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ORIGINAL ARTICLE

Molecular Characterization of Extended Spectrum β-Lactamases, Ampccephalosporinases and Carbapenemases in *Klebsiellapneumoniae* Causing Bacteremia at Charles Nicolle Hospital of Tunisia

Elaa Maamar[1], Samia Hammami[1,2,3], Sana Ferjani[1], Zaineb Hamzaoui[1], Asma Jlizi[4], Saidani M[1,2], Slim A[1,2], Boutiba-Ben Boubaker I[1,2]

[1]University of Tunis El Manar, Faculty of Medicine of Tunis -LR99ES09 Research Laboratory «Antimicrobial resistance», 1007 Tunis-Tunisia, [2]Charles Nicolle Hospital, Laboratory of Microbiology,1006-Tunis-Tunisia, [3]University of Gafsa, Faculty of Sciences of Gafsa-Tunisia, [4]Rabta University Hospital, Laboratory of Microbiology,1007-Tunis-Tunisia

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Elaa Maamar

ABSTRACT

Purpose of the Study: This study was conducted to detect and characterize the genes encoding extended spectrum β-lactamases and associated β-lactamases (carbapenemases and Ambler Class C β-lactamases).

Patients and Methods: In 2011, out of the 65 non-duplicative *Klebsiellapneumoniae* collected from blood culture at Charles Nicolle hospital of Tunisia, 36 were resistant to 3rd generation cephalosporin.

Results: All strains showed a double disk synergy test positive. They were mainly isolated in intensive care unit (31%). They were frequently resistant to most antibiotics tested, except colistin and tigecyclin. Five isolates (13%) showed reduced susceptibility to carbapenems. $bla_{CTX\text{-}M\text{-}15}$ was harbored by 35 strains and $bla_{SHV\text{-}12}$ by one. $bla_{CTX\text{-}M\text{-}15}$ were associated with $bla_{TEM\text{-}1}$ (n=21), $bla_{OXA\text{-}48}$ and $bla_{CMY\text{-}2}$ (n=1) and $bla_{OXA\text{-}48}$and $bla_{TEM\text{-}1}$ (n=4). The conjugation wassuccessfulfor4/5 strains (3 harboring $bla_{CTX\text{-}M\text{-}15}$ and one $bla_{SHV\text{-}12}$). The plasmids carrying the $bla_{CTX\text{-}M\text{-}15}$ were assigned to IncN or IncL/M only for 2 strains. The remaining $bla_{CTX\text{-}M\text{-}15}$-carrying plasmid was negative for all of the replicons tested as well as the $bla_{SHV\text{-}12}$-carrying plasmid.

Conclusion: Our results confirm the spread of CTX-M-15 in our institution. To our knowledge, this is the first report of *K. pneumoniae* coproducing CTX-M-15, CMY-2 and OXA-48. The implementation of preventive measures against the spread of these multiresistant bacteria is needed.

INTRODUCTION

Since their description in the mid-1980s, extended spectrum β-lactamase (ESBL)-producing organisms have become recognized as a worldwide problem.[1] Although ESBLs have been detected in a wide variety of Gram-negative bacteria, *Klebsiellapneumoniae* has been found to be the most common species to produce ESBLs.[2] Because ESBL-producing organisms are frequently multidrug resistant, therapeutic options for these infections are severely limited and treatment can be challenging. Also, there have been many reports of outbreaks caused by these organisms, and it has been demonstrated that ESBL production by infecting organisms adversely affects the clinical outcome with a significant morbidity and mortality.[3-6]

In Tunisia, epidemiological data of the spread of ESBL-producing *K. pneumoniae*strains showed a rapid diffusion since their first description in 1984.[7] In fact, their prevalence varies from 27.9% in 1999 to 51.5% in 2007[8] with a high incidence 87.5% in Mongi Slim Hospital followed by 19%[4] in Children's Hospital.

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Corresponding Author:
Elaa Maamar, University of Tunis El Manar, Faculty of Medicine of Tunis -LR99ES09 Research Laboratory «Antimicrobial resistance», 1007 Tunis-Tunisia. Phone: +21654236119, E-mail: alaa_maamar@hotmail.com

This study was conducted to detect and characterize ESBLs and associated β-lactamases (carbapenemases and Ambler Class C β-lactamases (AmpC)) produced by *K. pneumoniae* resistant to 3rd generation cephalosporin (3rd GC) causing bacteremia at Charles Nicolle Hospital, during 2011.

MATERIALS AND METHODS

Bacterial Isolates

Among the 65 non-duplicate *K. pneumoniae* isolated from blood cultures of 65 patients hospitalized at Charles Nicolle Hospital of Tunis from January to December 2011, 36 (55.3%) were resistant to 3rd GC. They were collected mainly from intensive care unit (31%), neonatology (26%) and surgery (23%) (Table 1).

Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing was determined by the disk-diffusion method on Mueller-Hinton (MH) agar plates (Bio-Rad, Marnes-la-Coquette, France) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[9] ESBL production was detected by the double disk synergy test (DDST) with or without cloxacillin as described previously.[10] Isolates with reduced susceptibility to carbapenems: imipenem (diameter zone ≤ 22 mm) and/or ertapenem (diameter zone ≤ 18 mm) were subjected to the modified Hodge screening test (MHT) for diffusible carbapenemase detection as previously described.[11] The minimal inhibition concentrations (MICs) of ceftazidime, ceftazidime-clavulanic acid, cefotaxime, cefoxitine and cefepime were determined by agar dilution technique. For imipenem, ertapenem and meropenem, MICs were determined by E-test (Bio-Mérieux SA, Marcy l'étoile, France). Results were interpreted according to the CLSI guidelines.[9]

Detection of β-lactamase-encoding Genes

K. pneumoniae isolates were screened by PCR for the following β-lactamases encoding genes: bla_{CTX-M} phylogenetic lineage groups 1, 2 and 9,[12-14] bla_{TEM}, bla_{SHV}, bla_{CIT}, bla_{ACC}, bla_{EBC}, bla_{MOX}, bla_{FOX}, bla_{DHA}, bla_{LAT}, bla_{ACT}, bla_{MIR},[15] bla_{OXA-48} and bla_{KPC}[16] as described previously.The PCR products were purified with a Purification Kit (BioMatik) and sequenced on an ABI PRISM 310 DNA Sequencer (Applied Biosystems). The nucleotide sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

β-Lactam Resistance Transfer Assays

Conjugation assays were performed on 5 isolates selected on the basis of their susceptibility to rifampin. It was carried out in brain heart broth (Bio-Rad), with *E. coli* J53-2 as the recipient. Transconjugants were selected on Mueller Hinton agar plates containing rifampicin (500 mg/L) and ticarcillin (125 mg/L). If not successful at the first attempt, mating experiments were repeated up to 3 times. Transconjugant's were submitted to bacterial identification with the API-20E system (Bio-Mérieux SA, Marcy l'étoile, France), antibiotic susceptibility testing and PCR amplification of the *bla* genes mentioned above.

Incompatibility Groups

Incompatibility groups (Inc) were determined by PCR-based replicon typing method as previously described.[17]

RESULTS

Antibiotic Susceptibility Tests

All isolates showed a positive DDST. They showed high level of resistance to cefotaxime (MIC_{50}= 512 μg/mL), ceftazidime (MIC_{50}=128 μg/mL) and cefepime (MIC_{50}=128 μg/mL). MICs of ceftazidime decreased when clavulanic acid is added. However, 1 strain showed high level of resistance to cefoxitin (MIC=>512 μg/mL) (Table 1). Five strains were resistant to ertapenem and showed a positive MHT. Three of them were resistant to imipinem and/or meropenem (Table 1).

All isolates were resistant to most antibiotics tested except colistin and tigecylin.They were frequently resistant to tobramycin (n=35), gentamicin (n=28), tetracycline (n=19), trimethoprim–sulfamethoxazole (n=30) and fluoroquinolones (n=26).

β-Lactamases Gene Characterization

The results of PCR and sequence analysis are summarized in Table 1. The bla_{SHV-1} gene was detected in all *K. pneumoniae* strains. ESBL genes were detected in all strains, $bla_{CTX-M-15}$ in 35 strains and bla_{SHV-12} in one. Moreover, 28 isolates harbored bla_{TEM-1}. Ertapenem resistant strains carried bla_{OXA-48}. The bla_{CMY-2} genewas detected in one strain that also coproduce 3 β-lactamases genes ($bla_{CTX-M-15}$, bla_{OXA-48} and bla_{TEM-1}).

Plasmid Replicon Type Determination

For 2 strains, the plasmids carrying $bla_{CTX-M-15}$ were assigned to the IncN and IncL/M replicon type. However, the remaining $bla_{CTX-M-15}$-carrying plasmid was negative for all of the replicons tested, as well as the bla_{SHV-12}-carrying plasmid (Table 1).

ESBL Transfer

Theconjugationwassuccessfulfor4/5 strains (3 harboring $bla_{CTX-M-15}$ and one bla_{SHV-12}). We also observed co-transfer, following conjugation, of resistance to other antibiotics, such as i.e. gentamicin (n=1), tobramycin (n=2), netilmicin (n=1), minocycline (n=4) and chloramphenicol (n=2).

Production of ESBLs was detected in all transconjugants by the DDST. The transfer of ESBL genes was confirmed

Table 1: Characteristics of extended spectrum β-lactamases *K. pneumoniae* producers isolated from blood cultures in 2011 at Charles Nicolle hospital

Strain	Ward	Date of isolation (day/month)	Antibioticresistance profil[a]	MIC (mg/L)[b]								DDST	β-lactamasesidentified	Replicontyping
				CAZ	CAZ-AC	CTX	FOX	FEP	ETP	IMP	MEM			
1	Urology	04/01	TM, NET, GM, AN, TE, MNO, NA, NOR, OFX, CIP, SXT	32	0,25	512	2	32	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
2	ICU	18/01	ETP, TM, NET, GM, NA, NOR, OFX, CIP, SXT	32	1	512	8	128	0,8	1	1	+	CTX-M-15; OXA-48; TEM-1	Negative
3	ICU	24/01	TM, NET, GM, MNO, NA, NOR, OFX, CIP, SXT	256	0,25	512	8	512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
4	ICU	10/03	TM, NET, GM, AN, C, TE, MNO, NA, NOR, OFX, CIP, SXT	256	0,25	512	8	128	Nd	Nd	Nd	+	CTX-M-15	Negative
5	Pediatrics	28/05	TM, NET, AN	256	0,5	512	2	32	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
6	Neonatology	28/03	TM, NET, GM, MNO, TE, NA, NOR, OFX, CIP, SXT	256	0,5	512	8	128	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
7	Urology	06/04	TM, NET, GM, TE, C, NA, NOR, OFX, CIP, SXT	512	0,25	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15	Negative
8	Pediatrics	06/04	TM, NET, GM, TE, MNO, NA, NOR, OFX, CIP, SXT	64	0,25	1024	8	128	Nd	Nd	Nd	+	CTX-M-15	Negative
9	Surgery	14/04	TM, NET, AN, TE, SXT	256	0,25	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	N
10	Surgery	16/05	TM, NET, GM, C, TE, NA, NOR, MNO, OFX, CIP, SXT, TZP	1024	0,25	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
11	Orthopedic	28/05	TM, NET, GM, TE, NOR, OFX, CIP, TZP, SXT	1024	1	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
12	ICU	27/06	C, NA, NOR, MNO, OFX, CIP, SXT, TZP, TE, TM, NET, GM TGC	1024	0,25	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
13	Surgery	14/07	TM, NET, GM, NA, NOR, OFX, CIP, SXT	32	0,06	1024	8	32	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
14	Neonatology	10/07	TM, GM, TE, MNO, CIP, TZP, SXT	128	0,01	512	8	16	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
15	Surgery	23/07	C, NA, NOR, MNO, OFX, CIP, SXT, TZP, TE, TM, NET, GM	512	0,25	512	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
16	ICU	05/09	TM, NET, GM, NA, NOR, CIP, TZP	256	8	1024	4	128	Nd	Nd	Nd	+	CTX-M-15	Negative
17	Urology	22/07	TM, NET, AN, GM, NA, NOR, OFX, CIP, TZP, SXT	32	0,06	1024	4	128	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
18	Neonatology	02/08	TM, NET, GM, FOS	128	32	2048	8	128	Nd	Nd	Nd	+	CTX-M-15	Negative
19	ICU	26/09	IMP, ETP, FOX, TM, NET, AN, GM, C, TE, MNO, NA, NOR, OFX, CIP, SXT	256	128	512	>512	>512	16	3	16	+**	CTX-M-15; OXA-48; CMY-2; TEM-1	Negative
20	Pediatrics	11/11	TM, NET, GM, TE, TZP, SXT	1024	0,5	>2048	4	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	L/M
21	Neonatology	12/10	TM, FOS, SXT	32	0,06	1024	4	128	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
22	ICU	10/10	TM, NET, GM, NA, NOR, OFX, CIP, TZP, SXT	128	1	512	8	64	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
23	Neonatology	17/11	TM, FOS, SXT	128	0,03	512	8	128	Nd	Nd	Nd	+	CTX-M-15	Negative
24	Surgery	15/11	TM, NET, GM, C, TE, MNO, NA, NOR, OFX, CIP, FOS, TZP, SXT	512	0,13	>2048	8	>512	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
25	ICU	19/11	TM, NET, GM, TE, NA, NOR, OFX, CIP, SXT	8	0,03	128	4	16	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
26	ICU	26/11	TM, NET, GM, TE, NA, MNO, NOR, OFX, CIP, TZP, SXT	64	0,06	512	8	32	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative

(Contd)...

Table 1: *(Continued)*

Strain	Ward	Date of isolation (day/month)	Antibiotic resistance profil[a]	MIC (mg/L)[b]							DDST	β-lactamases identified	Replicontyping
				CAZ	CAZ-AC	CTX	FOX	FEP	ETP	IMP	MEM				
27	Neonatology	11/12	TM, SXT	16	0,03	128	8	16	Nd	Nd	Nd	+	CTX-M-15	Negative
28	Neonatology	12/10	TM, NET, AN, GM, TE, MNO	256	16	>2048	2	256	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
29	ICU	27/12	ETP, IMP, TM, NET, AN, GM, MNO, NA, NOR, OFX, CIP, FOS, TZP, SXT	512	16	>2048	8	>512	>32	>32	>32	+	CTX-M-15; OXA-48; TEM-1	Negative
30	Surgery	23/11	C, TE, MNO, FOS	0,5	0,5	0,5	8	0,25	Nd	Nd	Nd	+	SHV-12	Negative
31	Surgery	13/07	ETP, TM, NET, GM, NA, NOR, OFX, CIP, SXT	32	1	256	8	128	2	0,5	2	+	CTX-M-15; OXA-48; TEM-1	Negative
32	Neonatology	12/02	TM, NET, GM, TE, MNO, NA, OFX, CIP, NOR, SXT	16	0,13	256	4	16	Nd	Nd	Nd	+	CTX-M-15	Negative
33	ICU	13/06	ETP, IMP, TM, NET, GM, NA, OFX, CIP, NOR, TZP, SXT	32	0,5	128	4	16	16	2	0,5	+	CTX-M-15; OXA-48; TEM-1	Negative
34	Surgery	19/05	TM, NET, GM, NA, OFX, CIP, NOR, TZP, SXT	64	1	256	4	64	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
35	Gastrology	26/12	TM, NET, MNO, NA, OFX, CIP, NOR	256	0,25	1024	8	256	Nd	Nd	Nd	+	CTX-M-15; TEM-1	Negative
36	Neonatology	03/09	TM, SXT	16	0,03	256	4	32	Nd	Nd	Nd	+	CTX-M-15	Negative

ICU: Intensive care unit, [a]TZP: Tazobactam, AN: Amikacin, GM: Ggentamicin, TM: Tobramycin, NET: Netilmicin, SXT: Cotrimoxazole, NA: Nalidixicacid, CIP: Ciprofoxacin, C: Chloramphenicol, TE: Tetracycline, MNO: Minocycline, OFX: Ofoxacin, NOR: Norfoxacin, FOS: Fosfomycin, [b]CAZ: Ceftazidime, CAZ AC: Ceftazidime clavulanicacid, CTX: Cefotaxime, FOX: Cefoxitin, FEP: Cefepime, IMP: Imipenem, ETP: Ertapenem, MEM: meropenem, DDST: Double disk synergy test, Nd: Not determined, **DDST positive when cloxacillin was added to the MH agar

in all transconjugants; $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}12}$ were detected in 3 and 1 transconjugants, respectively. Only one of the 4 transconjugants was typeable for the incompatibility groups (IncL/M) (Table 2).

DISCUSSION

ESBL producing *K. pneumonia*e is one of the most important pathogens responsible for life threatening infections.[4,18,19] In the present study, frequency of ESBL producing *K. pneumoniae* causing bacteremia is 47% which is lower than 87.5% reported in a Tunisian pediatric intensive care unit.[4] A study conducted in India reported 56% ESBL *K. pneumoniae* causing septicemiaamongneonates.[18]

In most European countries, Latin America, and East Asia, CTX-M variants have displaced TEM and SHV enzymesas the predominant β-lactamases produced by Gram-negative bacteria such as *K. pneumoniae*.[20] Our work shows that CTX-M-15 is the most common ESBL. CTX-M-15, first described in India in 1999,[21] has spread over the world, and seems to be the most common ESBL type.[22,23] In Tunisia, dissemination of CTX-M-15 has been reported in *K. pneumoniae*, *E. coli* and *Enterobacter cloacae* strains.[2,5,24,25] $bla_{CTX\text{-}M\text{-}15}$ gene is generally found on large conjugative plasmids and is located downstream of an IS*Ecp1* insertion sequence which explains its remarkable transmission success.[26] The large use of 3rdGC in clinical practice has significantly contributed to their selection.[27]

Although SHV-12 was found in one isolate of our collection, previous Tunisian studies showed that this enzyme was associated with *K. pneumoniae*involved in nosocomial outbreaks.[2,3,20,28,29] Worldwide, the distribution of SHV-ESBLs showed that SHV-2, SHV-4 and SHV-5 enzymes are the most prevalent in the United States and Europe.[30]

The ESBL-producing organisms additionally exhibited co-resistance against multiple antimicrobials from other classes, such as tetracycline, aminoglycosides and fluoroquinolones. This complexity in antimicrobials resistance combinations limits suitable drug of choice for antimicrobial therapy, leaving carbapenems the last options for treatment in some cases. However, the emergence of plasmidiccarbapenemases producers clearly compounds potential treatment options.[14] Carbapenem resistance was found in 5 of our strains. All harbored OXA-48. This class D carbapenemasewas first identified in *K. pneumoniae* from Turkey in 2003[16] and then extensively spread in other countries[31] and species. In Tunisia, recent studies reported the spread of OXA-48 producing *Enterobacteriaceae*.[32,33]

$bla_{CMY\text{-}2}$ is the most frequently encountered plasmid encoded AmpC-type β-lactamase gene found in *E. coli*

Table 2: Resistance profils and β-lactamases genes of donor strains and their transconjugants

Strain[a]	Otherresistanceprofil[b]	bla_{SHV-12}	bla_{TEM-1}	$bla_{CTX-M-15}$	Replicontyping
4	TM, NET, GM, AN, C, TE, MNO, NA, NOR, OFX, CIP, SXT	-	+	+	Negative
Tc 4	TM, GM, TE, MNO	-	+	+	Negative
20	TM, NET, GM, TE, TZP, SXT	-	+	+	L/M
Tc 20	TM, NET	-	-	+	L/M
22	TM, GM, NET, NA, OFX, CIP, NOR, SXT, C	-	+	+	Negative
Tc 22	C	-	-	+	Negative
30	TE, C, MNO, FOS	+	-	-	Negative
Tc 30	TE, C, MNO	+	-	-	Negative

[a]Tc: Transconjugant, [b]AN: Amikacin, GM: Gentamicin, TM: Tobramycin, NET: Netilmicin, SXT: Cotrimoxazole, NA: Nalidixicacid, CIP: Ciprofoxacin, C: Chloramphenicol, TE: Tetracycline, MNO: Minocycline, OFX: Ofoxacin, NOR: Norfoxacin, FOS: Fosfomycin

worldwide.[34-36] the first AmpC type β-lactamase to be identified in Tunisia was in 1996 in a *P. mirabilis* isolate, and was characterized as a CMY-4 enzyme.[37] In Tunisia, CMY-2 has already been detected in food samples[34] and fecal flora of healthy chickens and pets[38] but never in clinical isolates. In our study, bla_{CMY-2} was detected in one strain, which co-produced 3 plasmidicβ-lactamases (CTX-M-15, OXA-48 and TEM-1). The coexistence of several enzymes in the same strain is noteworthy and has been previously described in *K. pneumoniae* in Tunisia (VIM-4, CTX-M-15 and CMY-4).[39]

Identification and classification of plasmids harbouring β-lactamases is helpful to analyze their distribution in nature and their relationship to host cells and to discover their evolutionry origins.[40] In our study, incompatibility groups of $bla_{CTX-M-15}$ carrying plasmids were determined only in 2 strains (IncN and IncL/M). However, other studies reported that some $bla_{CTX-M-15}$ and bla_{SHV-12}-carrying plasmids were negative for all of the replicons tested,[2,28] as well as described in our study. Further research is necessary to provide more information about our plasmids.

In conclusion, our study reports a widespread diffusion of CTX-M-15 with the emergence of OXA-48 in *K. pneumoniae* in Tunisia which seriously limits therapeutic options in cases of clinical infections with these bacteria. In addition, coexistence of ESBLs production with cephalosporinase or carbapenemase is a serious public health problem and requires continuous surveillance, monitoring and revision of the antibiotic use policies.

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Disclosure Statement

All authors disclose no commercial associations that might create a conflict of interest in connection with this study.

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