Clonal Dissemination of a Multidrug-Resistant Strain of *Klebsiella pneumoniae* Producing OXA-48 Carbapenemase at a Tunisian Hospital.

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***Abstract*- Emergence and dissemination of carbapenem resistance among *Enterobacteriaceae* represent a significant threat to the management of nosocomial infections. *Klebsiella pneumoniae* had proved to be the most concerned as multidrug-resistant bacteria causing severe infections with limited treatment options. In the present study, we investigate the molecular epidemiology of 21 ertapenem-resistant strains of *Klebsiella pneumoniae* collected in Rabta hospital of Tunisia, between June 2010 and December 2011. The molecular epidemiology including the characterization of carbapenemases and others β-lactamases, pulsed-field gel electrophoresis, were carried out. Medicals records were reviewed to evaluate predictive factors for infection. All strains were multidrug resistant. The OXA-48 carbapenemase was identified in all strains and was associated with CTX-M-15 and CTX-M-14/CMY-4 in 11 and 1 cases, respectively. Pulsed-field gel electrophoresis typing indicated the clonal dissemination of an epidemic strain. The risk factors for acquired OXA-48 infections were: severity of underlying disease, longer length hospital stay and admission to an intensive care unit. Amplification of antibiotic resistance with expression of carbapenemase and extended-spectrum β-lactamase in the same strain requires continuous surveillance programs using molecular techniques as powerful tools for early detection and for prevention of dissemination of these pan-drug-resistant isolates.**

***Index Terms* : *Klebsiella pneumoniae*, Multidrug resistance, carbapenmaes, DNA sequencing, OXA-48, pulsed-field gel electrophoresis, extended-spectrum β-lactamase**

I. INTRODUCTION

Emergence of carbapenamase-producing *Enterobacteriaceae*, is currently a major public health concern worldwide [1]. *Klebsiella pneumoniae* (*K. pneumoniae*) had proved to be the most important enterobacterial species as a source of hospital spread of multidrug resistance [2].

The wide spread of this pathogen was related to its ability to carry plasmid encoded β-lactamases such as extended-spectrum β-lactamase (ESBLs) and carbapenemases with others determinants of resistance [3,4]. A large variety of carbapenemases has been identified in *K. pneumoniae* strains belonging to Ambler class A, B or D enzymes [5] which some occurred with close geographic associations [6,[7]. In Tunisia, the first carbapenemase producer was a VIM-4 metallo-β-lactamase *K. pneumoniae* strain, identified in 2006 [8]. Since then, many studies have reported the occurrence of oxacillinase OXA-48 [9-11]. In this purpose, a prospective study was undertaken to investigate the molecular epidemiology of 21 ertapenem-resistant strains of *K. pneumoniae* collected in Rabta hospital of Tunisia between June 2010 and December 2011 and to evaluate predictive factors for infection.

II. MATERIALS AND METHODS

A. Bacterial strains

This retrospective study was performed from June 2010 to December 2011, 2014 in the Microbiology Laboratory at Rabta University Hospital in Tunis (Tunisia). This collection consisted of 21 non-replicate carbapenem-resistant *K. pneumoniae* strains: 20 strains were recovered from invasive clinical specimens, including urine (n=7), catheters (n=5), tracheal aspirate (n=3), blood (n=3) and wounds (n=2) and one environmental strain recovered from an atomizer in Intensive Care Unit (ICU). The strains were obtained from patients hospitalized in various wards: ICU (ICU1), ICU from general surgery unit (ICU2), urology, nephrology, emergency, cardio-vascular surgery infectiology unit, general surgery, cardiology and private hospital.

B. Review of medical records

The medical records of 18 infected patients were collected by a prospective chart review including patient's age, sex, length of stay before infection, underlying diseases, admission to an intensive care unit, antimicrobial treatment during hospitalization and the final outcome of infection.

C. Microbial Identification and antibiotic susceptibility testing and phenotypic screening for production of carbapenemases

Isolates were identified by using a Vitek 2 Gram-negative Identification System (bio-Mérieux, Marcy l'Etoile, France).

Antibiotic susceptibility was tested by disk diffusion method (DDM) on Mueller-Hinton (MH) agar plates and results were interpreted according to the standards of the French Antibiogram Committee (CA-SFM) (http://www.sfm.asso.fr) for the following antibiotics:

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amoxicillin, amoxicillin-clavulanic acid, ticarcillin, piperacillin, cephalotin, cefsulodine, cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefepime, imipenem, ertapenem, streptomycin, kanamycin, gentamicin, tobramycin, amikacin, nalidixic acid, pefloxacin, ofloxacin, ciprofloxacin, levofloxacin, colistin, trimethoprim-sulfamethoxazole, tetracycline and tigecycline. Minimum inhibitory concentrations (MICs) of imipenem were determined by the E-test strips (bio-Mérieux) and the results were interpreted according to the breakpoints established by the guidelines of CA-SFM (susceptibility was defined by MICs ≤ 2 µg/ml and resistance was defined by MICs >8 µg/ml).

Extended spectrum ß-lactamase (ESBL) was detected by a double disk synergy test between amoxicillin-clavulanic acid and a third generation cephalosporin. An ertapenem disk (10ug, Biorad) was used because of its high sensitivity in detecting carbapenemase resistant strains. A reduced susceptibility to carbapenems was defined by an inhibition diameter around ertapenem disk less than 28 mm. The modified Hodge test was utilized for the detection of carbapenemase. *Escherichia coli* (*E. coli*) ATCC 25922 was used as reference strain for antimicrobial susceptibility testing.

D. Polymerase Chain Reaction (PCR) amplification and DNA sequence analysis of resistance genes

DNA extraction was performed by an alkaline lysis protocol as described previously [12]. The strains were screened for the presence of carbapenemase genes (bla_{KPC}, bla_{OXA-48}, bla_{IMP} and bla_{VIM}), extended-spectrum β-lactamases (bla_{CTX-M}, bla_{SHV} and bla_{TEM}) and plasmid-mediated AmpC cephalosporinases (bla_{CMY}, bla_{ACC} and bla_{DHA}) by PCR [5,8,13].

PCR products were purified using a purification Kit (Promega) according to the manufacturer's procedure and sequenced on an ABI Prism® 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were analyzed using software available at the website of the National Center of Biotechnology Information (http://www.ncbi.nlm.nhi.gov).

E. Pulsed-field gel electrophoresis analysis

All isolates were typed by using pulsed-field gel electrophoresis (PFGE) which was performed according to a previously described protocol [14]. After digestion with *Xba*I enzyme, the DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels and 0.5 Tris-borate-EDTA buffer using a CHEF DRIII apparatus (Bio-Rad Laboratories, Inc, Richmond, UA, USA). Electrophoresis conditions were 14°C at a gradient of 6V/cm for 22 h with switch times ranging from 2.2 to 54.2 seconds of linear ramping. The DNA fingerprints generated were analyzed with the fingerprinting^{TM}–II Software Version 3.00 (Bio-Rad, Germany). Strains showing 85 % or more of similarity were classified as genetically related and assigned to the same lineage.

III. RESULTS

A. Risk factors for infection

Clinical characteristics of infected patients were summarized in Table 1. All cases presumed to have been acquired during hospitalization. These patients consisted of 12 males and 6 females with the mean age of 56.1 years. All cases had severe underlying disease (renal insufficiency, diabetes, pulmonary disease, liver disease, receipt of an organ transplantation, trauma, and malignancy) and presented a prolonged hospital stay (over 17 days). Six patients died after a prolonged hospital stay in intensive care unit especially, with severe sepsis. The crude mortality was 44 % reflecting the critical status of the patients. For antimicrobial treatment, a greater proportion of cases was exposed to numerous inappropriate antibiotics before the onset of infection and then was treated with a combined-drug regimen including mainly imipenem and colistin.

B. Antibiotic susceptibility testing

Antimicrobial susceptibility testing of *K. pneumoniae* strains using the DD method and E-test for MIC determination revealed high rates of resistance to many drugs. All clinical isolates were resistant to amoxicillin, piperacillin, ticarcillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, piperacillin/tazobactam and ertapenem. Ninety percent of isolates were resistant to cefoxitin and to extended-spectrum cephalosporins including cefotaxime, ceftazidime, ceftriaxone and cefepime. Co-resistance to other antibiotics was frequent among these isolates with 100% of resistance to all fluoroquinolones tested, kanamycin and tobramycin. Streptomycin and amikacin still have activity against strains. Resistance rate to gentamicin, tetracycline, cotrimoxazole and tigecycline were respectively, 90%, 75%, 70%, and 45%. MICs determination revealed that 65% (n=13) of isolates were characterized as intermediate to imipenem (MICs were > 2 µg/ml and ≤ 8 µg/ml) and 35% were characterized as susceptible. All strains remained sensitive to colistin (MICs≤22 µg/ml). The environmental strain was resistant to all antibiotics tested except streptomycin, amikacin and colistin and was characterized as intermediate to imipenem (MIC = 6 µg/ml).

C. PCR amplification and DNA sequence analysis of resistance genes

PCR analysis of all *K. pneumoniae* isolates for relevant carbapenem-resistance genes (bla_{KPC}, bla_{IMP} and bla_{VIM}) was negative. PCR experiments followed by sequencing identified bla_{OXA-48} gene in all isolates, $bla_{CTX-M-15}$ in 11 isolates and $bla_{CTX-M-14}$ associated with bla_{CMY-4} in 1 isolate (Table 2).

D. Pulsed-field gel electrophoresis (PFGE) analysis

All isolates were assigned to 3 different pulsotypes (A, B and C) and the cutt-off value of 87% similarity was indicated by a dotted red line in Figure 1. One major PFGE type (A) was identified comprising 18 clinical isolates and the environmental strain (E1. Two OXA-48-producing *K. pneumoniae* were genetically distinct (pulsotype B and C).

All isolates recovered from ICU 1 belonged to a single molecular type indicating a clonal dissemination (pulsotype A in Figure 1).

IV. DISCUSSION

Increased prevalence of carbapenemase producing *Enterobacteriaceae* has been reported in many countries over the world, albeit with a great variability in the occurrence and distribution among different geographic areas [1,6].

The oxacillinase OXA-48 was first identified in a clinical *K. pneumoniae* isolate from Turkey in 2001 [15]. Since then, several others OXA-48-like producers have been reported in various enterobacterial species, mainly in *K. pneumoniae* isolates, from countries in the Middle East, North Africa and Europe [16].

OXA-48-producing *K. pneumoniae* cause serious infections which are often difficult to treat because of a multidrug resistance [16,17].

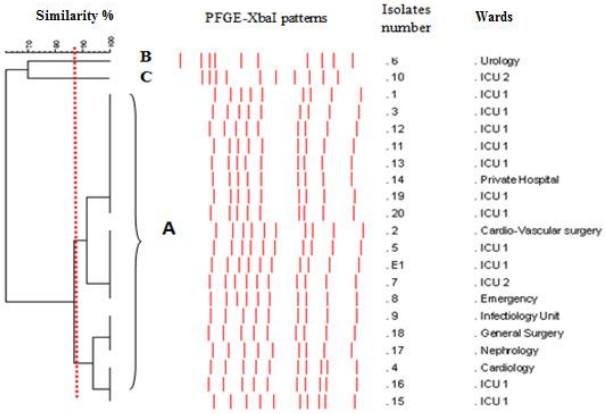

Fig 1. Dendrogram showing the relatedness of PFGE patterns of OXA-48-producing *Klebsiella pneumoniae.*
Legend: a cut-off of 87% similarity (vertical red line) was chosen for determination of clonal relatedness. The horizontal bar on the top left indicates the percentage similarity within the strains. The dendrogram demonstrates 3 clones (A, B and C). One major PFGE type (A) was identified comprising 18 clinical isolates and the environmental strain. All isolates recovered from ICU 1 belonged to a single pulsotype A.

Abbreviations: ICU1: intensive care unit 1, ICU2: ICU from general surgery unit (ICU2), E1: environmental strain from ICU1

In our study, infections due to OXA-48-producing *K. pneumoniae* affected seriously elderly patients with severe underlying diseases after a prolonged hospital stay and were associated with a significant mortality. It had been reported that combination therapy including a carbapenem with another active agent, preferentially an aminoglycoside or colistin, may be effective against carbapenemase-producing *Enterobacteriaceae* with a MIC value for meropenem and imipenem < 8 mg/l [18]. However, in our study, combination therapy failed to treat OXA-48 infections in eight cases. Indeed, published data regarding efficacy of carbapenems for treating OXA-48 infections remains debatable given that OXA-48-like strains producers exhibit variable resistance profiles [16].

In Tunisia, the OXA-48 β-lactamase has been spreading at an alarming rate with a prevalence of 13.7% among *Klebsiella pneumoniae* isolates exhibiting reduced susceptibility to extended-spectrum cephalosporins and or imipenem [9]. In our study, all 21 ertapenem-resistant strains carried OXA-48 carbapenemase. The large and still increasing proportion of OXA-48-producing *Klebsiella pneumoniae* seems to be due to the silent spread of this mobile determinant [11,19]. In fact, OXA-48-like producers have been reported to be difficult to detect since the level of acquired resistance to carbapenems may remain quite low [20,21] . Reduced susceptibility to imipenem but with the MIC still in susceptible range, as low as 2 µg/ml using E-test, was observed on 35% of isolates in our study. It is noteworthy that OXA-48 enzyme hydrolyze penicillins and, at a lower level imipenem, but spare extended-spectrum cephalosporin [20]. Multidrug resistance in OXA-48-producing strains often results from the coproduction of various resistance mechanisms, in particular Class A extended-spectrum β-lactamase (most often CTX-M-15 or SHV-12) and the narrow-spectrum β-lactamases making those isolates resistant to all β-lactams available [16]. In our study, OXA-48 carbapenemase was associated with TEM-1, SHV-1 and CTX-M-15 which was the most common CTX-M enzyme in *K. pneumoniae* isolates in Tunisia [22]. CTX-M-14 enzyme was rarely identified in nosocomial infections in Tunisia, even though it has been reported to causing an outbreak and to be associated with OXA-48/ CMY-4 producers [9]. The OXA-48 β-lactamase appear to have a greater ability to spread and cause outbreaks [16,17]. So, the incidence of OXA-48-producing *K. pneumoniae*, reported in this study, was attributed to the clonal dissemination of an epidemic strain. Contamination of medical equipment detected in the intensive care unit suggested that a potential common environmental source of infection was implicated in the spread of this bacterium.

This study underlines that *K. pneumoniae* is an enterobacterial species prone to cause hospital-based outbreak of strains carrying multidrug-resistant genes such as carbapenemase and ESBL determinants and highlights the importance of continuous surveillance programs using molecular techniques as powerful tools for early detection and prevention of dissemination of these pan-drug-resistant isolates.

Competing interests: we declare no competing interests

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Table 1. Clinical characteristics of infected patients.

Patient	Age (yr) gender	Underlying disease	Date of hospitalization	Days of stay before detection	Clinical status	Empirical antibiotic therapy	Oriented antibiotic therapy	Clinical outcome
1	76/M	Renal insufficiency, diabetes	07/06/2010	4	Nosocomial pneumonia and septicemia	Amoxicillin-clavulanate	Imipenem, colistin, ofloxacin,vancomycin	Death related (septic shock)
2	64/M	Diabetes	24/06/2010	7	Surgical wound infection	Amoxicillin-clavulanate, metronidazole	Cefotaxim, amikacin, metronidazole	Recovered
3	64/M	Necroziting fascitis	11/06/2010	48	Septicemia	Cefotaxim,imipenem, colistin, ciprofloxacin	Imipenem , colistin	Death related (septic shock)
4	64/F	Renal and heart insufficiency, diabetes	21/01/2011	45	Nosocomial pneumonia, urinary infection	Amoxicillin-clavulanate, metronidazole	Amoxicillin-clavulanate	Not Determined
5	60/M	Chronic obstructive pulmonary disease	01/03/2011	25	Nosocomial pneumonia, bloodstream infection	None	Imipenem , colistin	Recovered
6	55/F	Septic arthritis	10/06/2011	12	Surgical wound infection	Amoxicillin-clavulanate, ofloxacin, gentamicin	Piperacillin/tazobactam, ciprofloxacin	Recovered
7	55/F	Diabetes	15/09/2011	3	Urinary infection	Ciprofloxacin, gentamicin	Colistin	Recovered
8	56/M	Trauma, diabetes	28/08/2011	22	Nosocomial pneumonia	Amoxicillin-clavulanate	Imipenem , colistin	Recovered
9	31/M	Trauma	10/09/2011	10	Septicemia	Amoxicillin-clavulanate, teicoplanine, piperacillin-tazobactam	Imipenem , colistin	Death related (septic shock)
10	41/F	Pyelonephritis, diabetes	18/09/2011	15	Catheter-related Infection	Cefotaxim, ciprofloxacin	Imipenem , teicoplanine	Death
11	68/M	Chronic obstructive pulmonary disease	05/10/2011	2	Nosocomial pneumonia	None	None	Transferred
12	82/M	Bladder cancer	25/09/2011	19	Nosocomial pneumonia + Septicemia	Cefotaxim, gentamicin, metronidazole	Imipenem, gentamicin, tigecycline	Death related (septic shock)
13	49/F	Renal insufficiency	18/11/2011	8	Catheter-related bloodstream Infection	Piperacillin-tazobactam, ofloxacin, vancomycin	Imipenem , colistin	Death related (septic shock)
14	42/M	Tuberculosis meningoencephalitis	15/11/2011	14	Catheter-related Infection	Cefotaxim, metronidazole	Cefotaxim, colistin	Recovered
15	30/M	Renal insufficiency	Not mentionned		Persistent urinary Infection	Cefotaxim,ofloxacin	Ertapeneme	Recovered
16	58/M	Diabetes	21/11/2011	19	Urinary infection	Cefotaxim, gentamicin, metronidazole	Piperacillin-tazobactam	Recovered
17	46/F	Viral meningoencephalitis	12/12/2011	20	Catheter-related Infection	Cefotaxim, teicoplanine	Imipenem , colistin	Death
18	69/M	Renal and liver insufficiency	02/12/2011	29	Catheter-related bloodstream Infection	Piperacillin-tazobactam, ofoxacin	Imipenem, colistin, vancomycin	Death related (septic shock)

Table 2. Characteristics of OXA-48-producing *Klebsiella pneumoniae* strains.

Strain number	Date of isolation	Specimen	Ward	Phenotype	Characterization of β-lactamase genes					MIC $_{IPM}$ (µg/ml)	Pulsotype
					OXA-48	CTX-M	SHV	TEM	CMY		
1	10/06/2010	Tracheal aspiration	ICU 1	ESBL + Amp C	OXA-48	CTX-M-15	SHV-1	TEM-1		2	A
2	30/06/2010	Pus	Cardio-vascular surgery	Amp C	OXA-48		SHV-1			6	A
3	29/07/2010	Blood	ICU 1	ESBL + Amp C	OXA-48	CTX-M-15	SHV-1			2	A
4	07/03/2011	Urine	Cardiology	Amp C	OXA-48		SHV-1			8	A
5	26/03/2011	Blood	ICU 1	Amp C	OXA-48		SHV-1			4	A
E 1	18/04/2011	Atomizer	ICU 1	Amp C	OXA-48					6	A
6	19/04/2011	Urine	Urology	ESBL + Amp C	OXA-48	CTX-M-14	SHV-1		CMY-4	1,5	B
7	22/06/2011	Pus	ICU 2	Amp C	OXA-48		SHV-1			4	A
8	24/07/2011	Urine	Emergency	Amp C	OXA-48					8	A
9	18/09/2011	Urine	Infectiology Unit	ESBL + Amp C	OXA-48	CTX-M-15	SHV-1	TEM-1		4	A
10	19/09/2011	Tracheal aspirate	ICU 2	ESBL	OXA-48	CTX-M-15				0,38	C
11	19/09/2011	Blood	ICU 1	ESBL+ Amp C	OXA-48	CTX-M-15		TEM-1		3	A
12	01/10/2011	Catheter	ICU 1	Amp C	OXA-48		SHV-1	TEM-1		1,5	A
13	07/10/2011	Tracheal aspirate	ICU 1	ESBL + Amp C	OXA-48	CTX-M-15				1	A
14	14/10/2011	Urine	Private hospital	ESBL + Amp C	OXA-48	CTX-M-15				6	A
15	25/11/2011	Catheter	ICU 1	ESBL + Amp C	OXA-48	CTX-M-15		TEM-1		3	A
16	28/11/2011	Catheter	ICU 1	ESBL + Amp C	OXA-48	CTX-M-15		TEM-1		3	A
17	09/12/2011	Urine	Nephrology	ESBL + Amp C	OXA-48	CTX-M-15		TEM-1		2	A
18	10/12/2011	Urine	General surgery	ESBL + Amp C	OXA-48	CTX-M-15		TEM-1		2	A
19	31/12/2011	Catheter	ICU 1	Amp C	OXA-48					6	A
20	31/12/2011	Catheter	ICU 1	Amp C	OXA-48					3	A

LegendTable2: ESBL: Extended Spectrum Beta Lactamase , ICU1: intensive care unit

1, ICU2: ICU from general surgery unit (ICU2), E1: environmental strain from ICU1

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