Prevalence of β-lactams resistance among *Escherichia coli* clinical isolates from a hospital in Algiers

Y. Messai¹, T. Benhassine¹, M. Naim², G. Paul³ and R. Bakour¹

¹Laboratoire de Biologie Cellulaire et Moléculaire, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediène, Algiers; ²Laboratoire de Microbiologie, Hôpital Central de l’Armée, Algiers, Algeria; and ³Laboratoire de Microbiologie, UFR de Médecine Cochin–Port-Royal, Paris, France

**SUMMARY**

A high prevalence of β-lactams resistance among Enterobacteriaceae have been reported worldwide; however, there are not sufficient data on this issue in Algeria. β-Lactams susceptibility of 203 *Escherichia coli* clinical isolates was determined by agar diffusion method, and production of extended-spectrum β-lactamases (ESBL) was screened by double-disk synergy test. This analysis showed five well-defined phenotypes: 1) 62 isolates (30.5%) were susceptible to all β-lactams; 2) 135 isolates (66.5%) presented a broad-spectrum β-lactamases phenotype (BSBL); 3) three isolates (1.5%) were defined as producing β-lactamase resistance (BLEE) and their corresponding transconjugants. Conjugation assay showed transfer of β-lactamases and AmpC resistance phenotypes and their corresponding β-lactamases to recipient *E. coli* BM21 in association with plasmids of 71.4 kb for the AmpC isolates and from 40–56 kb for penicillinase isolates. This result showed that the AmpC phenotype is plasmid mediated. ESBL isolates were found not to transfer their resistance through conjugation experiment. Polymerase chain reaction (PCR) experiments using primers specific to *bla*<sub>TEM</sub>, *bla*<sub>AmpC</sub> and *bla*<sub>CTX-M</sub> genes showed specific amplification with *bla*<sub>TEM</sub> primer for two ESBL isolates; *bla*<sub>CTX-M</sub> for the remaining ESBL isolate; and *bla*<sub>AmpC</sub> for the remaining AmpC isolate.

**Key words**: β-lactams - Resistance - *Escherichia coli*

---

**RESUMEN**

La prevalencia de la resistencia a los betalactámicos entre las enterobacterias es alta en todo el mundo, pero en Argelia no se dispone de suficientes datos. Se determinó la sensibilidad a los betalactámicos de 203 cepas clínicas de *Escherichia coli* mediante difusión en agar y se analizó la producción de betalactameras de espectro extendido (BLEE) mediante la técnica de la sinergia del doble disco. Este análisis mostró cinco fenotipos bien definidos: 1) 62 cepas (30,5%) fueron sensibles a todos los betalactámicos; 2) 135 cepas (66,5%) presentaron un fenotipo caracterizado por betalactameras de amplio espectro (BLEA); 3) 3 cepas (1,5%) se definieron como productoras de BLEE; 4) 2 cepas (1%) fueron productoras de betalactameras de tipo AmpC; y 5) una (0,5%) presentó un fenotipo de disminución de la permeabilidad celular a los betalactámicos. La determinación del punto isoeléctrico mostró betalactameras con puntos isoeléctricos de 5,4 o 7,6 para las cepas productoras de cefalosporinasa AmpC y sus cepas transconjugantes correspondientes. El ensayo de conjugación mostró una transferencia de los fenotipos de resistencia de penicilinas y cefalosporinasa AmpC y correspondientes betalactameras a *E. coli* BM21 en asociación con plasmidos de 71,4 kb para las cepas productoras de cefalosporinasa AmpC y de 40-56 kb para las productoras de penicilinas. Este resultado mostró que el fenotipo productor de cefalosporinasa AmpC está mediado por plasmidos. Las cepas productoras de BLEE no transfirieron su resistencia mediante el ensayo de conjugación. La reacción en cadena de la polimerasa utilizando primers específicos para los genes *bla*<sub>TEM</sub>, *bla*<sub>AmpC</sub> y *bla*<sub>CTX-M</sub> mostró una amplificación específica con el primer para *bla*<sub>TEM</sub> en dos cepas productoras de BLEE; los primers para *bla*<sub>TEM</sub> y *bla*<sub>AmpC</sub> para la tercera cepa productora de BLEE; y los primers para *bla*<sub>CTX-M</sub> y *bla*<sub>AmpC</sub> para las cepas productoras de cefalosporinasa AmpC y sus cepas transconjugantes correspondientes. El estudio mostró una alta tasa de cepas productoras de penicilinas y una baja frecuencia de fenotipos productores de AmpC y BLEE. Las betalactameras productoras de cefalosporinasa AmpC estaban mediadas por plasmidos y las BLEE pertenecieron al tipo CTM-M.

**Palabras clave**: Betalactámicos - Resistencia - *Escherichia coli*
INTRODUCTION

Among Gram-negative bacteria, *Escherichia coli* is the most frequently isolated in hospitals and the most common cause of infections (1). This species is naturally susceptible to β-lactams despite its intrinsic chromosomal cephalosporinase, which is not produced in amounts high enough to reach clinical resistance. The extensive or inappropriate use of new β-lactams led to the emergence of new resistant phenotypes related mainly by amplification or mutation of old β-lactamases genes. This acquired resistance is mainly due to:

- Overproduction of AmpC-type cephalosporinases (chromosomal or plasmid encoded), which are characterized by their hydrolysis of extended-spectrum cephalosporins and cephemycins and resistance to clavulanic acid inhibition (2-4).
- Plasmid-mediated β-lactamases: 1) broad-spectrum β-lactamases (BSBLs) such as TEM-1 or TEM-2 and SHV-1, which can be hyperproduced and thereby confer resistance to aminopenicillins, carboxypenicillins, β-lactam--β-lactamase inhibitor combinations and to first- and second-generation cephalosporins; 2) extended-spectrum β-lactamases (ESBLs), which are clavulanate-susceptible enzymes capable of hydrolyzing oxyimino-cephalosporins and monobactams but not cephemycins and carbapenems. ESBLs have emerged as an important mechanism of resistance to β-lactams and create serious therapeutic problems. They have evolved mainly from the parental class A β-lactamases, TEM-1 or TEM-2, and SHV-1 by various amino acid substitutions around active site (5). Reports described the emergence of ESBLs in *E. coli* belonging to other families, such as PER, VEB, TLA-1 and IBC (ceftazidimase), and CTX-M (ceftaximases) derivatives (6-10). The CTX-M β-lactamases are rapidly increasing worldwide (10, 11); 3) inhibitor-resistant TEM β-lactamases (IRT) deriving from TEM-1 and TEM-2 by mutations which result in a loss of susceptibility to inhibition by β-lactamase inhibitors and thereby a resistance to β-lactam--β-lactamase inhibitor combinations such as amoxicillin– ticarcillin–clavulanic acid (12-14).

With worldwide reports on increasing resistance to β-lactams in members of *Enterobacteriaceae*, in particular in *E. coli*, and very few resistance data published in Algeria, the aim of this study was to determine the in vitro susceptibility to 15 β-lactams of 203 *E. coli* isolates recovered from a university hospital in Algiers.

MATERIALS AND METHODS

Bacterial strains

A total of 203 nonrepetitive *E. coli* clinical isolates were isolated at Microbiology Laboratory of Central Hospital of Army in Algiers from various pathological specimens. The majority of isolates (n = 176) were recovered from urine samples. The isolates were identified by the API 20E system (bioMérieux, France).

Antibiotic susceptibility testing

Antibiograms were done on Mueller Hinton agar plates with the disk-diffusion method according to standard procedures (15). Susceptibility patterns were interpreted according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (16), Livermore et al., (17) and Vedel (18). The following β-lactam disks (Sanofi Diagnostics Pasteur) were tested: amoxicillin (25 µg), amoxicillin–clavulanic acid (20/10 µg), ticarcillin (75 µg), ticarcillin–clavulanic acid (75/10 µg), mezlocillin (75 µg), mecillinam (10 µg), imipenem (10 µg), cefalotin (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), latamoxef (30 µg) and aztreonam (30 µg).

*E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing.

ESBL production was screened for using the double-disk synergy test (DDST) as a standard disk-diffusion assay on Mueller-Hinton agar. Disks containing aztreonam, ceftazidime, ceftriaxone and cefotaxime were placed at a distance of 30 mm (center to center) around a disk containing amoxicillin plus clavulanic acid. Enhancement of the inhibition zone toward the amoxicillin–clavulanic acid disk, indicating a synergy between clavulanic acid and any one of the test antibiotics, was taken as presumptive evidence of ESBL production (19).

The β-lactamase production was detected by iodometric technique in the presence of benzylpenicillin as substrate (15).

Conjugation experiments

Mating experiments were performed as previously described (20), with *E. coli* BM21 (nalidixic acid resistant) as a recipient (20). Exponential cultures of clinical isolates as donor (1 vol) and recipient (2 vol) were inoculated as a spot on Brain Heart Infusion Agar (BHIA). After overnight incubation at 37 °C, the bacteria were resuspended, diluted and plated onto BHIA containing relevant selective agents.
at the following concentrations: nalidixic acid (100 µg/ml) and amoxicillin (64 µg/ml). Samples from donor and recipient were used as control. Transconjugants growing in the selection plates were subjected to antibiotics susceptibility, DDST, isoelectric focusing and polymerase chain reaction (PCR) analysis.

**Plasmid analysis**

Plasmid DNA was extracted by the procedure described previously and analyzed by electrophoresis on 0.7% (wt/vol) agarose gels for four hours at 5 volts/cm (21). Plasmid size was estimated by using reference plasmids: RP4 (60 kb), pRK2013 (48 kb) and pBR322 (4.36 kb).

**Isoelectric focusing (IEF) assay**

β-Lactamases were characterized by isoelectric focusing of ultrasonicated bacterial extracts. Bacteria growing exponentially at 37 ºC in nutrient broth (Difco) were harvested, and cell-free lysates were prepared by sonication. β-Lactamases were analyzed by isoelectric focusing of cell extracts on polyacrylamide gels containing ampholytes with a pH range of 3.5–9.5 (Pharmacia) (22). The focused extracts on polyacrylamide gels containing ampholytes were as: initial denaturation at 94 ºC for 5 minutes followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 42 ºC (TEM), 60 ºC (CTX-M) and 65 ºC (AmpC) for 30 seconds, and elongation at 72 ºC for 30 seconds. The final elongation step was extended to 10 minutes at 72 ºC. The PCR products were separated on 1.5% agarose gels. Bands were visualized under ultraviolet light after being stained with ethidium bromide and photographed. Expected sizes of TEM, AmpC and CTX-M PCR products were, respectively, 850 bp, 510 bp and 550 bp.

**RESULTS**

The activities of 15 β-lactams against 203 *E. coli* isolates are shown in Table I. Sixty two (30.5%) of these isolates were susceptible to all β-lactams, and 100% of isolates were susceptible to latamoxef and imipenem, which were the two most effective drugs. The highest resistance rates were found for amoxicillin and ticarcillin (69.5% and 69%, respectively). Intermediate resistance rates were obtained for mezlocillin (18.7%), mecillinam (18.7%) and ceftalotin (10.8%). The lowest resistance rates were observed for cefuroxim (4.9%), amoxicillin–clavulanate (2%), ticarcillin–clavulanate (4.4%), cefoxitin (1.5%), cefotaxime (1.5%) and ceftriaxone (1.5%). None of the isolates was fully resistant to ceftazidime and aztreonam.

The plasmid profiles analysis have shown a relationship between carriage of plasmids and antimicrobial resistance; the number and the size of plasmids in antibiotic-resistant isolates (number and size plasmid range: 1–7 and 4.2–74.2 kb) appeared to be higher than in susceptible isolates (number and size plasmid range: 0–2 and 3.1–8.5 kb) (Fig. 1). With the exception of one isolate, β-lactamase production was detected in all resistant isolates; those were distributed into 17 different resistance patterns, which can be classified into four groups (Table II): 1) 135 isolates presented a resistance to penicillins and, in some cases, to amoxicillin–clavulanate and to narrow-spectrum cephalosporins (cefalotin and cefuroxime) with a negative DDST; 2) three isolates have shown a resistance to broad-spectrum cephalosporins (cefotaxime, ceftriaxone) with a positive DDST; 3) two isolates were resistant to cefotaxin and amoxicillin–clavulanate, intermediate to cefotaxime, ceftriaxone and ceftazidime, and negative for the DDST; 4) one isolate was resistant to amoxicillin, amoxicillin–clavulanate and cefoxitin, and negative for the DDST. According to Livermore et al., (17), Vedel (18) and Livermore (4), these phenotype groups are specific features, respectively, to BSBLs, ESBLs, AmpC cephalosporinase and decreased cell permeability to β-lactams.

Isoelectric focusing revealed β-lactamases with isoelectric points of 5.4 or 7.6 for isolates with BSBL phenotype; −9.0 for two ESBL isolates; 5.4, 7.6 and −9.0 for the remaining ESBL isolate; and 5.4 and −9.0 for the AmpC iso-
lates. The cefotaxime hydrolysis corresponds to the basic bands with an isoelectric point of $\sim$9.

Mating assays allowed the transfer of penicillinase and AmpC resistance phenotypes and their corresponding $\beta$-lactamases to recipient *E. coli* BM21, in association with plasmids of 71 kb for the AmpC isolates and 40–56 kb for penicillinase isolates. This result showed that the AmpC phenotype is plasmid mediated. The ESBL isolates were found not to transfer their resistance through conjugation experiment. Amplification by PCR using primers specific to *bla*$_{TEM}$, *bla*$_{AmpC}$ and *bla*$_{CTX-M}$ genes showed positive results for *bla*$_{CTX-M}$ for two ESBL isolates, *bla*$_{TEM}$ and

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>Breakpoint* (zone in mm)</th>
<th>Phenotype n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>$\geq 21$</td>
<td>$&lt; 14$</td>
</tr>
<tr>
<td>Amoxicillin–clavulanate</td>
<td>$\geq 21$</td>
<td>$&lt; 14$</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>$\geq 22$</td>
<td>$&lt; 18$</td>
</tr>
<tr>
<td>Ticarcillin–clavulanate</td>
<td>$\geq 22$</td>
<td>$&lt; 18$</td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>$\geq 21$</td>
<td>$&lt; 16$</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>$\geq 22$</td>
<td>$&lt; 18$</td>
</tr>
<tr>
<td>Cefalotin</td>
<td>$\geq 18$</td>
<td>$&lt; 12$</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>$\geq 22$</td>
<td>$&lt; 15$</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>$\geq 22$</td>
<td>$&lt; 15$</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>$\geq 21$</td>
<td>$&lt; 15$</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>$\geq 21$</td>
<td>$&lt; 15$</td>
</tr>
<tr>
<td>Latamoxef</td>
<td>$\geq 23$</td>
<td>$&lt; 17$</td>
</tr>
<tr>
<td>Imipenem</td>
<td>$\geq 22$</td>
<td>$&lt; 17$</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>$\geq 23$</td>
<td>$&lt; 17$</td>
</tr>
<tr>
<td></td>
<td>201 (99.0)</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

S: susceptible; R: resistant; I: intermediate.

*: Breakpoints and susceptibility were interpreted according to CA-SFM guidelines (16).

| Figure 1. Plasmid content of $\beta$-lactams–resistant and –susceptible *E. coli* isolates analyzed by agarose gels (0.7%) electrophoresis (for four hours at 5 volts/cm). At left: $\beta$-Lactams–resistant *E. coli* isolates: broad-spectrum $\beta$-lactamases (lanes 1–10), AmpC cephalosporinase (lanes 11–12), extended spectrum $\beta$-lactamases (lanes 13–15). At right: $\beta$-Lactams–susceptible *E. coli* isolates (lanes 1–10). M1 (pBR322, 4.36 kb), M2 (pRK 2013, 48 kb) and M3 (RP4, 60 Kb): plasmid size references.
DISCUSSION

Our results showed high rates of resistance in *E. coli* clinical isolates to amoxicillin (69.5%) and ticarcillin (69.0%); these rates are in agreement with those reported in Tunisia (24), while they are much higher than those given in France (25, 26). This high rate of resistance is probably due to the extensive use of penicillin–aminoside combinations in Algerian hospitals and the current empirical antibiotic therapy. The combination of amoxicillin and ticarcillin with clavulanic acid significantly restored their activity, the rates of resistance fell to 2% (amoxicillin–clavulanate) and 4.4% (ticarcillin–clavulanate); these figures are lower than those observed in studies carried out in Tunisia and Turkey (24, 27) and are consistent with those reported in France (about 5%) and in the United States (4.7%) (28, 29).

The first- and second-generation cephalosporins were more active than penicillins, while the third-generation cephalosporins were most powerful, with resistance rate of 1.5%. This rate is much lower than those reported in many countries, such as Korea and Turkey, where the *E. coli* cefotaxime resistance rates were 8.5% and 25%, respectively (30, 31); however, in studies conducted in the United States and Europe, the rates of cefotaxime resistance were in agreement with our results (32-34). This low resistance rate in Algeria is probably due to the fact that these new cephalosporins are not available in the community and not used extensively in hospital practice.

The resistant isolates harbor plasmids whose number and size are higher than those of plasmids of susceptible isolates; this shows that the resistance was acquired by genetic exchanges between bacteria in hospital environments and patients under antibiotics selective pressure.
The mechanism of resistance was mainly the production of β-lactamases, which was detected in all resistant isolates. One hundred and eighteen isolates were resistant to amoxicillin and ticarcillin, and susceptible or resistant to meccillinam and mezlocillin; these phenotypes indicate penicillinase production. In cefotaxin and amoxicillin–clavulanate-resistant isolates (n = 17), the resistance mechanism is a enhanced penicillinase production (35). Classic TEM (isoelectric point: 5.4) and SHV (isoelectric point: 7.6) β-lactamases were found in these isolates. These enzyme types were commonly observed in Klebsiella spp. and E. coli resistant to amoxicillin–clavulanate and to first- and second-generation cephalosporins (26, 36, 37).

ESBLs were produced in three isolates (1.5%), which had a resistance to cefotaxime and ceftriaxone, a susceptibility to amoxicillin–clavulanate and ticarcillin–clavulanate, a strong isolectric focusing band at isoelectric point ∼9.0, and a positive DDST. The prevalence of ESBLs varied among countries. Our isolates harbor ESBLs less frequently than in Turkey (27.7%), India (18%), Russia (15.8%), Cameroon (14.3%) and Korea (9.3%) (31, 38–41). In contrast, in agreement with our data, Canadian, European, Lebanese, Tunisian and U.S. investigations revealed that overall prevalences of ESBLs among E. coli isolates were 0.26%, 1.3%, 2%, 2.7% and 3.3%, respectively (42-44). ESBL producers observed in our study were found carrying the ESBL genes for CTX-M–type β-lactamases. As described previously, in contrast with the TEM- and SHV-type ESBLs, the CTX-M enzymes are much more active against cepotaxime and ceftriaxone than against cefazidime. During the past decade CTX-M–type ESBLs have emerged and increased worldwide in proportion to the other ESBL types, in single and epidemic clinical isolates (10, 11).

The detection of the bla_{CTX-M} genes in Algerian Salmonella enterica serotype Seftenberg isolates supports our results (46). The three CTX-M–producing isolates were from urine; E. coli CTX-M producers were described as the most common cause of urinary tract infections (47).

The ESBL phenotype may not have been transferable because these β-lactamases may be carried on small non-transferable or large transfer-deficient plasmids. A small plasmid (7 kb), without genes required for conjugation, harboring bla_{CTX-M} was described (48), and ceftriaxone and cefotaxime resistance with ESBL production mediated by a small plasmid of 3.2 kb was reported in S. enterica serotype typhimurium (49). However, the non-self-transmissible plasmids can be mobilized (50). In addition, different elements, like insertion sequences and integrons, were also involved in the mobilization of bla_{CTX-M} genes (51, 52).

Three isolates (1.5%) were resistant to cefoxitin. Two isolates (1%), which had a transferable cefoxitin, amoxicillin–clavulanate and ticarcillin–clavulanate resistance, as well as strong isolectric focusing bands at isoelectric point ∼9.0, were plasmid-encoded AmpC producers. Plasmid-mediated AmpC β-lactamases were detected in many countries, but in contrast to the findings for ESBLs, they are still rare; recent reports from the United States, Canada and China showed that, respectively, only 4%, 0.085% and 2% of E. coli strains contained this enzyme type (53-55). The plasmid-mediated AmpC β-lactamases are a significant cause of concern because their mobility allows them an easy diffusion within the E. coli species and possibly to other species and genera. In addition, the widespread use of the amoxicillin–clavulanate combination can contribute to the selection of AmpC–β-lactamase-producing strains.

The cefoxitin resistance (with susceptibility for extended-spectrum β-lactams) of one isolate (0.5%) could be due to an alteration in the cell permeability to cefoxitin, which is associated with a low expression of a 36 kDa outer membrane protein (56). However, the level of decreased susceptibility to extended-spectrum β-lactams is low (57).

The present study revealed high rates of penicillinase-producing E. coli isolates with resistance to penicillins and to first- and second-generation cephalosporins. The AmpC- and ESBL-resistant phenotypes were found at low frequencies, resulting from plasmidic AmpC or decreasing cell permeability and from non-transferable bla_{CTX-M} genes, respectively. The most active drugs were imipenem, lataxoxef, aztreonam and cefazidime.

ACKNOWLEDGEMENTS

We thank Liliane Gilly for technical assistance. This work is supported in part by grants from Institut National de la Santé et de la Recherche Médicale (INSERM, France) and Agence Nationale de la Recherche en Santé (ANDRS, Algeria).

Correspondence: Rabah Bakour, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediène, BP 32 El-Alia, Bab-Ezzouar 16111, Algiers, Algeria. Tel.: 213 21 24 79 13; Fax: 213 21 24 72; E-mail: rbakour@yahoo.fr

REFERENCES


