time at 94, thirty five cycles were performed for each reaction with the following temperature profile for each cycle: 94°C for 1 min, the proper annealing temperature for each primer (58°C for TEM, 52°C for SHV and 59°C for ampC) for 1 min and 72°C for 1 min. An additional 5-10 min incubation time was carried out at 72°C.

RESULTS

Susceptibility test results: Tables 1 and 2 show the susceptibility results for in and outpatients respectively. Using the disc diffusion method, all clinical isolates were resistant to ampicillin, amoxicillin, oxacillin and cephradine. Carbenicillin resistance was found in all isolates except one from an outpatient. Among the outpatients, 76% of the isolates were resistant to cephalothin and cefalexin and 80% were resistant to cefazoline. Of the bacteria isolated from the inpatients, 84% were resistant to cephalothin and cefalexin and 96% were resistant to cefazoline. On the other hand, the majority of the clinical isolates were susceptible to the third generation β-lactamases. Among the outpatients isolates, 100%, 84% and 88% were sensitive to cefoxitime, ceftriaxone and ceftazidime respectively. Among the inpatient isolates, 92% were susceptible to all three antibiotics. The use of amoxiclave discs rendered 20% of the amoxicillin resistant isolates from both patients groups susceptible (zone of inhibition > 18 mm). Sixty percent of the outpatient isolates and 80% of the inpatients clinical isolates became intermediately susceptible to coamoxiclave (zones of inhibition 14-17 mm). The double disc test results for extended spectrum β-lactamases showed that none of the fifty isolates carried ESBLs. The MIC determination for ampicillin showed that all isolates from both patients groups were resistant to >2000 μg/ml of ampicillin. For the third generation cephalosporins, the NCCLS guideline suggests that MIC of 8 μg/ml or below is considered susceptible. We found that among the inpatients isolates, 2 (8%) were resistant to ceftazidime, cefotaxime and ceftriaxon (MIC values of >256, 256 and 256 μg/ml respectively) and one outpatient isolate was resistant to ceftazidime, cefotaxime and ceftriaxone (MICs of 64, 128 and 256 μg/ml). All bacterial isolates were susceptible to imipenem (data not shown). Resistance to cefepime was only observed for the two inpatient isolates which were also resistant to the third generation cephalosporins (MICs of 64 and 128 μg/ml).

Conjugative transfer of β–lactam resistance: Transfer of β-lactams resistance occurred equally in *E. coli* isolates from both patients groups. Overall, plasmid transfer by conjugation took place in 72% of the isolates from each group. The best recipient was *E. coli* K12 where 56% of the outpatients and 40% of the inpatients isolates were able to transfer their β-lactamase mediated resistance into this host. *E. coli* HB101 also received β-lactamase mediated resistance plasmids by conjugation from 24 % and 16% of the in and out patients respectively. Only 8% of the isolates from inpatients and none of the strains from outpatients were able to transfer their resistance markers to *E. coli* XL1Blue. The transconjugants had the same antibiotic susceptibility patterns as the donors as well as the antibiotic markers of the recipient cells and produced β-lactamase as shown by the iodometric test. Conjugation did not occur between 20% of the inpatient and 24% of the outpatient isolates and any of the recipient hosts. Conjugation was not detectable in 8% of the inpatient and 4% of the outpatient isolates due to the resistance of these organisms to antibiotic markers present in recipient hosts.

PCR amplification of TEM, SHV and ampC genes: The PCR products for bla_{TEM}, SHV and ampC genes were 850, 800 and 1100 base pairs respectively. As shown in tables 1 and 2, 23/25 (92%) of the *E. coli* isolates from both groups of patients carried bla_{TEM} either alone, with SHV or ampC. Fifty two percent of the inpatients and 40% of the outpatient isolates carried the bla_{TEM} alone. SHV alone was only found in 8% of the inpatient and none of the outpatient isolates and ampC was found in 4% of the outpatient and none of the inpatient isolates. TEM and SHV together were present in 16% of the inpatient and 24% of the outpatient clinical isolates. The combination of TEM and ampC were found in 16% of the inpatient and 12% of the outpatients isolates and none of the clinical isolates carried the SHV/ampC combination. Finally, 8% of the isolates from inpatients and 20% of the outpatient isolates carried all three genes.

DISCUSSION

Resistance to β-lactam antibiotics among the urinary isolates of *Enterobacteriaceae* has posed a major problem for eradication of these common infections. To date, most of the studies have concentrated on the isolates obtained from nosocomial infections and very few have made any comparison between the isolates from the community infections and the hospitalized

Table1. Antibacterial susceptibility, PCR amplification of bla_{TEM}, SHV and ampC, and conjugative transfer of β-lactamase gene profiles of the urinary clinical isolates from hospitalized patients.

	Inhibition zone (mm)								MICs (µg/ml)			PCR gene	Conjugative
E. coli isolate no.	AMC	CF	CN	CZ	CD	CT	CRO	CAZ	CTX	CRO	CAZ	amplification	transfer into
I1	15	13	12	10	9	13	12	13	256	256	>256	TEM/SHV/AmpC	*E. coli*XL1Blue
I2	18	15	18	15	14	35	29	22	4	2	0.5	TEM	-
I5	14	13	13	11	7	13	12	13	256	256	>256	TEM/SHV/AmpC	ND
I6	16	17	15	12	10	33	23	18	0.125	0.25	0.25	TEM	-
I9	18	15	14	14	10	29	23	21	4	2	0.25	TEM	*E. coli* K12
I31	15	13	14	11	7	29	25	21	0.125	0.125	0.25	TEM/SHV	*E. coli* XL1Blue
I32	17	18	16	13	7	32	28	23	4	2	<0.125	TEM	-
I41	15	14	12	11	8	28	23	22	4	2	0.25	SHV	*E. coli* K12
I42	16	14	12	12	8	30	30	26	2	2	0.25	TEM/SHV	"
I47	14	14	9	12	9	30	30	22	0.125	2	0.25	TEM/AmpC	*E. coli* HB101
I61	15.5	19	18	14	10	33	22	18	0.125	0.125	0.5	TEM	-
I65	20	23	18	23	12	33	30	22	0.25	2	<0.125	TEM/SHV	*E. coli* K12
I67	14	16	12	14	8	32	23	26	0.25	2	0.5	TEM/SHV	*E. coli* HB101
I69	14	12	10	11	10	29	26	23	0.25	2	0.5	TEM	*E. coli* K12
I74	14	15	13	14	9	29	27	22	0.5	2	0.5	TEM	"
I81	17	12	11	10	11	33	26	21	0.25	2	0.5	TEM/AmpC	"
I84	17	12	12	12	10	30	27	23	0.125	0.125	0.125	TEM/AmpC	"
I86	16	15	11	13	10	30	33	29	"	"	"	TEM	ND
I89	16	15	14	12	11	30	24	22	2	2	0.5	SHV	*E. coli* HB101
I92	18	16	15	15	14	31	24	21	0.125	0.125	0.125	TEM	-
I98	15.5	12.5	15	12	10	29	28	26	<0.125	<0.125	0.25	TEM	*E. coli* HB101
I101	15	15	16	16	11	30	30	26	"	"	<0.125	TEM	*E. coli* K12
I105	17	13	12	12	12	30	25	24	"	0.25	0.5	TEM/AmpC	*E. coli* HB101
I110	16	18	18	16	14	35	30	27	"	"	<0.125	TEM	"
I112	18	14	15	13	13	31	30	26	2	1	2	TEM	*E. coli* K12

AMC, amoxiclav, CF, cephalothin, CN, cefalexin, CZ, cefazoline, CD, cefradin, CT, ceftizoxime, CTX, cefotaxime, CRO, ceftriaxon, CAZ, ceftazidime, and ND, not-Determined·

Table 2. Antibacterial susceptibility, PCR amplification of bla_{TEM-1}, SHV and ampC, and conjugative transfer of β-lactamase gene profiles among the urinary clinical isolates from outpatients.

	Inhibition zone (mm)								MICs (μg/ml)			PCR gene	Conjugative
E. coli isolate no.	AMC	CF	CN	CZ	CD	CT	CRO	CAZ	CTX	CRO	CAZ	amplification	transfer into
O1	17	17	19	16	9	30	32	24	<0.125	0.5	2	TEM	-
O3	15	15	8	14	7	30	26	24	<0.125	1	< 0.125	TEM/AmpC	*E. coli* K12
O6	11.5	10	11	7	7	31	30	24	2	1	2	TEM	*E. coli* HB101
O10	16	12	13	13.5	9	31	26	23	<0.125	1	2	TEM/SHV	-
O11	14	14	15	13	7	30	27	24	2	1	2	TEM/SHV	-
O12	16	14	16	13	10	31	31	21	2	2	< 0.125	TEM	*E. coli* K12
O13	15	16	17	15	10	31	28	20	1	0.5	<0.125	TEM	-
O14	18	18	14	18	7	28	19	17	8	8	8	TEM/AmpC	ND
O17	15	15	20	13	9	30	26	20	4	2	<0.125	TEM/SHV	*E. coli* K12
O18	12	21	20	17	11	30	27	24	2	2	<0.125	TEM/SHV	*E. coli* HB101
O23	15	14	13.5	12	9	29	25	21	<0.125	1	2	TEM	
O24	20	18.5	19.5	19	12	29	25	20	2	1	2	TEM/SHV	*E. coli* K12
O25	22	23	22	22	11	29	27	22	2	1	<0.125	TEM/SHV	*E. coli* K12
O26	12	23	23	22	7	30	20	20	2	2	2	TEM	-
O27	16	14	14	10	7	32	25	24	2	2	<0.125	TEM/SHV/Amp	*E. coli* K12
O28	9	8.5	8	7	7	20	12	13	128	256	64	TEM/SHV/Amp	-
O29	16	15	16	13	7	28	26	21	2	0.25	2	TEM	*E. coli* HB101
O30	17	13	13	13	12	29	25	22	2	<0.125	<0.125	TEM/Amp	*E. coli* K12
O31	16	14	12	10	7	30	20	21	8	8	8	TEM/SHV/Amp	*E. coli* K12
O32	21	21	20	20	12	32	30	22	1	<0.125	2	TEM	*E. coli* HB101
O33	13.5	15	15	15	7	32	26	24	1	2	2	TEM/SHV	*E. coli* K12
O34	14	8	10	14	13	32	27	22	0.5	1	1	AmpC	*E. coli* K12
O35	16	9	16	17	17	40	33	21	1	1	2	TEM	*E. coli* K12
O37	15	8	13	15	15	30	34	22	1	2	1	TEM/SHV/Amp	*E. coli* HB101
O39	19	8	17	14	16	35	28	28	1	1	1	TEM	-

AMC, amoxiclav, CF, cephalothin, CN, cefalexin, CZ, cefazolin, CD, cphradine, CT, ceftizoxime, CTX, cefotaxime, CRO, ceftriaxone, CAZ, ceftazidime, and ND, not-Determined·

subjects. In the current study, fifty clinical isolates of *E. coli* were studied from two groups of subjects, 25 from hospitalized patients and 25 from patients who attended the outpatient clinic at the same time. Majority of the isolates from both groups were resistant to the first generation cephalosporins and susceptibility to amoxicillin was enhanced by clavulanic acid. These results along with the fact that all organisms were highly resistant to ampicillin indicate the presence of penicillinases in all our isolates. In fact, the substrate hydrolysis profiles of β-lactamases from the isolates showed that all organisms hydrolyzed penicillin better than cephaloridine (over 30%) using a UV spectrophotometric assay (data not shown). Only 2 inpatient isolates and one isolate from an outpatient showed resistance to the third generation cephalosporins. The NCCLS guidelines suggest that MIC breakpoint of > 2 μg/ml for some third generation cephalosporins could mean that the organism is a potential ESBL producer (NCCLS, 2000c). We used a double disc method to identify the ESBL producers and found none among our isolates. Investigators have shown that loss or reduction of outer membrane proteins in clinical isolates may lead to an increase in MIC to ceftazidime (Wu *et al*., 2001). Overproduction of the ampC β-lactamase is also known to cause false negative results for ESBL producers (Bradford, 2001). Numerous surveys on β-lactamase mediated resistance among the family *Enterobacteriaceae* around the world have shown that the rate of resistance has been increasing to an alarming degree and the prominent β-lactamases are TEM and SHV types and their derivatives (Bradford, 2001; Heritage *et al*., 1999; Medeiros, 1984; Sanders and Sanders, 1992). More recently, AmpC type β-lactamase which was originally found in *Klebsiella spp.* has been also detected in *E. coli* and some other genera of *Enterobacteriaceae* (Coudron *et al*., 2000 and Livermore, 1995). Many methods have been employed for typing β-lactamases, among which PCR has become the method of choice almost universally. This method has the advantage of showing the presence of multiple resistance genes in bacteria. The PCR amplification results of our study showed that the majority of the isolates carried bla_{TEM} either alone or along with SHV or ampC genes. The two isolates from the inpatients and one outpatient isolate which were highly resistant to the third generation cephalosporins carried all three β-lactamase genes. Simultaneous presence of more than one β-lactamase gene has been previously reported (Sanders and Sanders, 1992; Spanu, *et al*., 2002; Tenover, *et al*., 2003; Thomson et al., 1999). Another important point of this study was that the conjugative transfer of β-lactamases occurred in 72% of all clinical isolates. Since this was found equally in both subject groups, the importance of transmission of resistance genes to nonpathogenic strains is stressed upon in the community. Rapid transfer of β-lactamase markers have been shown to occur between different Gram negative rods such as *Salmonella spp., Klebsiell, spp., Escherichia coli* (both pathogenic and nonpathogenic), *Enterobacter aerogenes, Citrobacter ferundii* and *Serratia spp.*(Brun-Buisson *et al*., 1987; Jarlier *et al*., 1988; Siu *et al*., 1997; Winokur *et al*., 2001). Transfer of the resistance genes from the highly resistant organisms into susceptible nonpathogenic strains of *E. coli* or other susceptible hosts may play an important role in the ecology of resistance and clinical infectious diseases.

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