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Prevalence and molecular typing of extended-spectrum β-lactamases in *Escherichia coli, Enterobacter cloacae and Citrobacter freundii* isolates from Laghouat Hospital, Algeria

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The antibiotic resistance of enterobacteriacae knows a worldwide worrying evolution with an increase of the extended-spectrum β-lactamases. The present study was to determine the prevalence and molecular typing of extended-spectrum β-lactamases (ESBLs) in clinical isolates of *Escherichia coli, Enterobacter cloacae,* and *Citrobacter freundii*, isolated between January 2010 and December 2012, at the Laghouat "Ahemida Ben Adjila" hospital, Algeria. Antimicrobial susceptibility testing was determined by disk diffusion on Mueller Hinton agar. Genetic transfers were performed by conjugation and plasmid DNA was extracted by the alcalin-lysis method. The characterization of ESBL genes were examined using PCR amplification and DNA sequencing and the clonal relatedness was investigated by ERIC-PCR. During the study period, twenty-one (8.23%) isolates were found to produce ESBLs, distributed as follows: 13 isolates of *E. coli (*61.9%), 6 isolates of *E. cloacae* (28.57%) and 2 isolates of *C. freundii* with 9.52 %. The CTX-M-15 ESBL were predominant (95.24%), followed by TEM-4 (14.28%) and SHV-12 (4.76%)*.* ERIC-PCR analysis showed that the isolates are genetically unrelated and conjugation experiments showed that *bla*$_{CTX-M-15}$ gene was transferred on a conjugative plasmid of high molecular weight (≈130 kb). This study indicated a high prevalence of CTX-M-15 enzymes among *E. coli, E. cloacae* and *C. freundii* in Laghouat hospital, Algeria.

Key words: Multiresistant bacteria, extended spectrum β-lactamase, CTX-M-15, genotyping, Algeria.

INTRODUCTION

ESBL-producing bacteria are responsible for many local, national and international outbreaks which originated from the different hospital wards and mostly in intensive care units (Rodriguez-Villalobos and Struelens, 2006). Infections due by these strains have been associated with high mortality in affected patients, and represent an

increased risk of therapeutic failure and are associated with longer duration of hospital stay and higher hospital charges (Soraas et al., 2013).

Production of extended-spectrum β-lactamases (ESBLs) is the principal mechanism of resistance to oxyimino-cephalosporins evolved by members of the family Enterobacteriaceae (Marco et al., 2013). These enzymes can hydrolyse penicillins, first, second and third-generation cephalosporins, and aztreonam, but do not hydrolyse cephamycins or carbapenems (Chanal et al., 1992). The activity of ESBL can be inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson, 2000). This family of plasmid-mediated ESBL belongs to Ambler class A and group 2be of the Bush-Jacoby and Medeiros classification.

In the recent years, a new family of plasmid-mediated ESBLs called CTX-M which preferentially hydrolyze cefotaxime has emerged. CTX-M enzymes are not closely related to TEM or SHV β-lactamases, as they only show approximately 40% similarity in sequence (Shahid et al., 2009). These ESBLs have been classified into five phylogenetic families on the basis of their amino acid identities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTXM-25 (Bradford, 2001; Bonnet, 2004).

CTX-M genes might be associated with insertion-sequence ISEcp1 or related insertion sequences involved in their expression and mobilization (Poirel et al., 2003). It is encoded by transferable plasmids that vary in size from 7 to 160 kb (Bonnet, 2004).

Now, CTX-M-15 β-lactamase, which was first described in 2001, it is recognized as the most widely distributed CTX-M enzyme (Livermore et al., 2007). This β-lactamase is found mainly in Enterobacteriaceae and was recently named "plasmids of resistance responsible for outbreak" because of their capacity to acquire genes of resistance and to transfer among bacteria (Coque et al., 2008).

The majority of previous studies on ESBL-producing isolates have been reported at the west, centre and east of Algeria (Baba Ahmed-KaziTani et al., 2013; Ramdani-Bouguessa et al., 2006; Gharout-Sait et al., 2012). But no study has been performed at southern regions of Algeria.

In this context, the aim of this study was to determine the prevalence and molecular typing of extended-spectrum β-lactamases (ESBLs) producing clinical isolates of *Escherichia coli, Enterobacter cloacae,* and *Citrobacter freundii* isolated in the Laghouat "Ahemida Ben Adjila" hospital, Algeria.

MATERIALS AND METHODS

Clinical isolates

During a three years period from January 2010 to December 2012,

255 non-repetitive clinical isolates of *E. coli*, *E. cloacae* and *C. freundii* were isolated from patients hospitalized at the Laghouat "Ahemida Ben Adjila" Hospital, Algeria. These isolates were obtained from various clinical specimens (including urine, pus, blood-culture, catheters and rectal swabs), and only one isolate per patient was investigated. The samples were performed on hospitalized patients from different wards: General surgery, orthopedics, women medicine, men medicine, pulmonology, obstetrics-gynecology, pediatrics ward and intensive care unit. All clinical isolates of enterobacteria were identified with the API 20E® system (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing and ESBL detection

The susceptibility to 32 antibiotics was determined by the standard disk diffusion method on Mueller-Hinton agar recommended by the Antibiogram Committee of the French Society for Microbiology (CASFM, 2010).

The following antibiotics (Oxoid, England) were used: 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), trimethoprim/sulfamethoxazole (25 µg), colistin (50 µg), and ceftazidime/clavulanic acid (30/10 µg).

Extended spectrum β-lactamase (ESBL) production was phenotypically confirmed using the double-disk synergy test, described by Jarlier et al. (1988). Synergy was determined between a disc of amoxicillin-clavulanate (20/10 µg) and a 30-µg disc of each third-generation cephalosporin test antibiotic (cefotaxime, ceftriaxone, ceftazidime, and aztreonam) placed at a distance of 20 mm from center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate. Clear extension of the edge of the inhibition zone of cephalosporin toward the augmentin disc was interpreted as positive for ESBL production (Bradford, 2001; Giriyapur et al., 2011). *E. coli* ATCC 25922 was used as quality control strains.

Isoelectric focusing (IEF)

The β-lactamases isolated from clinical isolates were characterized by isoelectric focusing (IEF) according to the protocol determined by Bonnet and Coll (2000). IEF of β-lactamases was performed with polyacrylamide gels containing Ampholines with a pH range of 3.5 to 10. Thus, the following β-lactamases: CTX-M-1 (pI 8.4), CTX-M-14 (pI 7.9), CTX-M-15 (pI 8.6), TEM-3 (pI 6.3) and SHV-2 (pI 7.6) were used as the reference bands of known β-lactamases.

Extraction of bacterial DNA

Total DNA of ESBL-producing isolates was extracted by boiling to100°C for 10 min a suspension of the strains in 200 µl of distilled water and centrifugation for 7 min at 13,000 × g, then the supernatant obtained was stored at -20°C. PCR experiments were performed with these crude lysates.

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Table 1. Primers used in PCR for detection of *bla*- genes.

Target	Primer	Sequence	Annealing temperatures (°C)	References
TEM	TEM A TEM B	5' - TAA AAT TCT TGA AGA CG - 3' 5' - TTA CCA ATG CTT AAT CA - 3'	44	Heritage et al. (2001)
SHV	SHV 105q SHV 149p	5' - TTA GCG TTG CCA GTG CTC GAT - 3' 5' - CGC TTC TTT ACT CGC CTT TAT - 3'	54	Rasheed et al. (1997)
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)
ERIC–PCR	ERIC2	5' - AAG TAA GTGACT GGG GTG AGC G - 3'	64	Dumarche et al. (2002)

Molecular characterization of *bla* genes

Genes encoding for extended-spectrum β-lactamases: bla_{CTX-M}, bla_{SHV}, bla_{TEM} were identified by PCR method (Polymerase Chain Reaction) (De Champs et al., 2004).

Primers used to amplify β-lactamases genes and annealing temperatures are shown in Table 1, thus the operating conditions and assay methods were performed to all ESBL producing isolates as previously described in detail (Lagha et al., 2014). The PCR products were visualized using UV after migration in agarose gel 1% and staining with ethidium bromide.

DNA sequencing was performed with the dideoxy chain termination method (Sanger et al., 1977), in GATC Biotech AG (European Custom Sequencing Centre, Gottfried-Hagen-Stra ße 20, 51105 Köln). The nucleotide and deduced protein sequences were analyzed using the Codon Code Aligner software and compared to sequences available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

The all ESBL producing clinical isolates were analyzed by enterobacterial repetitive intergenic consensus PCR (ERIC2-PCR) as previously reported (Lagha et al., 2014). DNA was amplified using the only one primer ERIC-2: 5'AAGTAAGTGACTGGGGTGAGCG3' (Dumarche et al., 2002), as shown in Table 1. Amplified products were visualized on agarose gels 1.5% stained with ethidium bromide. The profiles of tested clinical isolates were considered different when at least one band differed (Khan et al., 2002).

Conjugation experiments

Conjugation experiments were performed as previously described (Sambrook et al., 1989) with *E. coli* C600 Rif R (Rifampicin resistant) as a recipient strain. A donor strain (clinical isolates) and a recipient strain were grown separately in Brain Heart Infusion broth (Oxoid) at 37°C for overnight. Then the transconjugants were selected on Mueller Hinton agar (Oxoid) containing rifampicin (300 µg/L) and cefotaxime (1 µg/L). All transconjugants were subjected to antimicrobial susceptibility testing and double-disk synergy test.

Plasmid analysis

Plasmids DNA from ESBL strains and their transconjugants were extracted by the method of Kado and Liu (Kado and Liu, 1981). Plasmid profiles were analyzed by electrophoresis in agarose gel at

Figure 1. Positive result of a double-disk synergy test for *E. coli* Ec6 (Synergism between amoxicillin-clavulanate and cefotaxime, ceftriaxone, ceftazidime, and aztreonam).

0.7% and the sizes of the plasmids were determined by comparison with that of plasmids-size standards: Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and (180 kb) as previously described (Robin et al., 2005).

RESULTS

During the study period, 255 non-duplicate clinical strains of enterobacteriaceae were identified. Among these strains, 21 (8.23%) were ESBL positive by double synergy test (Figure 1). The prevalence of ESBL production per species among these tested clinical isolates was the following: 6.91% (13/188) *E. coli*, 10.34% (6/58) *E. cloacae*, 22.22% (2/9) *C. freundii* (Table 2).

The clinical and genetic characteristics, including isolation date, specimen and ward distribution of the 21 ESBL producing isolates are shown in Table 3.

These ESBL-producing strains were isolated from 21 individual patients, consisting of 10 men (47.61%) and 11 women (52.38%) with a mean age of 45.42 years. The patients have been hospitalized during periods between 9

Table 2. Distribution of extended-spectrum β-lactamase producing strains isolated in Laghouat hospital by species and years.

ESBL isolate	2010		2011		2012	
	%	n	%	n	%	n
Escherichia coli	4	3/69	7	4/54	9	6/65
Enterobacter cloacae	0	0/16	9	2/22	20	4/20
Citrobacter freundii	0	0	0	0/5	50	2/4
Total	3.53	3/85	7.4	6/81	13.48	12/89

Table 3. Clinical and genetic characteristics of extended-spectrum β-lactamase-producing *E. coli*, *E. cloacae* and *C. freundii*.

Isolate	Code	Period of isolation	Wards	Sample origin	Sex	Age (years)	β-lactamase pI	β-lactamase gene	Conjugaison
E. coli (n = 13)	Ec1	24/01/2010	Intensive care unit	Catheter	M	53	5.4 + 8.6	CTX-M15	+
	Ec2	18/04/2010	Women medicine	Urine	F	34	7.5 + 8.6	CTX-M15	+
	Ec3	02/05/2010	Intensive care unit	Catheter	F	54	7.5 + 8.6	CTX-M15	-
	Ec4	20/02/2011	Men medicine	Urine	M	46	8.6	CTX-M15	+
	Ec5	06/03/2011	Orthopedics	Urine	M	51	7.5 + 8.6	CTX-M15	-
	Ec6	10/04/2011	Women medicine	Urine	F	32	7.5 + 8.2 + 8.6	CTX-M15, SHV-12	-
	Ec7	07/08/2011	Orthopedics	Pus	M	37	5.4 + 8.6	CTX-M15	+
	Ec8	08/04/2012	Obstetrics-Gynecology	Rectal	F	49	7.5 + 8.6	CTX-M15	-
	Ec9	22/04/2012	Women medicine	Urine	F	39	7.5 + 8.6	CTX-M15	-
	Ec10	24/06/2012	Orthopedics	Urine	M	58	8.6	CTX-M15	-
	Ec11	12/08/2012	Intensive care unit	Catheter	M	56	5.6 + 8.6	CTX-M15, TEM-4	-
	Ec12	02/09/2012	Orthopedics	Urine	F	52	5.6 + 7.5 + 8.6	CTX-M15, TEM-4	+
	Ec13	30/09/2012	Obstetrics-Gynecology	Urine	F	28	7.5 + 8.6	CTX-M15	+
E. cloacae (n = 6)	En1	06/11/2011	Orthopedics	Urine	F	57	5.4 + 7.5 + 8.6	CTX-M15	+
	En2	25/12/2011	Intensive care unit	Blood	F	42	5.4 + 8.6	CTX-M15	+
	En3	08/01/2012	Intensive care unit	Pus	M	55	5.4 + 7.5 + 8.6	CTX-M15	-
	En4	19/08/2012	Orthopedics	Catheter	M	52	5.4 + 7.5 + 8.6	CTX-M15	-
	En5	25/11/2012	Pediatrics	Rectal	F	8 mois	5.4 + 7.5 + 8.6	CTX-M15	+
	En6	09/12/2012	Pulmonology	Urine	M	56	8.6	CTX-M15	+
C. freundii (n = 2)	Cf1	22/01/2012	Women medicine	Urine	F	48	5.4 + 7.5 + 8.6	CTX-M15	-
	Cf2	12/02/2012	Orthopedics	Blood	M	54	5.6	TEM-4	-

and 123 days.

The principal source of isolation was urine (52.38%); following of the catheters source (19.04%), then pus, blood and rectal sources with a percentage of isolation 9.52% for each one.

However, 33.33% of the patients were

Table 4. Antibiotic susceptibility of ESBL-producing clinical isolates (n=21).

Antibiotic	Susceptibility of ESBL-producing isolates: n (%).			Total
	E. coli (n = 13)	*E. cloacae* (n = 6)	*C. freundii* (n = 2)	
Amoxicillin	0 (0)	0 (0)	0 (0)	0 (0)
Ticarcillin	0 (0)	0 (0)	0 (0)	0 (0)
Piperacillin	0 (0)	0 (0)	0 (0)	0 (0)
Cephalotin	0 (0)	0 (0)	0 (0)	0 (0)
Amoxicillin/clavulanic acid	3 (23.07)	1 (16.67)	0 (0)	4 (19.05)
Ticarcilline/clavulanic acid	2 (15.38)	1 (16.67)	0 (0)	3 (14.28)
Cefotaxime	1 (7.69)	1 (16.67)	0 (0)	2 (9.52)
Ceftazidime	1 (7.69)	1 (16.67)	0 (0)	2 (9.52)
Aztreonam	0 (0)	1 (16.67)	0 (0)	1 (4.76)
Cefepime	7 (53.84)	3 (50)	1 (50)	11 (52.38)
Cefpirome	0 (0)	0 (0)	0 (0)	0 (0)
Cefuroxime	0 (0)	1 (16.67)	0 (0)	1 (4.76)
Cefixime	2 (15.38)	1 (16.67)	(0)	3 (14.28)
Piperacillin + tazobactam	11 (84.61)	6 (100)	2 (100)	19 (90.48)
Ceftazidime/clavulanic acid	9 (69.23)	6 (100)	0 (0)	15 (71.42)
Cefoxitin	8 (61.54)	1 (16.67)	0 (0)	9 (42.86)
Imipenem	13 (100)	6 (100)	2 (100)	21 (100)
Kanamycin	8 (61.54)	2 (33.33)	0 (0)	10 (47.62)
Tobramycin	8 (61.54)	2 (33.33)	0 (0)	10 (47.62)
Amikacin	13 (100)	6 (100)	2 (100)	21 (100)
Gentamicin	9 (69.23)	2 (33.33)	0 (0)	11 (52.38)
Netilmicin	9 (69.23)	2 (33.3)	0 (0)	11 (52.38)
Nalidixic acid	6 (46.15)	3 (50)	1 (50)	10 (47.62)
Ofloxacin	4 (30.76)	3 (50)	2 (100)	9 (42.86)
Ciprofloxacin	7 (53.84)	3 (50)	2 (100)	12 (57.14)
Chloramphenicol	3 (23.07)	1 (16.67)	0 (0)	4 (19.05)
Tetracycline	4 (30.76)	3 (50)	1 (50)	8 (38.09)
Colistin	13 (100)	6 (100)	2 (100)	21 (100)
Trimethoprim	6 (46.15)	1 (16.67)	1 (50)	8 (38.09)
Trimethoprim/sulfamethoxazole	6 (46.15)	1 (16.67)	1 (50)	8 (38.09)
Fosfomycin	13 (100)	6 (100)	2 (100)	21 (100)

hospitalized at the orthopedics ward, followed by an intensive care unit (23.8%) and the ward of women medicine (19.04%). In opposite to other hospital wards (General Surgery, Men Medicine, Pulmonology, Obstetrics-Gynecology, Pediatrics), where the prevalence of ESBL-producing strains is low, including one strain was isolated by ward (4.76%).

Antibiotic susceptibilities of ESBL-producing strains showed that all isolates were resistant to amoxicillin, ticarcillin, piperacillin, cephalotin, and cefpirome. Thus high levels of resistance were founds to cefotaxime (90.48%), cefuroxime (95.24%), cefixime 85.72%), ceftazidime (90.48%), aztreonam (95.24%), amoxicillin-clavulanic acid (80.95%) and ticarcilline-clavulanic acid (85.72%).

All ESBL-producing isolates were also multidrug-resistant to others antibiotics and most of them were resistant to: Chloramphenicols (80.95%), trimethoprim (61.91%), and sulfonamides (66.67%). It should be noted that almost half of the isolates were resistant to aminoglycosides (47.62% to gentamicin, 52.08% to tobramycin and kanamycin), and fluoroquinolones (57.14% ofloxacin and 42.86% ciprofloxacin).

Moreover, it is worth noting that all 21 strains (100%) were susceptible to imipenem, amikacin, colistin and fosfomycin. Results of antimicrobial susceptibility testing are shown in Table 4.

In this study, all 21 ESBL-producing isolates were analyzed for their β-lactamases content by isoelectric focusing. It showed the presence of one to five β-lactamase bands with different pIs. The first band of pI5.4 corresponded to the TEM-1-type chromosomal penicillinase. The following bands were of pI: 7.5 (compatible with the OXA-1-type oxacillinase); and pI:
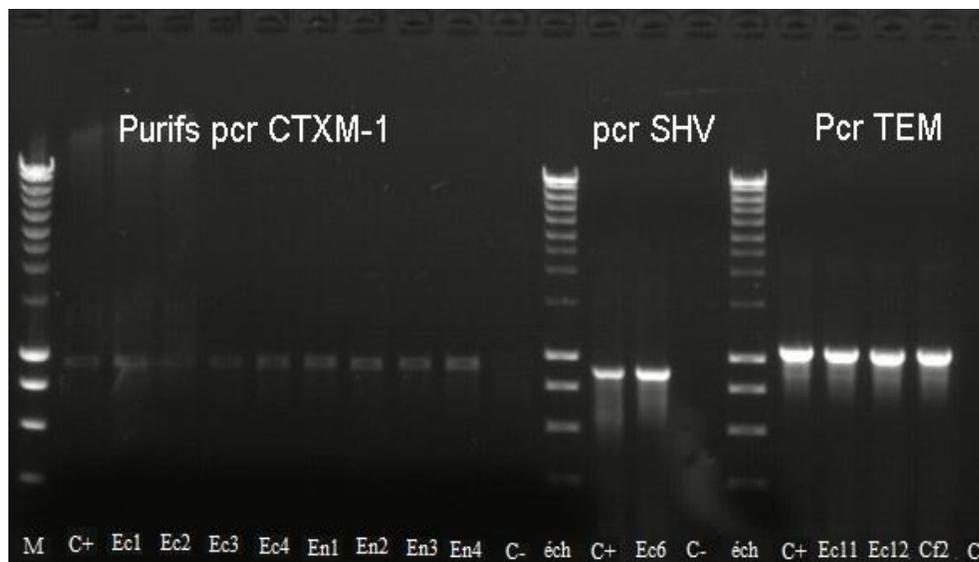
Figure 2. PCR amplification products of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes (M: molecular weight marker; C+: positive control; C-: negative water control; Ec: *E. coli*; En: *E. cloacae*; Cf: *C. freundii*).
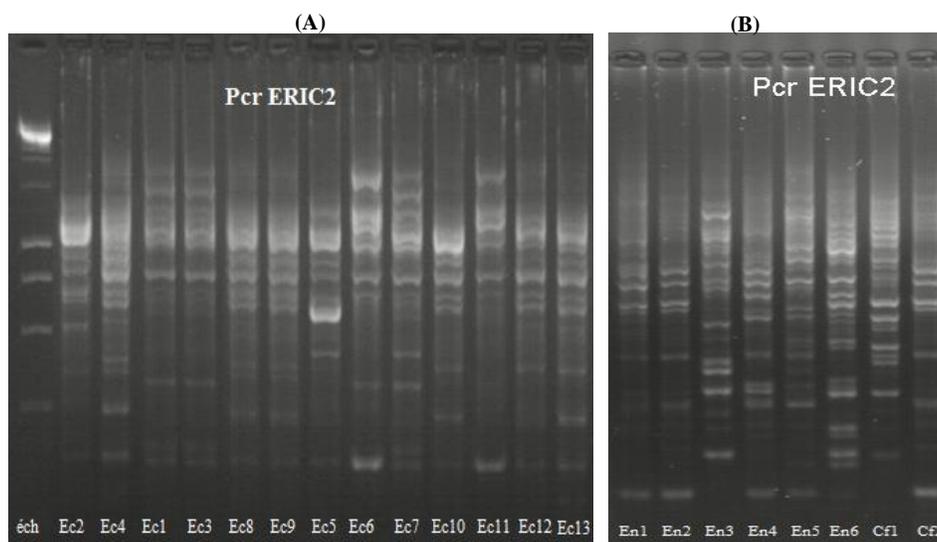
Figure 3. ERIC-PCR profiles of the extended-spectrum β-lactamase-producing isolates obtained with the primer ERIC-2. **(A)** *E. coli* (Ec); **(B)** *E. cloacae* (En) and *C. freundii* (Cf).

5.6, pI: 8.2 and pI: 8.6 corresponded to the different ESBL-types detected.

The genotypic analysis by PCR showed the presence of bla_{SHV}, bla_{TEM}, and $bla_{CTX-M-1}$ group on the chromosomal DNA of strains (Figure 2). Nucleotide sequence analysis of the bla_{CTX-M} genes showed the presence of CTX-M-15 in the twenty ESBL-producing isolates (95.24%). Whereas TEM-4 was detected in three isolates (14.28%) mainly in *E. coli and C. freundii,* and SHV-12 was identified in only one strain (04.76%) of *E.*

coli isolated from the women medicine ward. The distribution of the different ESBL types for each strain is shown in Table 3.

ERIC-PCR analysis of genomic DNA from the 21 ESBL-producing clinical isolates revealed that there were 11 different patterns among the 13 *E. coli* isolates (Figure 3A), whereas all strains of *E. cloacae* and *C. freundii* have been different (8 distinct ERIC-PCR patterns were seen in the 8 isolates) (Figure 3B). This indicated clearly heterogeneity in isolates genetic profiles.

Concerning the conjugation assays, 10 ESBL-producing transconjugants were obtained from the 21 ESBL isolates selected, which consisted of six *E. coli* and four *E. cloacae.* All transconjugants also expressed resistance to aminoglycosides (gentamicin and tobramycin), fluoroquinolones, sulfamid and trimethoprim-sulfamethoxazole.

After gel electrophoresis, comparison of the plasmidic content of isolates *E. coli* and *E. cloacae* and their transconjugants showed the presence for each strain, one to four bands, different sizes, between 5 and 180 kb. A common band was observed in all extracts which isolates a high molecular weight > 85 kb (≈130 kb); it indicated that the $bla_{CTX-M-15}$ gene is carried by this plasmid and transferred between strains.

DISCUSSION

Extended-spectrum β-lactamases producing Enterobacteriaceae (ESBL-E) are emerging worldwide in hospitals and in the community (Bradford, 2001). Their incidence varies according to countries, regions or even hospitals (Arnaud et al., 2015).

In our study, ESBLs were found in 21 (8.23%) of 255 clinical isolates with *E. coli* being the major ESBLs produce (13/21), following of *E. cloacae* (6/21) and *C. freundii* (2/21). This rate of ESBLs isolates is in accordance with those reported in Kingdom of Saudi Arabia (8.9%) (El-Khizzi and Bakheshwain, 2006), but even higher than the rate observed in recent European studies, from 2.4% to 5.1% in France (Toubiana et al., 2016), and 2.9% in Sweden (Kaarme et al., 2013). Studies conducted in Ethiopia (Mulisa et al., 2016) reported the prevalence rate of 25% of ESBLs producers respectively among Enterobacteriaceae family. These prevalence rates are high as compared to our study which may be attributed to variation in drug management policies or follow other control programs.

Additionally, Latin America, the Middle East, Europe, and the South Pacific displayed a prevalence of ESBL of approximately 10 to 35% (Morrissey et al., 2013; Fernandez-Reyes et al., 2014). However, more than 40% of clinical isolates from Asia were ESBL producers in 2011 (Lukac et al., 2015).

At Laghouat hospital, the patients were hospitalized more frequently in orthopedics wards (33.33%), intensive care unit (23.8%) and the women medicine wards (19.04%). As they were previously-shown, that orthopedic surgical site infections are often associated with substantial morbidity and exorbitant costs, and are challenging to treat, especially in case of multi-resistant pathogens or presence of implants (Martinez-Pastor et al., 2010). Additionally, many studies in Intensive care units have established risk factors for the acquisition of infection due to ESBL producing *E. coli* and even to other bacterial species (Oteo et al., 2013).

Moreover, it is worth noting that, the principal source of isolation the ESBL-producing isolates in our study was urine with the proportion 52.38%; our results are in accordance with other recent studies realized on a group of 124 Enterobacteriaceae isolates resistant to third generation cephalosporin, and collected in distinct health care facilities of different Portuguese regions, which have even described a 58.9% clinical strains were isolated from urine (Jones-Dias et al., 2014). However, studies have shown that ESBL producing uropathogens have their reservoir in the digestive tract (Anil Kumar and Babu, 2013).

The major risk factors found in this study were length of hospitalization, the hospital ward where the ESBL-strains was isolated (orthopedics, intensive care unit and the women medicine wards), urinary tract, and antibiotic therapy, as previously reported (Dayan et al., 2013)

Our antimicrobial susceptibility analysis of the all ESBL-producing isolates found highly prevalent resistances against to the majority of β-lactams, and even to others tested antibiotics: Gentamicin, tobramycin, ofloxacin, chloramphenicols, trimethoprim, and sulfonamides, which confirmed the presence of multidrug-resistant isolates in this hospital. Unfortunately, comparable results of the susceptibility rate to these molecules were also reported by Rakotonirina et al. (2013).

This correlates with other studies, where many ESBL producers are multi-resistant to non-β-lactam antibiotics, including fluoroquinolones and aminoglycosides (Livermore et al., 2007), trimethoprim, tetracyclines, sulfonamides, and chloramphenicol, which are often encoded by the same plasmids that determine the ESBL (Karisik et al., 2006).

Consequently, effective antibiotic therapy for treating these infections is limited to a small number of drugs, such as carbapenems and thus increasing the chance of resistance to carbapenems among the Enterobacteriaceae (Pitout, 2010).

In this study, among the twenty one ESBL-producing clinical strains isolated, $bla_{CTX-M-15}$ was the most commonly detected genotype in all clinical isolates (95.24%).

The blaCTX-M-15 ESBL gene is considered to be the most prevalent ESBL worldwide, as it is found in a Tunisian study analyzed 32 ESBL *E. coli* isolates collected during a 10-month period, which reported the emergence of CTX-M-15 in 97% (31/32) of isolates; and 81% (26/32) also harbored TEM-1 (Réjiba et al., 2011).

According to the previously studies realized, among ESBL-producing Enterobacteriaceae in Algeria County, CTX-M-15 with CTX-M-3 enzymes were most frequently reported in the west (Baba Ahmed-KaziTani et al., 2013), centre (Ramdani-Bouguessa et al., 2006) and east of Algeria (Gharout-Sait et al., 2012). The rapid dissemination of CTX-M-15 producing Enterobacteriaceae were reported in a number of countries is a significant public health concern (Bonnet,

2004; Livermore et al., 2007).

Thus, in our study, 14.28% of the clinical isolates harbored bla_{TEM-4}, followed bla_{SHV-12} which is also distributed, but only in a single strain of *E. coli* with rate of 4.76%. This SHV-12 type extended-spectrum β-lactamase is most often found in Asia (Kim et al., 1998) and including Africa (Kasap et al., 2010). It is already found in Algeria, as shown previously in *E. cloacae* (Iabadene et al., 2008), and *K. pneumonia* (Berrazeg et al., 2013).

However, the spread of TEM-4 enzyme is very rare in Algeria; their presence is reported only in one study by Kermas et al. (2012) where this enzyme is detected in *Salmonella enterica*.

The molecular typing by ERIC-PCR revealed that the majority of ESBL-producing isolates from our hospital showed distinct genetic profiles, with the presence of 19 different patterns among the 21 genetic profiles. These isolates were considered genetically unrelated. This property explains the easy horizontal dissemination of $bla_{CTX-M-15}$ -harboring plasmids, a high molecular weight > 85 kb (≈130 kb); and their emergence between different strains.

This result correlates with previous studies in Algeria, where showed that $bla_{CTX-M-15}$, was carried by conjugative plasmid of high molecular weight, vary from 85 kb (Messai et al., 2008), to ≥ 125 kb (Nedjai et al., 2012). Already, Messai et al. (2008) showed that the CTX-M-15 enzyme was developed from CTX-M-3 under Algerian clinical context.

This dissemination of the CTX-M-15-type ESBLs is not restricted to the nosocomial setting but also involves the community. This phenomenon is acting to modify the epidemiology of ESBLs, whereas those enzymes were, previously, mostly restricted to the nosocomial setting (Rossolini et al., 2008).

This study is the first report conducted on ESBL-producing strains of *E. coli*, *E. cloacae* and *C. freundii*, isolated from various clinical specimens in Laghouat hospital, Southern Algeria; that demonstrates such a high prevalence of CTX-M-15 enzymes among clinical strains. Although, TEM-4 and SHV-12 are the less frequent enzymes isolated in some strains of *E. coli* and *C. freundii*. All this indicates the dissemination of multidrug resistant isolates in Laghouat, in different hospital wards and probably in the community.

Conflict of Interests

The authors have not declared any conflict of interests.

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REFERENCES

Anil Kumar V, Babu R (2013). Fecal Carriage of Extended-Spectrum β-Lactamase Producing Enterobacteriaceae. J. Med. Microbiol. Diagn. 2(3):1000e119.

Arnaud I, Maugat S, Jarlier V, Astagneau P (2015). Ongoing increasing temporal and geographical trends of the incidence of extended-spectrum beta-lactamase-producing Enterobacteriaceae infections in France, 2009 to 2013. Eur. Surveill. 20(36):1-7.

Baba Ahmed-Kazi Tani Z, Decré D, Genel N, Boucherit-Otmani Z, Arlet G, Drissi M (2013). Molecular and epidemiological characterization of enterobacterial multidrug- resistant strains in Tlemcen Hospital (Algeria) (2008–2010). Microbiol. Drug Resist. 19:185-190.

Berrazeg M, Drissi M, Medjahed L, Rolain JM (2013). Hierarchical clustering as a rapid tool for surveillance of emerging antibiotic resistance phenotypes in *Klebsiella pneumoniae* strains. J. Med. Microbiol. 62:864-874.

Bonnet R (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48:1-14.

Bonnet R, Sampaio JL, Chanal C, Sirot D, De Champs C, Viallard JL, Labia R, Sirot J (2000). A novel class A extended-spectrum beta-lactamase (BES-1) in *Serratia marcescens* isolated in Brazil. Antimicrob. Agents Chemother. 4(11):3061-3068.

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

CASFM (2010). Antibiogram Committee of French Society for Microbiology. Communique 2010. Paris. France. http://www.sfm-microbiologie.org/UserFiles/files/casfm_2010.pdf.

Chanal C, Poupart MC, Sirot D, Labia R, Sirot J, Cluzel R (1992).Nucleotide sequences of CAZ-6, and CAZ-7 beta-lactamase genes. Antimicrob. Agents Chemother. 36:1817-1820.

Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L, Baquero F, Cantón R, Nordmann P (2008). Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg. Infect. Dis. 14:195-200.

Dayan N, Dabbah H, Weissman I, Aga I, Even L, Glikman D (2013). Urinary tract infections caused by community-acquired extended-spectrum beta-lactamase producing and nonproducing bacteria: a comparative study. J. Pediatr. 163:1417-1421.

De Champs C, Chanal C, Sirot D, Baraduc R, Romaszko JP, Bonnet R, Plaidy A, Boyer M, Carroy E, Gbadamassi MC, Laluque S (2004). Frequency and diversity of class A extended-spectrum β-lactamases in hospitals of the Auvergne, France: a 2 year prospective study. J. Antimicrob. Chemother. 54(3):634-639.

Dumarche P, De Champs C, Sirot D, Chanal C, Bonnet R, Sirot J (2002). TEM derivative-producing *Enterobacter aerogenes* strains: dissemination of a prevalent clone. Antimicrob. Agents Chemother. 46:1128-1131.

El-Khizzi NA, Bakheshwain SM (2006). Prevalence of extended spectrum β-lactamases among Enterobacteriaceae isolated from blood culture in a tertiary care hospital. Saudi Med. J. 27:37-40.

Fernandez-Reyes M, Vicente D, Gomariz M, Esnal O, Landa J, Onate E, Pérez-Teallero E (2014). High rate of fecal carriage of extended-spectrum β-lactamase-producing *Escherichia coli* in healthy children in Gipuzkoa, northern Spain. Antimicrob. Agents Chemother. 58:1822-1824.

Gharout-Sait A, Touati A, Benallaoua S, Guillard T, Brasme L, Madoux J, De Champs C (2012). CTX-M from community-acquired urinary tract infections in Algeria. Afr. J. Microbiol. Res. 6:5306-5313.

Giriyapur RS, Nandihal NW, Krishna BVS, Patil AB, Chandrasekhar MR (2011). Comparison of Disc Diffusion Methods for the Detection of Extended-Spectrum Beta Lactamase-Producing Enterobacteriaceae. J. Lab. Physicians. 3(1):33-36.

Heritage J, Ransomeb N, Chambersa PA, Wilcox MH (2001). A comparison of culture and PCR to determine the prevalence of ampicillin-resistant bacteria in the faecal flora of general practice patients. J. Antimicrob. Chemother. 48(2):287-289.

Iabadene H, Messai Y, Ammari H, Ramdani-Bouguessa N, Lounes S, Bakour R, Arlet G (2008). Dissemination of ESBL and Qnr determinants in *Enterobacter cloacae* in Algeria. J. Antimicrob Chemother. 62:133-136.

Jarlier V, Nicolas MH, Fournier G, Philippon A (1988). Extended-broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10:867-878.

Jones-Dias D, Manageiro V, Ferreira E, Louro D, Antibiotic Resistance Surveillance Program in Portugal (ARSIP) Participants, and Manuela Canica (2014). Diversity of Extended-Spectrum and Plasmid-Mediated AmpC β-Lactamases in Enterobacteriaceae Isolates from Portuguese Health Care Facilities. J. Microbiol. 52(6):496-503.

Kaarme J, Molin Y, Olsen B, Melhus A (2013). Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in healthy Swedish preschool children. Acta Paediatr. 102:655-660.

Kado CI, Liu ST (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

Karisik E, Ellington MJ, Pike R, Warren RE, Livermore DM, Woodford N (2006). Molecular characterization of plasmids encoding CTX-M-15 β-lactamases from Escherichia coli strains in the United Kingdom. J. Antimicrob. Chemother. 58:665-668.

Kasap M, Fashae K, Torol S, Kolayli F, Budak F, Vahaboglu H (2010). Characterization of ESBL (SHV-12) producing clinical isolate of Enterobacter aerogenes from atertiary care hospital in Nigeria. Ann. Clin. Microbiol. Antimicrob. 9(1):1.

Kermas R, Touati A, Brasme L, Le Magrex-Debar E, Mehrane S, Weill FX, De Champs C (2012). Characterization of extended-spectrum β-lactamase-producing Salmonella enterica serotype Brunei and Heidelberg at the Hussein Dey hospital in Algiers (Algeria). Foodborne Pathog. Dis. 9:803-808.

Khan AA, Mc Carthy S, Wang RF, Cerniglia CE (2002). Characterization of United States outbreak isolates of Vibrio parahaemolyticus using enterobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3: K6 isolates. FEMS. Microbiol. Lett. 206:209-214.

Kim J, Kwon Y, Pai H, Kim JW, Cho DT (1998). Survey of Klebsiella pneumonia strains producing extended-spectrum beta-lactamases: prevalence of SHV-12 and SHV-2a in Korea. J. Clin. Microbiol. 36:1446-1449.

Lagha N, Abdelouahid DE, Hassaine H, Robin F, Bonnet R (2014). First characterization of CTX-M-15 and DHA-1 β-lactamases among clinical isolates of Klebsiella pneumoniae in Laghouat Hospital, Algeria. Afr. J. Microbiol. Res. 8(11):1221-1227.

Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Poirel L, Woodford N (2007). CTX-M: changingthe face of ESBLs in Europe. J. Antimicrob. Chemother. 59(2):165-174.

Lukac PJ, Bonomo RA, Logan LK (2015). Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae in Children: Old Foe, Emerging Threat. Clin. Infect. Dis. Adv. 60(9):1389-1397.

Marco MD, Fabio A, Lucia P, Gian MR (2013). CTX-M-type β-lactamases: A successful story of antibiotic resistance. Int. J. Med. Microbiol. 303(6):305-317.

Martinez-Pastor JC, Vilchez C, Pitart C (2010). Antibiotic resistance in orthopaedic surgery: acute knee prosthetic joint infections due to extended-spectrum beta-lactamase-producing Enterobacteriaceae. Eur. J. Clin. Microbiol. Infect. Dis. 29:1039-1041.

Messai Y, Iabadene H, Benhassine T, Alouache S, Tazir M, Gautier V, Arlet G, Bakour R (2008). Prevalence and characterization of extended-spectrum beta-lactamases in Klebsiellapneumoniae in Algiers hospitals (Algeria). Pathol. Biol. 56(5):319-325.

Morrissey I, Hackel M, Badal R, Bouchillon S, Hawser S, Biedenbach D (2013). A review of ten years of the Study for Monitoring Antimicrobial Resistance Trends (SMART) from 2002 to 2011. Pharmaceuticals 6:1335-1346.

Mulisa G, Selassie LG, Jarso G, Shiferew T, Zewdu A, Abebe W, Belachew F, Sewunet T (2016). Prevalence of Extended Spectrum Beta-lactamase Producing Enterobacteriaceae: A Cross Sectional Study at Adama Hospital, Adama, Ethiopia. J. Emerg. Infect. Dis. 1(1):2-6.

Nedjai S, Barguigua A, Djahmi N, Jamali L, Zerouali K, Dekhil M, Timinouni M (2012). Prevalence and characterization of extended spectrum beta-lactamases in Klebsiella-Enterobacter-Serratia group bacteria, in Algeria. Med. Mal. Infect. 42:20-29.

Oteo J, Cercenado E, Vindel A, Bautista V, Fernandez-Romero S, Saéz D, Padilla B, Zamora E, Campos J (2013). Outbreak of multidrug-resistant CTX-M-15-producing Enterobacter cloacae in a neonatal intensive care unit. J. Med. Microbiol. 62:571-575.

Paterson DL (2000). Recommendation for treatment of severe infections caused by Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs). Clin. Microbiol. Infect. 6:460-463.

Pitout JD (2010). Infections with extended-spectrum β-lactamase producing Enterobacteriaceae: changing epidemiology and drug treatment choices. Drugs 70:313-333.

Poirel L, Decousser JW, Nordmann P (2003). Insertion sequence ISEcp1B is involved in expression and mobilization of a blaCTX-M β-lactamase gene. Antimicrob. Agents Chemother. 47:2938-2945.

Rakotonirina HC, Garin B, Randrianirina F, Richard V, Talarmin A, Arlet G (2013). Molecular characterization of multidrug-resistant extended-spectrum β-lactamase-producing Enterobacteriaceae isolated in Antananarivo, Madagascar. BMC Microbiol. 13:1-10.

Ramdani-Bouguessa N, Mendonca N, Leitao J, Ferreira E, Tazir M, Canica M (2006). CTX-M-3 and CTX-M-15 extended-spectrum β-lactamases in isolates of Escherichia coli from a hospital in Algiers, Algeria. J. Clin. Microbiol. 44:4584-4596.

Rasheed JK, Jay C, Metchock B, Berkowitz F, Weigel L, Crellin J, Steward C, Hill B, Medeiros AA, Tenover FC (1997). Evolution of extended-spectrum beta-lactam resistance (SHV-8) in a strain of Escherichia coliduring multiple episodes of bacteremia. Antimicrob. Agents Chemother. 41:647-653.

Réjiba S, Mercuri PS, Power P, Kechrid A (2011). Emergence and dominance of CTX-M-15 extended-spectrum β-lactamase among Escherichia coli isolates from children. Microb. Drug Resist. 17:135-140.

Robin F, Delmas J, Chanal C, Sirot D, Sirot J, Bonnet R (2005). TEM-109 (CMT-5), a Natural Complex Mutant of TEM-1 β-Lactamase Combining the Amino Acid Substitutions of TEM-6 and TEM-33 (IRT-5). Antimicrob. Agents Chemother. 49(49):4443-4445.

Rodriguez-Villalobos H, Struelens MJ (2006). Extended-spectrum β-lactamases mediated bacterial resistance: Implications for the intensivist. Rev Réanimation. 15(3):205-213.

Rossolini GM, D'Andrea MM, Mugnaioli C (2008). The spread of CTX-M type extended-spectrum β-lactamases. Clin. Microbiol. Infect. Rev. 14(1):33-41.

Sambrook J, Tritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2nd ed. 1: 1659.

Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Shahid M, Sobia F, Singh A, Malik A, Khan HM, Jonas D, Hawkez PM (2009). Beta-lactams and beta-lactamase-inhibitors in current or potential clinical practice: A comprehensive update. Crit. Rev. Microbiol. 35(2):81-108.

Soraas A, Sundsfjord A, Sandven I, Brunborg C, Jenum PA (2013). Risk Factors for Community-Acquired Urinary Tract Infections Caused by ESBL Producing Enterobacteriaceae –A Case–Control Study in a Low Prevalence Country. PLoS One 8(7):e69581.

Toubiana J, Timsit S, Ferroni A, Grasseau M, Nassif X, Lortholary O, MD, Zahar JR, MD, Chalumeau M (2016). Community-Onset Extended-Spectrum β-Lactamase- Producing Enterobacteriaceae Invasive Infections in Children in a University Hospital in France. Med. J. 95(12):e3163.