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Multidrug resistance of the extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolated in Tizi-Ouzou (Algeria)

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Abstract: The emergence and spread of multidrug-resistant bacteria is a major public health concern. This study sought to investigate the phenotypic and genotypic characteristics of clinical isolates of ESBL-producing *Klebsiella pneumoniae*, at University Hospital of Tizi-Ouzou. Antibiotic susceptibility testing of the strains was carried out by the disc diffusion method, the ESBL production was screening by the Double Disc Synergy Test and confirmed by the Phenotypic Confirmatory Disc Diffusion Test. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit mini kit (Qiagen) according to the manufacturer's instructions.PCR targeting the genes *bla*CTX-M, *bla*TEM, *bla*SHV, *bla*VEB, *bla*GES, *bla*PER, *bla*BEL, *bla*VIM, *bla*IMP, *bla*KPC, *bla*NDM and *bla*OXA48 was performed. A CTX-M PCR-based grouping method was carried out using primers specific to the groups 1, 2 and 9. Conjugative transfer of plasmids was carried out using sodium azide-resistant recipient strain *Escherichia coli* K12. The phylogenetic relationship was determined by ERIC-PCR. All strains of *K. pneumoniae* tested shared ESBL producer's genes belonging to the CTX-M group 1. These strains showed a high level of resistance to ß-lactams, aminoglycosides, fluoroquinolones and trimethoprim/ sulfamethoxazole. Resistance to fosfomycin was also detected in one strain of *K. pneumoniae*. Only one carbapenem-resistant strain was isolated. Phylogenetic analysis showed 49 different genetic profiles of *K. pneumoniae* strains, showing the absence of clonality. This study revealed a high prevalence of ESBL belonging to the CTX-M group 1 in *K. pneumoniae* tested. The emergence of resistance to carbapenem and fosfomycin, could seriously limits the therapeutic choices options.

Key words: Klebsiella pneumonia; Extended-spectrum beta-lactamase; Susceptibility; Antibiotics.

Introduction

The resistance of bacteria to antibiotics is constantly changing in terms of both susceptibility profile and incidence. The emergence and spread of multidrug-resistant bacteria are therefore of a major public health concern. The incidence of resistant strains has become alarming and requires the adoption of adequate strategies based on the epidemiological data collected from different countries of the world (1, 2). *Klebsiella* is an opportunistic genus belong to the *Enterobacterales* order and the *Enterobacteriaceae* family. *Klebsiella* are non-motile, rod-shaped, gram-negative bacteria with a prominent polysaccharide capsule. Some species of klebsiella are responsible for respiratory tract infections, septicaemia and urinary tract infections (3,4). *K. pneumoniae* is ranked as priority 1 (Critical) in the WHO Priority Pathogens for New Antibiotics Research and Development List in 2017. *Klebsiella pneumoniae* is an enterobacteriaceae of *Klebsiella* genus and known for its capacity to acquire multidrug resistance and virulence factors (capsular polysaccharide, lipopolysaccharide, adherence factors, urease, siderophore and biofilm formation). The combined presence of various antibiotic virulence and multidrug resistance factors in *Klebsiella pneumoniae* makes this species a high health risk germ. It is responsible for endemic and epidemic communal and nosocomial infections, particularly with the appearance of hypervirulent serotypes and multiresistant clones such as the ST11 clone of *Klebsiella pneumoniae* (5). The widespread use of antibiotics in clinical practice has led to the rapid appearance and spread of resistance to broad-spectrum beta-lactam antibiotics via the production of extended-spectrum beta-lactamase (ESBL) and carbapenemases. Resistance acquisition is mainly due to the horizontal transfer among clinical strains of genetic determinants associated to the antimicrobial resistance (6, 7).

Several studies have shown that the resistance of *K. pneumoniae* to antibiotics by enzymatic mechanism is by far the most frequent (8, 9, 10). More recently, resistance to fosfomycin and tigecycline, has been reported worldwide (11). ESBL belong to Ambler class A and D serine proteases. They are characterized for their ability to hydrolyze third and fourth cephalosporins and monobactams. ESBL encoding genes are generally located in plasmids. Beta-lactamases sharing the classical ESBL phenotype are structured into groups, according to sequence similarities. The most common include TEM, SHV, CTX-M, GES and VEB (class A) and OXA (class D) families. The prevalence of these enzymes varies by country and hospital. ESBL types

CTX-M (cefotaximase) are considered the most common type in the world (12, 13). CTX-M is classified into 6 distinct phylogenetic groups, composed of 172 different CTX-M isoforms: CTX-M-1, CTX-M 2, CTX-M-8, CTX-M-9, CTX-M-25 and CTX-M-45 (14). The CTX-M-15 variant belongs to the CTX-M-1 subgroup and is derived from CTX-M-3 by a mutation at the Asp to Gly^{240} position, which leads to an increased hydrolysis rate of ceftazidime compared to that of cefotaxime. Clavulanic acid and tazobactam show good inhibition of CTX-M-15 enzymes. Now the CTX-M15 concerns all *Enterobacteriaceae*, notably *K. pneumoniae*, and remains by far the most epidemiologically dominant in several countries in the world including Algeria (15, 16, 17). The treatment of nosocomial and/or community-acquired infections caused by *K. pneumoniae* producing ESBL and/or carbapenemases is becoming more and more difficult because the carbapenemases enzymes have hugely impacted the utility of carbapenems (often considered as last resort drugs) which are used for the management of multi-resistant Gram-negative bacilli, leading to therapeutic failures (18). The recent appearance of *K. pneumoniae* strains resistant to fosfomycin and tigecycline represent a new threat that further complicates the situation and shows that *K. pneumoniae* remains a very serious causative agent of therapeutic failure (19).

The aim of this study was to determine the antimicrobial resistance pattern of 58 non-duplicate clinical strains of *K. pneumoniae* resistant to third-generation cephalosporins, obtained from the Microbiology Laboratory of the University Hospital Center of Tizi-Ouzou during 2013 and 2014, screening and molecular characterization of ESBL, Conjugative potential of plasmids embedding resistance determinants and epidemiological relatedness of the strains will also be investigated.

Materials and Methods

Bacterial isolates

58 non-repetitive clinical strains of *K. pneumoniae* resistant to third-generation cephalosporins were obtained from the Microbiology Laboratory of the Tizi-Ouzou University Hospital Center. 27 strains were obtained during the period from January to September 2013 and 31 strains during the period from January to July 2014. The strains were isolated on Hektoen agar and incubated at 37°C during 24h. Identification was carried out with API20E (Biomerieux, France) and confirmed by MALDI-TOF (BRUKER, Microflex). 52 strains were from nosocomial origin (infectious diseases, pediatrics, pediatric emergency, medical reanimation, neonatology, haematology, medical emergencies, nephrology, surgical emergencies, urology and neurosurgery) versus 6 of community origin. The strains were collected from the following biological samples: urine (n = 29), blood (n = 7), pus (n = 16), bronchial fluid (n = 5) and horny fluid (n = 1).

Antimicrobial susceptibility testing

The antibiogram was performed with the standard method of diffusion in Muller-Hinton agar plates, according to CLSI standards (2014). Commercially available antibiotics discs (Biorad – France) included in this study were: Beta-lactamins : Ampicillin (10 µg), Cefoxitin (30 µg), Amoxicillin/Clavulanic acid (40 µg), Cefepime (30 µg), Piperacillin/Tazobactam (110 µg), Cefuroxime (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Ertapenem (10 µg), Meropenem (10 µg), Imipenem (10 µg), Aztreonam (30 µg). Aminosides : Gentamicin (10 µg), Amikacin (30 µg). Fluoroquinolones : Ciprofloxacin (5 µg). Cyclines : Minocycline (30 µg), Tigecycline (15 µg). Sulfamides : Trimethoprim/Sulfamethoxazole (25 µg) and Fosfomycin (200 µg). The classification of strains into sensitive, intermediate or resistant phenotypes is carried out according to the 2014 CLSI Standardization Manual. The standards used for tigecycline are EUCAST 2013 respectively. *Escherichia coli* strain ATCC25922 was used as a control strain.

Phenotypic detection of ESBL production

Isolates with reduced susceptibility (intermediate by CLSI criteria) to any of the 3GC were considered as potential ESBL producers. ESBL production by *K. pneumoniae* strains was screening by the Double Disc Synergy Test and confirmed by the Phenotypic Confirmatory Disc Diffusion Test as described previously (20, 21). A 5 mm increase in the area around the combined disk of the inhibition zone compared to the single disk indicates the production of an ESBL. The interpretation is performed according to the 2013 and 2014 CLSI Standardization Manual.

Molecular detection of ESBL production

Total DNA of the 58 clinical strains was extracted using a DNeasy blood and tissue kit (Quigen, Germany). The DNA was then subjected to different end-point multiplex PCR schemes targeting genes belonging to the beta-lactamase family's *bla*CTX-M, *bla*TEM, *bla*SHV *bla*VEB, *bla*GES, *bla*PER and *bla*BEL. A search for the *bla*VIM genes, *bla*IMP, *bla*KPC, *bla*NDM, *bla*OXA48 was carried out solely on the DNA of the S197 strain due to its resistance to carbapenems.

The amplification conditions was as follows: initial denaturation 95°C/15min followed by 25 cycles of 95°C/30s, 57°C/90s and 72°C/90s, followed by a final at 72°C/10min. The amplification products are subjected to capillary electrophoresis using a QIAxcel system (Qiagen, Germany). The primers sequences used in this study and their respective references are given in Table 1.

Molecular determination of the CTX-M group

Specific primers of the groups 1, 2 and 9 were used (Table 1). The amplification conditions are as follows: initial denaturation 94°C/5min followed by 25 cycles of denaturation at 94°C/25s, hybridization of the primers 53°C/40s and elongation at 72°C/50s. An additional elongation step is performed at 72°C/10min. The amplification products are subjected to capillary electrophoresis and revealed with QIAxcel (Qiagen).

ESBL resistance transfer assay

Conjugation experiments were performed using the sodium azide resistant *E. coli* K12 strains as recipient strain. Overnight cultures of donor and recipient strains grown in Mueller–Hinton broth at 37°C were mixed to-

Table 1. Primer sequences, amplicon sizes, targeted genes and related publications.

PCR	Primers	Sequence oligonucleotide 5' 3'	Gene type	Size product (pb)	References
PCR 1	TEM 5'	GTG CGG TAT TAT CCC GTG TT	bla_{TEM}	416	(22)
	TEM 3'	AA TTT ATC CGC CTC CAT CC			
	SHV5'	GGA AAC GGA ACT GAA TGA GG	bla_{SHV}	301	(22)
	SHV3'	ATC CCG CAGA TAA ATC ACC A			
	CTX5'	CG(CT) TTT (GC)C(AGCT) ATG TGC AG(CT) AC	bla_{CTX-M}	524	(22)
	CTX 3'	TC(AGCT) CCG CTG CCG GTY TTA TC			
PCR 2	PERFW	AGT GTG GGG GCC TGA CGA T	bla_{PER}	725	(23)
	PERRV	GCA ACC TGC GCA ATR ATA GCT T			
	VEBFW376	CGA CTT CCA TTT CCC GAT GC	bla_{VEB}	376	(23)
	VEBRV376	TGT TGG GGT TGC CCA ATT TT			
	GESFW600	CTG GCA GGG ATC GCT CAC TC	bla_{GES}	600	(23)
	GESRV600	TTC CGA TCA GCC ACC TCT CA			
PCR3	BEL1F	CGA CAA TGC CGC AGC TAA CC	bla_{BEL}	448	(23)
	BEL1R	CAG AAG CAA TTA ATA ACG CCC			
	IMP2012A	ACA-YGG-YTT-RGT-DGT-KCT-TG	bla_{IMP}	387	(23)
	IMP2012B	GGT-TTA-AYA-AAR-CAA-CCA-CC			
	VIM1-437Fw	TGT-CCG-TGA-TGG-TGA-TGA-GT	bla_{VIM}	437	(23)
	VIM1-437Rv	ATT-CAG-CCA-GAT-CGG-CAT-C			
	NDM-1Fw	ACT-TGG-CCT-TGC-TGT-CCT-T	bla_{NDM}	603	(23)
	NDM-1Rv	CAT-TAG-CCG-CTG-CAT-TGA-T			
	KPCF	TCG-CCG-TCT-AGT-TCT-GCT-GTC-TTG	bla_{KPC}	353	(23)
	KPCR	ACA-GCT-CCG-CCA-CCG-TCA-T			
	OXA-48F2	ATG-CGT-GTA-TTA-GCC-TTA-TCG	bla_{OXA48}	265	(23)
	OXA-48R2	CAT-CCT-TAA-CCA-CGC-CCA-AAT-C			
	ERIC2	AAG TAA GTG ACT GGG GTG ACGC	Enterobacterial repetitive consensus sequence	-	(24)
PCR 4	CTX-M G1 F	AAA AAT CAC TGC GCC AGT TC	$bla_{CTX-M\ group\ 1}$	415	(25)
	CTX-M G1 R	AGC TTA TTC ATC GCC ACG TT			
	CTX-M G2 F	CGA CGC TAC CCC TGC TAT T	$bla_{CTX-M\ group\ 2}$	552	(25)
	CTX-M G2 R	CCA GCG TCA GAT TTT TCA GG			
	CTX-M G9 F	CAA AGA GAG TGC AAC GGA TG	$bla_{CTX-M\ group\ 9}$	205	(25)
	CTX-M G9 R	ATT GGA AAG CGT TCA TCA CC			

gether at 1:10 (v/v) proportion and incubated for 4h at 37°C without shaking. Part of the mixture was diluted (1:10 and 1:100) and 0.1 mL was plated on Muller-Hinton supplemented with 8µg/mL of Cefotaxim and 300µg/mL of sodium azide (26). The transconjugants were maintained on the selection plates and then subjected to identification by MALDI-TOF, antibiotic susceptibility testing, double disc synergy test and PCR to confirm the acquisition of ESBL genes. Conjugation experiments were repeated three times for each donor strain.

Molecular Typing

Phylogenetic relationship between the 58 strains of *K. pneumoniae* was determined by ERIC-PCR (Enterobacterial Repetitive Consensus PCR) using the ERIC2 primers (Table 1). PCR was performed following cycling conditions 95°C/3 min, then 40 cycles of 92°C/30s, 52°C/1 min, 72°C/8 min and once at 72°C/16min. The electrophoretic profiles of the amplification products were compared by the Bionumeric software version 6.5 (Applied Maths, Belgium). Two profiles are considered different if they differ by at least one band.

Results

In this study, 58 non-repetitive strains of *K. pneumoniae* were obtained from different clinical specimens from the Microbiology Laboratory of the Tizi-Ouzou University Hospital Center. All the strains were resistant to Cefotaxime and Ceftriaxone. A resistance rate of 67.24%; 63.79%; 70.69%; 67.24% was observed to amoxicillin- clavulanic acid; ceftazidime; cefepime and aztreonam respectively. Table 2 shows the result of the AST (Antimicrobial susceptibility testing) analysis for the 58 strains of *K. pneumoniae*.

The phenotypic detection test for the production of ESBL was positive for 57 strains (98.27%). The results of the synergy test showed the appearance of areas of synergy between Amoxicillin/Clavulanic acid and Cef-

Table 2. Percentage of resistance to antimicrobials agent of the 58 ESBL *Klebsiella pneumoniae* clinical strains.

Antimicrobials (Disc load)	**Breakpoints Inhibition zone (mm)**		**Phenotypes (%)**		
Antimicrobials	R	S	S	I	R
Ampicilline (10µg)	≤13	≥17	00	00	100
Amoxicillin+Clavulanic acid (20+10µg)	≤13	≥18	18.96	13.79	67.24
Piperacillin+Tazobactam (110µg)	≤17	≥21	43.10	43.10	13.79
Cefoxitin (30µg)	≤14	≥18	98.27	00	01.72
Cefuroxim (30µg)	≤14	≥18	00	00	100
Ceftriaxon (30µg)	≤19	≥23	00	00	100
Cefotaxim (30µg)	≤22	≥26	00	00	100
Ceftazidim (30µg)	≤17	≥21	08.62	27.58	63.79
Cefepim (30µg)	≤18	≥25	00	29.31	70.69
Ertapenem (10µg)	≤18	≥22	98.27	00	01.72
Imipenem (10µg)	≤19	≥23	98.27	00	01.72
Meropenem (10µg)	≤19	≥23	98.27	00	01.72
Aztreonam (30µg)	≤17	≥21	05.17	27.58	67.24
Gentamicin (10µg)	≤12	≥15	13.80	00	86.20
Amikacin (30µg)	≤14	≥17	98.38	00	08.62
Ciprofloxacin (05µg)	≤15	≥21	20.68	15.51	63.79
Trimethoprim/Sulfamethoxasol (25µg)	≤10	≥16	10.35	00	89.65
Minocyclin (30µg)	≤12	≥16	79.31	18.96	01.72
Tigecyclin (15µg)	< 15	≥18	83.18	16.82	00
Chloramphenicol (30µg)	≤12	≥18	86.21	00	13.79
Fosfomycin (200µg)	≤12	≥16	95.55	1.72	01.72

S: sensitive, I: intermediate, R: resistant.

tazidime, Ceftriaxone and cefepime. Areas of synergy are also observed between cefepime and piperacillin/tazobactam. The presence of ESBL was confirmed by the confirmatory test and an increase of more than 5 mm in the inhibition zone is observed for the 57 positive strains.

The results of the antibiograms also show a relatively high percentage of resistance to antibiotics of other families such as aminoglycosides (Gentamicin 86.20%), fluoroquinolones (Ciprofloxacin 63.79%) and the combination trimethoprim + sulfamethoxazole (SXT 89.65%). At the same time resistance levels chloramphenicol (13.79%) and fosfomycin (1.72%) remain low. The strain S197 is resistant to all ß-lactams (cefoxitin included, which supposes the existence of a chromosomal or plasmidic cephalosporin) including carbapenems, aminoglycosides, fluoroquinolones, and trimethoprim + sulfamethoxazole intermediate with respect to fosfomycin. The synergistic zone was not observed for this strain. However, the *bla*CTX-M gene was detected by PCR. These results showed that 100% of our strains were ESBL positive. Molecular of the strain S197 shows the absence of carbapenemase genes such as *bla*VIM, *bla*IMP, *bla*KPC, *bla*NDM, *bla*OXA48. The strain S1115 was resistant to fosfomycin. The multiplex PCR amplifications of ß-lactamases showed the presence of the *bla*TEM, *bla*SHV and *bla*CTX-M genes. The combinaison *bla*SHV, *bla*TEM and *bla*CTX-M was found 48 strains, *bla*SHV and *bla*CTX-M in 8 strains and *bla*TEM and *bla*CTX-M in 2 strains respectively. There was no strain containing the beta-lactamase genes namely *bla*VEB, *bla*GES, *bla*PER, *bla*BEL. The PCR amplification carried out on the total DNA of the 58 strains of *K. pneumoniae* using the primers specific for different groups of the gene *bla*CTX-M showed that all strains belong to the phylogenetic group CTX-M1.

The results of the conjugation experiments carried out on ten (10) strains *K. pneumoniae* were selected on the basis of their antibiotic resistance profiles and biological origin, these strains were S197, S825, S2042, S1212, S1216, S1766, S4683, S4936, S1115 and S364 for plasmid transfer essay experiments. Eight (08) transconjugants were obtained. The susceptibility testing of recipient strains showed a resistance to aminoglycosides, fluoroquinolones, trimethoprim + sulfamethoxazole and chloramphenicol associated with resistance to ß-lactamines. The phenotypic analysis also showed the transfer of ESBLs, as confirmed by PCR, particularly the *bla*CTX-M gene. Table 3 shows the results obtained on transconjugants. Molecular genotyping obtained by ERIC-PCR showed the presence of forty-nine (49) different amplification profiles, which is interpreted as an absence of clonality between the isolated strains.

Discussion

K. pneumoniae ESBL is considered as one of the main agents of nosocomial infections at the hospital level (27). The prevalence of *K. pneumoniae* ESBL isolates at the Tizi-Ouzou CHU was 23% in 2013 and 30% in 2014 (28, 29). Epidemiological studies have shown similar results in other countries (30, 17). These studies showed a very high percentage of resistance to ß-lactams except carbapenems with a low percentage of resistance. The percentage of resistance to beta-lactams is significantly higher than those previously reported

Table 3. Resistance pattern and cotransfered resistance of *Klebsiella pneumoniae* (n = 8) and their transconjugants.

Isolates	Specimen	Resistance pattern	bla β-lactamase	Transferred resistance	bla β-lactamase
S197	Urine	AMC-CAZ-FEP-CTX- ATM-GMN -AKN-SXT-CHL	SHV-TEM-CTX-M	CTX-GMN	SHV-CTX-M
S825	Urine	AMC-CAZ-FEP-CTX-ATM-GMN-AKN-SXT-CIP-CHL	SHV-TEM-CTX-M ATM-CHL	CAZ$^{(I)}$-CTX-FEP$^{(I)}$-ATM-CHL	SHV-CTX-M
S2042	Blood	AMC-CAZ-CTX-ATM-GMN-AKN-SXT-CHL	SHV-TEM-CTX-M	CAZ$^{(I)}$-CTX -GMN	SHV-CTX-M
S1115	Bronchial	FEP-CAZ-CTX-ATM-SXT- CIP-FOS- GMN	SHV-TEM-CTX-M	CAZ$^{(I)}$-FEP$^{(I)}$-CTX-ATM$^{(I)}$-GMN	SHV-CTX-M
S1212	cornea	AMC-CAZ-CTX-GMN- SXT-CIP	SHV-TEM-CTX-M	AMC$^{(I)}$-CTX- CAZ$^{(I)}$-GMN -CIP$^{(I)}$-SXT	SHV-CTX-M
S4683	Pus	FEP-CTX-ATM-GMN-CIP-CHL	SHV-TEM-CTX-M	FEP$^{(I)}$-CTX-GMN	SHV-CTX-M
S1766	Urine	CAZ-CTX-ATM-GMN-CIP-CHL	CTX-M-TEM	CTX-GMN	TEM-CTX-M
S4936	Pus	AMC-CAZ-FEP-CTX-ATM-GMN-AKN-SXT-CIP	SHV-TEM-CTX-M	CAZ-FEP$^{(I)}$-CTX-ATM$^{(I)}$	SHV-CTX-M

AMC : Amoxicilline/Acide clavulanic ; CTX :Cefotaxime ; CAZ : Ceftazidime ; FEP :Cefepime ; ATM : Aztréoname ; GMN : Gentamicine ; AKN : Amikacine ; CIP : Ciprofloxacine ; SXT : Trimethoprim/sulfamethoxasol; CHL : Chloramphenicol ; FOS : Fosfomycine . (I) : intermediare.

in Algeria (1); Iran (31); Brazil (32) and in India (33). However, it is rather similar to those reported in Morocco (34); Sri Lanka (13); in South Africa (11) and in Pakistan (35). The absence of a synergistic zone for strain S197 could be explained by the presence of a second beta-lactam resistance mechanism such as the production of a plasmid-mediated cephalosporinase or a hyperproduction of a chromosomal cephalosporinase, which could be supported by the resistance of this strain to cefoxitin. Plasmids harbouring cephalosporinase conferring resistance to cefoxitin were characterized in *K. pneumoniae* in Algeria (36) and worldwide (37). The resistance to carbapenems of the strain S197 might not be related to an enzymatic mechanism such as carbapenemases, as suggested by the negative results of amplification of the *bla*VIM, *bla*IMP, *bla*KPC, *bla*NDM and *bla*OXA48 genes. It has been reported that the production of certain beta lactamases such as cephalosporinases or ESBL associated with a mechanism of impermeability by the loss of porins can lead to carbapenem resistance of some strains of *K. pneumoniae* (38).

The high prevalence of resistance of strains of *K. pneumoniae* to antibiotics of the beta-lactam family could be explained by the extensive and abusive use of antibiotics of this family in hospitals. Moreover, the clinical *K. pneumoniae* ESBL isolates showed high percentage of resistance to other classes of antibiotics such as aminoglycosides and fluoroquinolones. This could also be explained by the location of determinants of resistance to these antibiotics on the same mobile genetic element as plasmids of *bla*CTX-M gene (30). The use of Fosfomycin and tigecycline are restricted to hospitals. These are the last resort drugs for the treatment of infections caused by *K. pneumoniae* ESBL and/or carbapenemases. In recent years, resistance to fosfomycin and tigecycline have emerged and are increasing in *K. pneumoniae* strains worldwide (39, 40). The isolates of *K. pneumoniae* obtained during this study showed a low percentage of resistance to fosfomycin. However, the percentage of tigecycline-intermediate phenotype strains may predict a shift towards the tigecycline resistance phenotype. The results of percentage resistance in our study were significantly lower than those reported by several authors around the world concerning tigecycline (32, 11). Overexpression of the AcrAB efflux pump is the most important mechanism for the resistance of *K. pneumoniae* strains to tigecycline (41). Resistance to fosfomycin was reported in several strains of *K. pneumoniae* worldwide, these strains show several resistance mechanisms such as enzymatic inactivation, target modification and decreased permeability of fosfomycin (42). The acquisition of resistance to fosfomycin and tigecycline by clinical isolates of *K. pneumoniae* sharing ESBL and/or carbapenemases of represents a serious problem for the treatment of infections because these antibiotics were utilized in last resort for treatment of ESBL and/or carbapenemases *K. pneumoniae* infections (8). The results of PCR amplification of the ß-lactamase genes *bla*TEM, *bla*SHV and *bla*CTX-M showed that all the strains harbour a *bla*CTX-M gene. This may explain the high level of resistance to third- and fourth-generation cephalosporins (43). Strains of *K. pneumoniae* carrying CTX-M group 1 enzymes are frequently isolated from the hospital environment. Similar results are reported by some authors in Algeria (1, 36) and also in Tunisia (44). The high percentage of resistance to ceftazidime in some strains of *K. pneumoniae* could be explained by the presence of the *bla*CTX-M15 gene. Indeed, several studies carried out in Algeria show the predominance of the *bla*CTX-M15 and *bla*CTX-M3 genes in some province in the north of the country such as Algiers, Tlemcen, Bejaia and Annaba (1, 45, 9, 46, 47, 16). The results of our study show that the *bla*CTX-M gene is easily transferable by conjugation. Intermediate resistance phenotypes are observed in some transconjugants against certain antibiotics such as ceftazidime, cefepime, aztreonam, amoxicillin-clavulanic acid and ciprofloxacin, this could be explained by the structural difference of the cell wall and plasma membrane between *K. pneumoniae* and *E. coli* K12. Indeed, the decrease in permeability is one of the resistance mechanisms of strains of *K. pneumoniae* with regard to antibiotics belonging to the family of beta-lactams, aminoglycosides and fluoroquinolones (48). These intermediate resistance phenotypes could also be explained by the level of expression of ESBL- genes

in *E. coli* K12. Indeed, during bacterial conjugation, a single plasmid molecule might be transferred therefore reducing the effective amount of enzyme produced by the transconjugant strain compared to that of the wild-type strain, reducing the catalytic efficiency of ESBLs and resistance (15). Co-transfers have been described for resistance to aminoglycosides, fluoroquinolones, sulfonamides and phenicols with beta-lactams, indicating that the genetic determinants of these resistances are carried by the same conjugative plasmid. The co-transfer of these resistances in *K. pneumoniae* is reported by several authors (49, 50, 1). The ERIC-PCR results show different profiles of *K. pneumoniae* strains which show the absence of a particular clone.

Multidrug-resistant strains of *K. pneumoniae* are still a serious public health problem. Their dissemination requires the use of carbapenems, thus inducing the appearance and spread of resistance to them. The onset of resistance to fosfomycin could lead to very serious therapeutic impasses in the treatment of infections caused by these ESBL strains and/or resistant carbapenems. In view of all these results, a much more rational use of antibiotics in hospitals is more than recommended, and local, regional and international surveillance is needed regarding the evolution of the resistance of these strains that become more and more resistant. Performing systematic and accurate identification of ESBL strains and/or resistant carbapenems producers in clinical microbiology laboratory is an important first step and provides key evidence for control of theses strains. The early identification of ESBL strains and/or resistant carbapenems producing isolates in clinical infections should be mandatory to prevent the development of untreatable infections. On the other hand, national and international campaigns to educate health-care providers, patients and lay persons may be warranted to limit the over-use and abuse of antibiotics in humans and agriculture. It would be interesting to continue this study by determining the type of CTX-M by sequencing, the study of the genetic environment and the determination of resistance genes to other families of antibiotics as well as their genetic supports.

References

1. Messai Y, Iabbaden H, Benhassine T, Alouache S, Tazir M, Gautier V, Bakour R. Prevalence et caracterisation des β-lactamases à spectre élargi chez *Klebsiella pneumoniae* dans les hopitaux d'Alger (Algérie). Pathologie Biologie. 2008 ; 56 : 319-325.

2. Bin LI, Yong YI, Wang QI, Partick CYW, Lin T, Hua J, George FG, Cui HL. Analysis of drug resistance determinants in *Klebsiella pneumoniae* isolates from Tertiary-Care Hospital in Beijing, CHINA. PLoS One. 2012; 7(7) : e42280.

3. Stone PW, Gufta A, Loughrey RN, Della-latta PH, Cimiotti RN, Larson E, Rubenstrein D, Saiman L. Attribuable Coast and Length of stay of an Extended Spectrum β-Lactamases Producing *Klebsiella pneumonia* Outbreak In Neonatal Intensive Care Unit. Infect Control Hosp Epidemiol. 2003; 24 (8) : 601-6.

4. Kumar V, Sun P, Vamathevan J, Li Y, Ingraham K, Palmer L, Huang J, Brown J. Comparative genomic of *Klebsiella pneumoniae* strains with different antibiotics resistance profils. Journal of Antimcrobial Chemotherapy. 2011; 55 : 4267-76.

5. Min Wu and Xuefeng Li. *Klebsiella pneumoniae* and *Pseudomonas aeroginosa*. Molecular Medical Microbiology. Chapter 27. Doi: http: //dx.doi.org/10.1016/B978-042-397169-02-00087-1.

6. Francis SC and Eric SD. Carbapenem resistance: A Review. Med Sci. 2018; 6(1).1.

7. Kelly LW and Holt KE. *Klebsiella pneumonia* as a key traffiker of drug genes from environmental to clinically important bacteria. Curr Opin Microbiol. 2018; 45 : 131-139.

8. Cheng YH, Lin TL, Pan YJ, Wang YP, Lin YT, Wang JT. Colistin resistance mechanisms in *Klebsiella pneumonia* strains from TAIWAN. Antimicrob Agents Chemother. 2015; 59. Number 5.

9. Nedjai S, Berguigua A, Djahmi N, Jamali L, Zerouali K, Dekhil M, Timimouni M. Prevalence and characterization of extended spectrum β-lactamases in *Klebsiella-Enterobacter-Serratia* group bacteria in Algeria. Med Mal Infect. 2012; 42 : 20-29.

10.Cao X, Xu X, Zhang Z, Shen H, Chen J, Zhang K. Molecular characterization of clinical multi-drug resistant *Klebsiella pneumonia* isolates. Ann Clin Microbiol Antimicrob. 2014; 13 : 16

11.VASAIKAR S, Obi L, Morose I, Bisi-johnson M. Molecular characteristics and antibiotics resistance profils of *Klebsiella pneumonia* isolates in Mthatha, East Cape Province, South Africa. Int J Microbiol. 2017; ID8486742. 7

12.Robin F, Hennequin C, Gniadkowski M, Beyrouthy R, Empel J, Gibold L, Bonnet R. Virulence factors and TEM type β-lactamases Produced by two isolates of an Epidemic *Klebsiella pneumonia* strain. Antimicrob Agents Chemother. 2012; 56(2) : 1101-1104.

13.Fernando MMPSC, Luke WANV, Mithinda JKND, Wickramasinghe RDSS, Sebastiampilla BS, Gunathilake MPML, Silva FHDS, Premaratna R. Extended Spectrum β-lactamases producing organisms causing urinary infections in SRI-LANKA and their antibiotic susceptibility pattern. A Hospital based cross sectional study. Infectious Diseases. 2017; 17 : 138.

14.Muddser A, Arif TJ, Qazi MRH. *bla*CTX-M152, a Novel Variant of CTX-M group 25, identified in a study Performed on the Prevalence of Multidrug Resistance among Naturel Inhabitants of River Yamnna, India. Front Microbiol. 2016; 7 : 176.

15.Poirel L, Gniadkowski M, Nordmann P. Biochemical analysis of ceftazidime-hydrolyzing Extended Spectrum β-lactamases CTX-M-15 and its structurally related β-lactamases CTX-M-13. J Antimicrob Chemother. 2002; 50: 1013-1034.

16.Baba Ahmed-Kazi TZ, Decré D, Genel N, Boucherit-Otmani Z, Arlet G, Drissi M. Molecular and epidemiological characterization of enterobacterial multidrug resistant strains in Tlemcen Hospital (Algeria) (2008-2010). Microb Drug Resist. 2013; (19) : 185-90.

17.Bevan RE, Jones AM, Hawkey PM. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. J Antimicrob Chemother. 2017; 72 (8) : 2145-2155.

18.Falagas ME, Lourida P, Poulikakos P, Rafailidis PI, Tansarki GS. Antibiotic Treatement of infections due Carabapenem-resistant Enterobactereaceae: Systematic Evaluation of the Avaible Evidence. Antimicrob Agents Chemother. 2013; 58(2) : 654-663.

19.Veeraraghavam B, Perumalla KS, Ragupathi KDN, Sethuvel MP, Inian S, Inbanathan Y. Coexistence of fosfomycin and colistin resistance in *Klebsiella pneumonia*: Whole-genome shotgun sequencing. Genome Annonc. 2016; 4 (6).

20.Clinical and Laboratory Standard Institut. Performance standards for Antimicrobial Susceptibility testing ; Twenty-third. Informational Supplement. Document M100-S23. CLSI : Wayne, PA, USA. 2013.

21.Clinical and Laboratory Standard Institut. Performance standards for Antimicrobial Susceptibility testing ; Twenty-third. Informational Supplement. Document M100-S24. CLSI : Wayne, PA, USA. 2014.

22.Rodriguez-Villalobos H, Malaviolle V, Frankard J, de Mendonça R, Nonhoff C, Struelens MJ. In vitro activity of temocillin against extended spectrum beta-lactamase-producing *Escherichia*

coli. J. Antimicrob. Chemother. 2006; 57: 771–774 http://dx.doi.org/10.1093/jac/dkl046.
23.Bogaerts P, Rezende de Castrol R, de Mendonça R, Huang TD, Denis O, Glupczynski Y. Validation of carbapenemase and extendedspectrum beta-lactamase multiplex endpoint PCR assays according to ISO 15189. J. Antimicrob. Chemother. 2013; 68: 1576–1582. http://dx.doi.org/10 .1093/jac/dkt065.
24.Decré D, Burghoffer B, Gautier V, Petit JC, Arlet G. Outbreak of multi-resistant *Klebsiella oxytoca* strains with Extended Spectrum β-lactamases and strains with extended spectrum activity of chromosomal β-lactamase. J Antimicrob Chemother. 2004; 54 : 881-8.
25.Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR rapid detection of gene encoding CTX-M Extended Spectrum β-lactamases. J. Antimicrob. Chemother. 2005; doi: 10.1093/jac/dki412
26.Abbassi MS, Torres C, Achour W, Vinué L, Sáenz Y, Costa D, Bouchachi O, Ben hassen A. Genetic characterization of CTX-M 15 producing *Klebsiella pneumonia* and Escherichia strains isolated from stem cell transplant patients in Tunisia. Int J Antimicrob agents. 2008; 32 : 308-314.
27.Navon-venezia S, Kondratyera K, Carattoli A. *Klebsiella pneumonia*: a major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol Rev. 2017; 41(3) : 252-275.
28.Azzam A. Caracterisation moléculaire des BLSE chez *Klebsiella pneumonia* isolées au CHU de Tizi-Ouzou en 2013.
29.Azzam A. Caracterisation moléculaire des BLSE chez *Klebsiella pneumonia* isolées au CHU de Tizi-Ouzou en 2014
30.Alibi S, Ferjani A, Boukadida J. Molecular characterization of Extended Spectrum β-lactamases produced by *Klebsiella pneumonia* clinical strains from a Tunisian Hospital. Med Mal Infect. 2015; 45 : 139-143.
31.Davod M, Mohammad M, Jamal S, Balak S, Azad K. Antibiotic susceptibility pattern and identification of Extended Spectrum β-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumonia* from Shiraz, Iran. Iran J Microbiol. 2016; 8(1) : 55-61.
32.Santana RC, Gaspar GG, Vilar FC, Bellissimo-Rodrigues F, Martinez R. Secular trends in *Klebsiella pneumonia* isolated in a tertiary carre hospital: increasing prevalence and accelerated declin in antimicrobial sesceptibilty. Rev Soc Bras Med Trop. 2016; 49(2) : 177-182.
33.Vemula S and Vadde R. Prevalence of ESBL- producing *Klebsiella pneumonia* osolates in Tertiary Care Hospital. International Scholary research Network, ISRN Microbiology, 2011; Volume 2011, doi : 5402/2011/318348.
34.Natoubi S, Berguigua A, Zerioul BS, Baghdad N, Timimouni M, Hilali A, Amghar S, Zerouali K. Incidence of Extended Spectrum β-lactamases producing *Klebsiella pneumonia* among patients and the environment of Hassan II hospital, settat , Morocco. Adv Microbiol. 2016; 6 : 152-161.
35.Batool A, Baig H, Qumar MU. Extended Spectrum β-lactamases producing *Escherichia coli* and *Klebsiella pneumonia* causing urinary tract infection. Afr J Microbiol Res. 2016; 10(42) : 1775-1778.
36.Iabaden H, Messai Y, Ammari H, Alouache S, Verdet C, Bakour R, Arlet G. Prevalence of plasmid-mediated AmpC β-lactamases among Enterobacteriaceae in Algiers hospitals. Int J Antimicrob Agents. 2009; 34 : 340-342.
37.Grover CN, Sahni AKB, Bhattacharya CS. Therapeutic challenges of ESBLs and AmpC beta lactamase producers in a tertiary care center. Med J Armed Forces India. 2013; 69 : 4-13.
38.Martinez-Martinez L, Conejo MC, Pascual A, Hernández-Allés S, Ballesta S, Ramírez de Arellano-Ramos E, Benedi VJ, Perea EJ. Activities of imipenem and cephalosporins against clonally related strains of *Escherichia coli* hyperproduction chromosomal β-lactamases and showing altered porin profils. Antimicrob Agents Chemother. 2000; 2534-2536.
39.Al-Tawfik JA, Laxminarayan R, Mendelson M. How should we respond to the emergence of plasmid-mediated colistin resistance in humains and animals? Int J Infect Dis. 2017; 54 : 77-84.
40.Pragasam KA, Shankar C, Veeraraghavan B , Biswas I, Nabarro LEB, Inbanathan FY, George B, Verghese S. Molecular mechanisms of colistin resistance in *Klebsiella pneumonia* causing bacteremia from Indian, A first report. Front Microbiol. 2017; doi : 10.3389/fmicb.2016.02135.
41.Sheng ZK, Hu F, Wang W, Guo Q, Chen Z, Xu X, Zhu D, Wang M. Mechanisms of tigecycline resistance among *Klebsiella pneumonia* clinical isolates. Antimicrob Agents Chemother. 2014;58(11) : 6982- 6985.
42.Karageorgopoulos DE, Wang R, Hong X, Falagas ME. Fosfomycin : evaluation of the published evidence on the Gram-negative pathogens. J Antimicrob Chemother. 2012; 67(2) : 255-268.
43.Poirel N T, Nordmann P. Genetic support of Extended Spectrum β-lactamases. Clin Microbiol Infect. 2008; 14 : 75-81.
44.Mamlouk K, Boubaker IB, Gautier V, Vimont S, Picard B, Ben Redjeb S, Arlet G. Emergence and Outbreaks of CTX-M β-lactamases producing *Escherichia coli* and *klebsiella pneumonia* strains in Tunisian hospital. J Clin Microbiol. 2006; 4049-4056.
45.Ramdane-Bouguessa N, Manageiro V, Jones-Dias D, Ferreira E, Tazir M, Carica M. Role of SHV beta-lactamase variants in resistance of clinical *klebsiella pneumonia* strains to beta-lactamins in Algerian hospital. J Med Microbiol. 2011; 60 : 983-7.
46.Touati A, Medboua C, Touati D, Denine R, Brasme L, De Champs C. CTX-M 15 producing Enterobcateriaceae isolats causing bloodstream infections at Beni-Messous hospital Algeries(Algeria). Int Res J Microbiol. 2012; 3: 181-5.
47.Gharout-Sait A, Touati A, Guillard T, Brasme L, De Champs C. Molecular characterization and epidemiology of cefoxitin resistance among Enterobacteriaceae laking inductible chromosomal AmpC genes from hospitzalized patients in Algeria: description of new sequence type in *klebsiella pneumonia.* Braz J Infect Dis. 2015; 19(2) : 187-195.
48.Chevalier j, Pagés JM, Eyraud A, Mallea M. Membrane permeability modifications are involved in antibiotic resistance in *klebsiella pneumonia*. Biochem Biophys Res Commun. 2000; 274(2) : 496-9.
49.Pons JM, Vubil D, Guirad E, Jaintilal D, Fraile O, Soto SM, Sigauque B, Nhampossa T, Aide P, Alonso PL, Vila J, Mandomando I, Ruiz J. Characterization of Extended Spectrum β-lactamases among *klebsiella pneumonia* isolates causing bacteraemia and urinary tract infection in Mozambique. J Glob Antimicrob Resist. 2015; 3(1) : 19-25.
50.Souza RD, Pinto NA, Hwang I, Younjee H, Cho YL, Kim H, Yong D, Choi J, Lee K, Chong Y. Molecular epidemiology and resistance analysis of multidrug-resistance ST1 *klebsiella pneumonia* strain containing multiple copies of Extended Spectrum β-lactamases gene using whole-genome. New Microbiol. 2017; 40(1) : 38-44.