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First characterization of CTX-M-15 and DHA-1 β-lactamases among clinical isolates of *Klebsiella pneumoniae* in Laghouat Hospital, Algeria

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Extended-spectrum β-lactamases (ESBL) are a problem of great concern owing to the potential transmission of resistance to others bacterial species. Our study aims to investigate the ESBL and plasmid-mediated β-lactamases produced by *Klebsiella pneumoniae* strains isolated from 2010 to 2012, among patients hospitalized in "Ahemida Ben Adjila" hospital, Laghouat, Algeria, and to seek a possible clonal dissemination. Antimicrobial susceptibility testing was performed by the agar diffusion method, the characterization of resistance genes to β-lactam antibiotics was performed by PCR amplification and gene sequencing, and molecular typing was performed by ERIC-PCR. In total, of 112 clinical strains of *K. pneumoniae* isolated, nine isolates produced an ESBL. Antibiotics susceptibility testing showed a complete resistance to the majority of third-generation cephalosporins and a very frequent resistance to aminoglycosides and fluoroquinolones resistance. PCR analysis and sequencing showed that all isolates produced ESBL CTX-M-15; three strains of them also produced the cephalosporinase DHA-1. Molecular typing showed that most strains were not related; only three strains that produced CTX-M-15 and DHA-1 had identical profiles suggesting a clonality link. This study revealed the dissemination of ESBL CTX-M-15 and plasmid-mediated AmpC cephalosporinase DHA-1 in *K. pneumoniae*. This is a first report of these enzymes at the Laghouat hospital.

Key words: *Klebsiella pneumoniae*, resistance, CTX-M-15, DHA-1, genotyping, Algeria.

INTRODUCTION

During the past 30 years, extended-spectrum β-lactamases (ESBL) diffused in most Enterobacteriaceae species, especially in *Klebsiella pneumoniae.* Among ESBL CTX-M enzymes, are a major problem because

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Table 1. Sequences of the primers used to detect bla- genes.

Target	Primer	Sequence	Annealing temperatures (°C)	Reference
CTX-M-1	CTXM1 A2 CTXM1 B2	5' - CTT CCA GAA TAA GGA ATC - 3' 5' - CCG TTT CCG CTA TTA CAA - 3'	48	De Champs et al. (2004)
DHA-1	AmpR-AmpcF AmpCR	5' - GGTAAAACTGAGATGACGGGC - 3' 5' - TTATTCCAGCGCACTCAAAAT - 3'	56	Hennequin et al. (2012)
ERIC–PCR	ERIC2	5' - AAG TAA GTGACT GGG GTG AGC G - 3'	64	Dumarche et al. (2002)

they have been involved in many nosocomial outbreaks and are associated with increased mortality (Edelstein et al., 2003; Lebessi et al., 2002; Podschun and Ullmann, 1998). CTX-M β-lactamases enzymes belong to class A. They preferentially hydrolyze cefotaxime more than ceftazidime (Bonnet, 2004). Currently, the CTX-M group 1, particularly CTX-M-15, appear to be more widespread and prevalent in many countries in the world (Carrer and Nordmann, 2011; Lavigne et al., 2007), while other ESBLs of the TEM and SHV subgroups seem to decrease. However, resistance to third generation cephalosporins in *K. pneumoniae* is no longer exclusively due to class A beta-lactamases. The acquisition of AmpC cephalosporinases is associated with a high level of cephalosporin resistance (Hanson, 2003). The earliest report of AmpC beta-lactamases was in 1990, with the identification of MIR-1 beta-lactamase (Papanicolaou et al., 1990). These enzymes are classified in Bush's Group 1 (Ambler's class C). They are generally highly expressed and confer resistance to most β-lactams, except for carbapenems (Philippon et al., 2002). The main objectives of this study were to determine the frequency of resistance to 3rd generation cephalosporins of *K. pneumoniae* strains isolated from patients hospitalized in Laghouat hospital, Algeria, to characterize the β-lactamases involved and to highlight the genetic diversity of these strains.

MATERIALS AND METHODS

Bacterial strains

This is a retrospective study over a period of three years from 1 January 2010 to 31 December 2012. It focused on 112 nonrepetitive *Klebsiella pneumoniae* strains isolated from patients hospitalized in "Ahemida Ben Adjila" hospital, Laghouat, Algeria. All patients hospitalized for more than 48 hours were included in the study. The samples correspond to different colonization sites (urine, catheters, rectal swabs and pus), and essentially isolated from services: Women Medicine; General Surgery, Orthopedics, Pulmonology and Intensive Care Unit. Some epidemiological data such as age, gender and origin of service were recorded in the first time for each patient. Species' biochemical identification was performed using the API 20E® identification system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility and synergy testing

The antibiotics susceptibilities were determined on Mueller–Hinton agar by the standard disk diffusion procedure as described by the Antibiogram Committee of the French Society for Microbiology (CASFM, 2010). The following antibiotics were tested: Amoxicillin (25 µg), amoxicillin/clavulanic acid (30 µg), ticarcillin (75 µg), ticarcilline/clavulanic acid (85 µg), piperacillin (75 µg), piperacillin + tazobactam (85 µg), cephalotin (30 µg), cefuroxime (30 µg), cefixime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefpirome (30 µg), imipenem (10 µg), aztreonam (30 µg), cefoxitin (30 µg), gentamicin (15 µg), tobramycin (10 µg), amikacin (30 µg), nalidixic acid (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), kanamycin (30 µg), fosfomycin (50 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulfonamide (200 µg), netilmicin (30 µg), trimethoprim (5 µg), sulphamethoxazole/trimethoprim (25 µg), colistin (50 µg), and ceftazidime/clavulanic acid (30/10 µg). The antibiotic disks were obtained from Oxoid, England.

All isolates were phenotypically screened for the production of ESBLs using the double-disk synergy test (DDST) (Jarlier et al., 1988). *Escherichia coli* ATCC 25922 was used as a control strain.

Analytical isoelectric focusing (IEF)

IEF was performed to determine the isoelectric point of the different β-lactamases of each strain as previously described by Bonnet and coll (Bonnet et al., 2000). β-lactamases with known pI were used as standards: CTX-M-1 (pI 8.4), CTX-M-14 (pI 7.9), CTX-M-15 (pI 8.6).

β-Lactamase characterization

The DNA extraction was conducted by preparing a suspension of the strains to be studied in 200µl of distilled water. After boiling for 10 min of suspension and centrifugation for 7 min at 13, 000 × g, and the supernatant was collected in a new 1.5 ml Eppendorf tube and stored at -20°C.

The detection of genes encoding the β-lactamase ESBL and plasmid-mediated AmpC cephalosporinases (DHA-1) was performed by Polymerase Chain Reaction (PCR), the operating conditions and primers used were described in Table 1. The PCR reactions mixture consists of: 5 µl of each primer (10 picomoles/µl), 1 µl of dNTP, 10µl of PCR reaction buffer 5X and 0.25 µl of Taq DNA polymerase (Promega). The DNA was amplified in a final volume of 50 µl, in a thermocycler: either Biometra T Personal (Labgene) or the Primus 96 plus (Biotech). The PCR products were separated in 1% agarose gels. When PCR was positive, the amplicon was purified and sequenced in GATC Biotech AG (European Custom Sequencing Centre, Gottfried-Hagen-Straße 20, 51105 Köln), to identify precisely the desired β-lactamases.

Table 2. Characteristics of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates.

Isolate	Wards	Date of isolation (day/month/year)	Sample origin	Sex	Age (years)	β-lactamase pI	β-lactamase gene
Kp3	Orthopedics	13/02/2010	Pus	F	32	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp9	Orthopedics	19/07/2010	Rectal	F	85	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp18	Women Medicine	04/03/2012	Rectal	F	70	5.4 + 7.5 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1
Kp20	Pulmonology	12/03/2011	Urine	H	60	5.4 + 7.7 + 8.6	CTX-M15
Kp31	General Surgery	22/05/2011	Rectal	F	27	5.4 + 7.7 + 8.6	CTX-M15
Kp51	Intensive care unit	04/09/2011	Rectal	F	49	5.4 + 7.7 + 8.6	CTX-M15
Kp55.2	Women Medicine	25/04/2012	Pus	F	29	5.4 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1
Kp57	Intensive care unit	07/08/2012	catheters	H	30	5.4 + 7.5 + 7.7 + 8.6	CTX-M15
Kp73	Intensive care unit	29/11/2012	Rectal	F	39	5.4 + 7.5 + 7.7 + 7.8 + 8.6	CTX-M15, DHA-1

Conjugation transfer experiments

Conjugation experience was performed with *E. coli* C600 Rif R (resistant to rifampicin) as the recipient strain. The strains were inoculated into a brain-heart infusion broth (BHIB) and incubated overnight at 37 C°. The transconjugants were selected on Mueller-Hinton agar containing rifampicin (300 µg/L) and cefotaxime (1 µg/L). After, the transconjugants pushed into the selection boxes were tested vis-à-vis the antibiotics to detect the presence of resistance phenotype towards 3rd generation cephalosporins and cefoxitin.

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The total DNA of each strain was tested by ERIC-PCR (Repetitive enterobacterial intergenic consensus). For ERIC-PCR reaction, DNA was amplified using the only one primer ERIC-2: 5' AAGTAAGTGACTGGGGTGAGCG 3' (Table 1), with the following program: 5 min at 94°C and 1 min at 36°C, then 36 cycles of 3 min at 72°C, 1 min at 94°C, and 1min/30 s to 36°C, and ending with 10 min at 72°C.

The preparation conditions of ERIC-PCR mix were as follows: distilled H_2O: 28.4 µL, dNTP (Eurogentec): 3.2 µl, $MgCl_2$: 3 µl, 5X Buffer: 5 µl, eric2 Primer: 5 µl, Go taq (Promega): 0.4 µl. After, 5µl mixed was added to the DNA crude extract diluted to 1/100 th with sterile distilled water. Fingerprints were visually compared and the patterns differing by at least one amplification band were classified as different.

RESULTS

During the period of our study, 215 patients were included and 112 strains of *Klebsiella pneumoniae* were isolated. The double synergy test was positive for nine strains of *K. pneumoniae,* suggesting the probable production of ESBL. These nine strains produced extended-spectrum β-lactamases (ESBLKp) (frequency of 8%). The majority of patients were women (78%) and their ages ranged from 27 to 85 years. Some hospital services appeared more concerned with the problem of resistance due to ESBL production, including the intensive care unit of which 33% of cases were noticed, followed by the orthopedics and women medicine service with 22% for each (Table 2).

The nine isolates tested were resistant to extended-spectrum cephalosporins, amoxicillin, ticarcillin, amoxicillin/clavulanic acid and ticarcilline/clavulanic acid. Only 33% of the strains were susceptible to piperacillin-tazobactam. In addition, three strains resistant to cefoxitin. For these three strains induction was observed between imipenem and third generation cephalosporins, suggesting the production of an inducible céphalo-sporinase. All strains remained susceptible to imipenem (Figure 1).

The ESBL phenotype is associated with resistance to aminoglycosides: kanamycin (100%), tobramycin (100%), gentamicin (78%), and amikacin (55%); sulfonamides (100%), and fluoroquinolones (89%). All strains were susceptible to colistin and fosfomycine. The determination of the isoelectric point (pI) showed that the nine strains produced β-lactamases of different pI: 5.4 (compatible with a TEM-1-type penicillinase), 7.5 (compatible with a OXA-1-type oxacillinase), 7.7 (compatible with the chromosomal penicillinase SHV-1) and 8.6 (compatible with a CTX-M-type ESBL). In addition to these β-lactamases, the three strains Kp18, Kp55.2 and kp73 also produced one β-lactamase of a pI: 7.8 (Figure 2).

Molecular characterization of β-lactamases by polymerase chain reaction (PCR) and sequencing revealed that the nine strains of *K. pneumoniae* producing ESBL from hospitalized patients in different departments in the hospital carried the same gene $bla_{\text{CTX-M-15}}$. Moreover, we noticed the presence of plasmid-mediated AmpC cephalosporinase by $bla_{\text{DHA-1}}$ gene in three strains Kp18, Kp55.2 and kp73.

Following the conjugation, antimicrobial susceptibility of the nine transconjugants showed resistance towards 3rd generation cephalosporins with the presence of synergy,

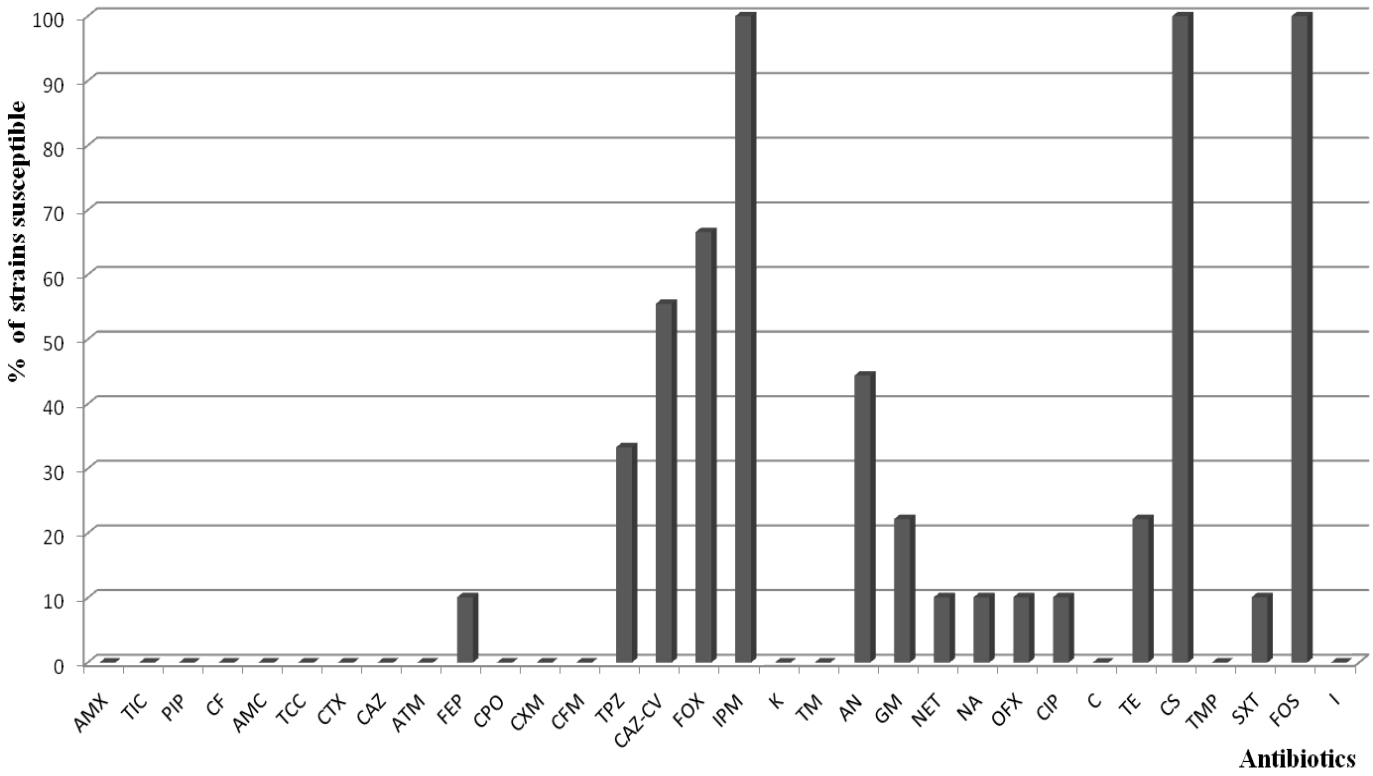

Figure 1. Susceptibility to antimicrobials of ESBL-producing *Klebsiella pneumoniae* isolates. AMX: amoxicillin, TIC: ticarcillin, PIP: piperacillin, CF: cefalotin, AMC: amoxicillin/clavulanic acid, TCC: ticarcilline/clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, ATM: aztreonam, FEP: cefepime, CPO: cefpirome, CXM: cefuroxime, CFM: Cefixime, TPZ: piperacillin + tazobactam, CAZ-CV : ceftazidime/clavulanic acid, FOX: cefoxitin, IPM: imipenem, K: kanamycin, TM: tobramycin, AN : amikacin, GM: gentamycin, NET: netilmicin, NA: nalidixic acid, OFX: ofloxacin, CIP: ciprofloxacin, C: chloramphenicol, TE: tetracycline, CS: colistin, TMP: trimethoprime, SXT: sulphamethoxazole/trimethoprim, FOS: fosfomycin, I: sulfonamide.

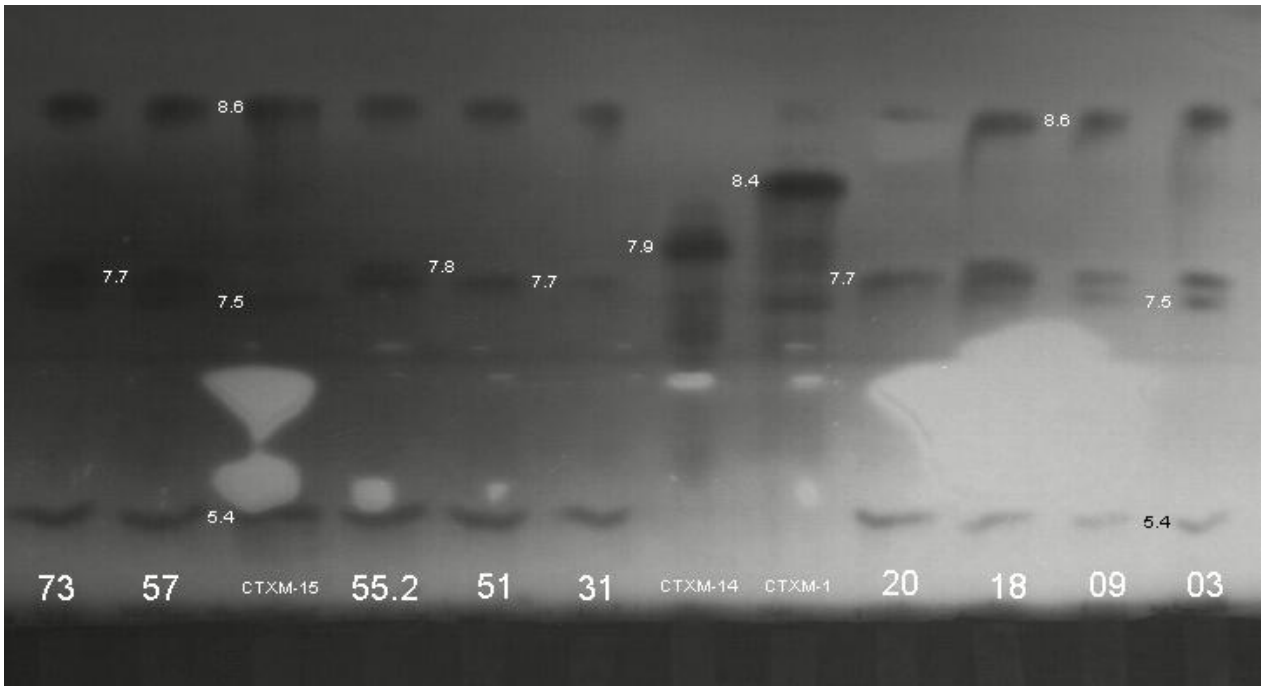

Figure 2. The isoelectric point pI of the extended-spectrum β-lactamase-producing *Klebsiella pneumoniae.*

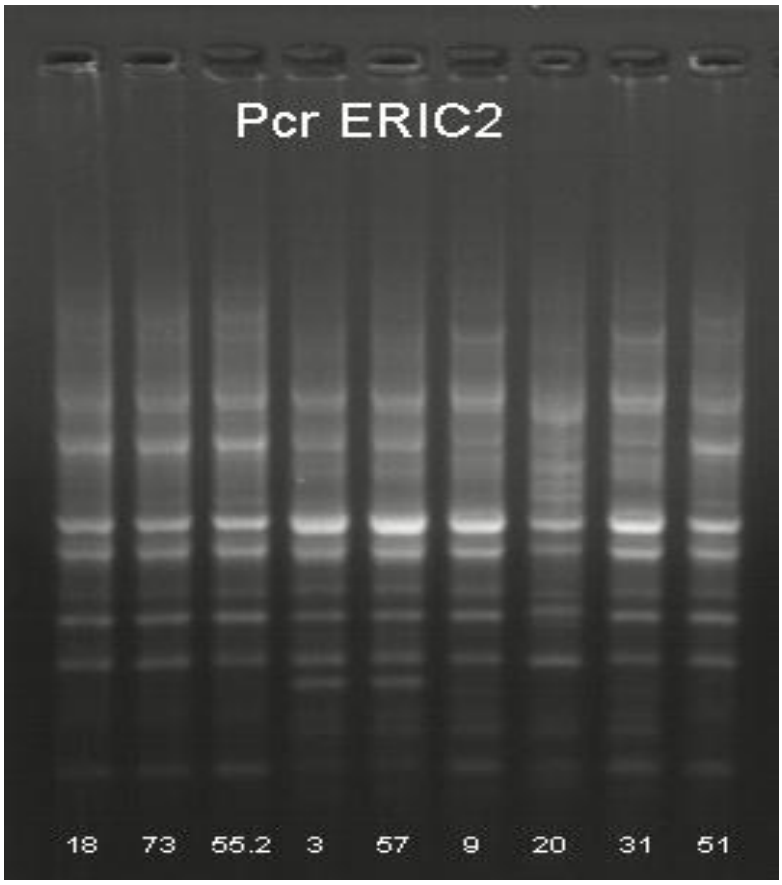

Figure 3. ERIC-PCR profiles of the extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* obtained with the primer ERIC-2.

aminoglycosides, kanamycin, and fluoroquinolones. Thus, the resistance to céfoxitine was observed in 3 transconjugants. This explained that the gene $bla_{CTX\text{-}M\text{-}15}$ and the gene $bla_{DHA\text{-}1}$ gene have been carried by a plasmid, and transferred to the transconjugants by conjugation. Molecular typing by ERIC-PCR showed the presence of seven different ERIC profiles. One profile was common to the 3 strains co-producing CTX-M-15 and DHA-1, suggesting a clonal relatedness (Figure 3).

DISCUSSION

In our study, the prevalence of ESBL producing *Klebsiella pneumoniae* isolates was 8%. The overall prevalence of ESBL production varied considerably according to geographic areas of countries and the different hospital structures. ESBL prevalence rate in this study is still lower compared to those reported in northern Algeria (19.9%) (Messai et al., 2008) and Tunisia (20.2%) (Ben Haj Khalifa and Khedher, 2009). However, higher prevalence rates of ESBL produced by *Klebsiella* were detected in South America (45.4% to 51.9%) (Villegas et al., 2008) and Saudi Arabia (55%) (Al-Agamy et al., 2009). In Pakistan, the prevalence of ESBLs produced in *K. pneumoniae* reached a very high alarming rate with 70% (Shah et al., 2004).

The antibiotics susceptibility profile of the nine strains studied showed resistance to most β-lactams tested except cefoxitin (30% of strains were resistant), and imipenem remained active in all strains studied. Thus, cross-resistance is observed with aminoglycosides and fluoroquinolones, and this could be related to the misuse of broad-spectrum antibiotics (penicillins, cephalosporins, chloramphenicol, tetracyclines, fluoroquinolones, and aminoglycosides).

The proportions found by Ben Haj Khalifa and Khedher in a study of ESBLs produced in uropathogen *Klebsiella* spp, isolated in Tunisian university hospital, are closer to those we observed (100% to 3rd generation cephalosporins, 92.5% to gentamicins, and 67.5% to fluoroquinolones) (Ben Haj Khalifa and Khedher, 2009).

It is now proven that the use of antibiotics, especially 3rd generation cephalosporins for therapeutic purposes, is the most important risk factor in the development of bacterial resistance (Rubin and Samore, 2002). It has become a major public health problem.

We found, the same ESBL CTX-M-15 in the 9 ESBLKp

strains studied. Currently, the ESBL CTX-M are the most frequently isolated in western and eastern Algeria (Ahmed et al., 2012; Nedjai et al., 2012), Tunisia (Elhani et al., 2011), Portugal (Mendonça et al., 2009), China (Liu et al., 2009), in Taiwan (Shu et al., 2010) and many countries worldwide.

In our study, the phenotype of the DHA-1-producing strains was characterized in three cefoxitin-resistant strains, two strains (Kp18, Kp55.2) were isolated in the women medicine service, and one strain (kp73) was isolated in the intensive care unit (ICU).

These enzymes have been recently detected among Enterobacteriaceae such as *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *P. mirabilis* and *Salmonella* spp. These plasmid-mediated cephalosporinases are genetically very close to chromosomal AmpC cephalosporinases, and the transfer of the genes coding these enzymes requires mobile genetic elements (Philippon et al., 2002; Bush et al., 1995).

Our results are comparable with those of other studies in Spain, China, and France, which observed the diffusion of DHA-1 plasmid-mediated cephalosporinases in *K. pneumoniae* strains (Hennequin et al., 2012; Guo et al., 2012; Tobes et al., 2013). There are few studies concerning the cephalosporinases AmpC diffusion in this species in Algeria. Only one study was conducted in three hospitals of Algiers, where there is the detection of the production of cephalosporinases CMY-2 in *K. pneumoniae* and DHA-1 was detected in *Enterobacter cloacae* strains (Iabadene et al., 2009). So, this is the first detection of DHA-1 in *K. pneumoniae* in Algeria.

Typing ESBL by ERIC-PCR showed a single profile for the three strains producing CTX-M-15 and DHA-1, and a genetic diversity for other strains that produced only CTX-M-15. This suggests that the distribution of DHA-1 is the result of the spread of a single clone among patients in the two services: Women Medicine and Intensive Care Unit, hospital.

The acquisition of the gene blaCTX-M-15 by strains of *K. pneumoniae* probably occurred by horizontal transfer from strains of *E. coli*. Several studies have described that the gene *bla*CTX-M-15 has been carried by transferable plasmids. A study in Spain showed that the gene blaCTX-M-15 is carried by the same plasmid with a size of 180kb (Valverde et al., 2008). On the other hand, in another study, the size of plasmid was 150kb, on the basis of which it is associated with genes encoding for aminoglycosides resistance (Mesko et al., 2009).

Our study is the first report made to "Ahemida Ben Adjila" Laghouat Hospital, Algeria. It allowed us to reveal the distribution of β-lactamase CTX-M-15 and DHA-1 in *K. pneumoniae* strains which produce the extended-spectrum β-lactamases, with the first detection of plasmid-mediated cephalosporinases DHA-1 produced by *K. pneumoniae* in Algeria.

Nowadays, controlling antibiotics use is essential to monitor the increasing spread of genes resisting to antibiotics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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