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Prevalence of Carbapenemase-Producing Enterobacteriaceae in a University Hospital in Rabat, Morocco: A 19-Months Prospective Study

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Abstract

Objectives: This study aims to assess the prevalence of clinical isolates of *Enterobacteriaceae* with carbapenem resistance, and to characterize the types of carbapenemases produced.

Methods: Non-duplicate *Enterobacteriaceae* (*Klebsiella spp*, *Enterobacter spp* and *E. coli*) clinical isolates collected over 19 months (May 2009 through December 2010) were included in this study. The modified Hodge test was performed on strains showing reduced ertapenem susceptibility. Isolates that tested positive were sent to the French reference center on emerging resistance for molecular characterization and clone determination.

Results: A total of 463 strains were investigated: *E. coli* 63.9%, *Klebsiella spp* 27.9% and *Enterobacter spp* 8.2%. Carbapenemase production occurred in 13 isolates (2.8%). Ten strains produced class D carbapenemases (OXA-48). These included 6 *K. pneumoniae*, 1 *K. oxytoca* and 3 *E. cloacae*. New Delhi metallo-β-lactamase-1 (NDM-1) was produced by three *K. pneumoniae* strains.

Conclusion: The high prevalence of carbapenemases producing *Klebsiella* and *Enterobacter* among clinical isolates and the detection of NDM-1 carbapenemase is a source of concern in Morocco. This feature requires setting up an emerging resistance monitoring and surveillance scheme at national level.

Keywords: Carbapenemases; Enterobacteriaceae; *Klebsiella;* Prevalence; Resistance.

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Introduction

Over the past ten years, carbapenem resistant *Enterobacteriaceae* have been reported worldwide (1). The first carbapenemase producer in *Enterobacteriaceae* (NmcA) was identified in 1993 (2), since then, a large variety of carbapenemases has been identified in *Enterobacteriaceae* belonging to 3 classes of β-lactamases: the Ambler class A, B and D.

The most frequently circulating carbapenemase among Mediterranean countries was the Ambler class D: OXA-48 type β-lactamase (3).The OXA-48 type was initially identified in 2004 in a carbapenem resistant *Klebsiella pneumonia* isolate from Istanbul (4), and progressively disseminated to other Mediterranean areas including Lebanon,Tunisia, France and Morocco. The first case of *K. pneumoniae* OXA-48 resistant in Morocco was reported in 2009 (5). Other isolates of OXA-48 producing *Enterobacteriaceae* were discovered in France and

Belgium, from patients who were transferred from Morocco (6,7). Recently a nosocomial occurrence of OXA-48 producing enterobacterial isolates was reported in Morocco and which showed the increased spread of OXA-48 *Enterobacteriaceae* in this country (8).

New Delhi metallo β-lactamase (NDM-1) is the latest carbapenemase to be discovered. It was first described in 2008 in *K. pneumoniae* and *E. coli* isolated in Sweden from an Indian patient transferred from a New Delhi hospital (9). Most positive NDM-1 bacterial isolates have shown epidemiological links to India and Pakistan (10). Recently, in 2010, three isolates of NDM-1 *K. pneumoniae* were reported in Morocco (11).

This study aims to assess the prevalence of carbapenemase producing strains in a main Moroccan hospital and to characterize the enzymes produced.

Material and Methods

Study location

This prospective study carried out over the period (May 2009 to December 2010) at the Microbiology Laboratory of Cheikh Zaid University Hospital in Rabat, Morocco. The hospital has 250 beds and an annual admission rate of nearly 3,000.

Bacterial strains

All non-duplicate *Klebsiella spp*, *E. coli*, and *Enterobacter spp* clinical isolates from inpatients were included in this study. All isolates were identified to species level using (API 20 E®, bioMérieux, Marcy-l'Étoile, France).

Phenotype screening for carbapenemase production

All clinical isolates with a decreased inhibition diameter (≤26mm of 10µg ertapenem disk) using the diffusion method had their Minimum Inhibitory Concentration (MIC) evaluated using the E-TEST method (bioMérieux, Marcy-l'Étoile, France). Isolates that were found to show no susceptibility to ertapenem (MIC >0.5µg/ml; according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (12) were tested using the modified Hodge test, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (13).

Phenotype determination of carbapenemase classes

All strains resistant to ertapenem and which were positive in the Hodge test underwent tests for carbapenemases activity inhibition. Class A enzyme production was investigated using the technique described by Doi et al. (14), which is based on 3-aminophenylboronic acid inhibition of carbapenemase activity. Class B carbapenemases were detected using the EDTA inhibition test. The inhibition diameters around a 10µg imipenem disk for the tested strains were compared to the diameters around another imipenem disk, to which had been added 750µg EDTA. An increase in the zone of inhibition to greater than or equal to 7mm was regarded as positive (15).

Molecular investigation

Strains that tested positive with the Hodge test were sent to the international expertise center for emerging resistance to antibiotics: INSERM Unit 914 for the molecular study, using PCR and sequencing to search for b-lactamase genes, in addition to the multilocus sequence typing and the pulsed-field gel electrophoresis to determine the clonal relationships between the isolates (8,11).

First, specific primers were used for the detection of of the following carbapenemase genes: *bla*NDM, *bla*VIM, *bla*IMP, *bla*KPC and *bla*OXA-48 (8,11).

For those isolates positive for the *bla*OXA-48, all β-lactamase genes that had previously been identified in OXA-48-producing *K. pneumoniae* isolates (*bla*TEM, *bla*SHV and *bla*CTX-M) were investigated together with the AmpC β-lactamase genes *bla*DHA and *bla*CMY, and corresponding amplicons were subsequently sequenced. Plasmid DNA extraction was performed according to the Kieser technique (16). The genetic environment of the *bla*OXA-48 gene was determined by PCR mapping (8).

All negative isolates for the *bla*OXA-48 were screened for 16S rRNA methylase-encoding genes using a multiplex PCR approach. Amplified DNA fragments were purified with the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France). Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide and deduced protein sequences were analyzed with a software available over the Internet from the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov) (11).

Antibiotic susceptibility profile determination

The MICs of carbapenems producing strains (imipenem, ertapenem and meropenem) was determined using the E-Test method. Susceptibility to other antibiotics was studied by the agar diffusion method according to EUCAST recommendations (12).

Results

Prevalence

A total of 463 clinical isolates were investigated, and *E. coli* isolates were accounted for 63.9% (296), followed by *Klebsiella spp* for 27.9% (129) and *Enterobacter spp* for 8.2% (38). Thirteen isolates (2.8%) were positive in modified Hodge test but they were non-susceptible to ertapenem (MIC >0.5µg/ml). These include 10 *Klebsiella* (9 *K. pneumoniae,* 1 *K. oxytoca*) and 3 *Enterobacter cloacae* strains which were represented a genus prevalence of 7.5% and 7.8%, respectively (**Table 1**). The isolates were recovered from 12 different patients; including two carbapenemase producing strains (one *K. pneumoniae* and one *E. cloacae*) were isolated from two different sampling sites of the same patient.

Carbapenemase classes produced

Non of the strain that tested positive in the Hodge test has produced any Class A carbapenemases . Also, three *K. pneumoniae* strains exhibited positive results in the EDTA inhibition test (**Table 2**). A total of 10/13 (76.9%) strains produced Class D (OXA-48) carbapenemases. Of these 7 were s *Klebsiella* (6 *K. pneumoniae*, 1 *K. oxytoca)* and 3 *E. cloacae* strains. The other 3 *K. pneumoniae* strains (23%) produced Class B carbapenemases (NDM-1) .

Antibiotic susceptibility

Antibiotic susceptibility is shown in **Table 2.** 10 out 13 strains (76.9%) were susceptible to imipenem and meropenem. Production of BLSE was found in all examined strains. Most isolates were nearly complete resistance to the other antibiotic families, except for colimycin and fosfomycin (**Table 3**).

Discussion

In recent years worldwide, there is increased emerging of resistance to carbapenem among *Enterobacteriaceae* clinical isolates. These isolates were resistance to several antibiotic families and were associated with high mortality (17,18). The

Table 1. Distribution of Hodge test positive isolates according to their isolation dates and the source of sampling.

Strains	Isolation date	Nature of sampling	Bacterial species
1	07/03/2009	Blood culture	*E. cloacae*
2	09/03/2009	Pus (postoperative wound)	*E. cloacae*
3	09/04/2009	Subcutaneous fluid collection	*K. pneumoniae*
4	09/24/2009	Urine	*K. pneumoniae*
5	12/22/2009	Pus (postoperative wound)	E. cloacae
6	01/27/2010	Urine	*K. pneumoniae*
7	08/13/2010	Catheter	*K. pneumoniae*
8	09/28/2010	Urine	*K. pneumoniae*
9	11/06/2010	Blood culture	*K. pneumoniae*
10	12/01/2010	Urine	*K. pneumoniae*
11	12/13/2010	Blood culture	*K. pneumoniae*
12	12/20/2010	Pus (pancreas abscess)	*K. pneumoniae*
13	12/28/2010	Urine	*K. oxytoca*

Table 2. MICs and molecular study of Hodge test positive strains.

Strains	MIC (µg/ml)			PCR	Clones
	IMP	ERT	MER		
1	0.5	1	0.25	OXA-48	EcA
2	0.5	1	0.25	OXA-48	EcA
3	0.5	3	0.38	OXA-48	Kp1
4	>32	>32	16	OXA-48	Kp2
5	2	16	2	OXA-48	EcB
6	1	2	0.5	OXA-48	Kp1
7	>32	>32	>32	OXA-48	Kp7
8	>32	>32	>32	OXA-48	Kp8
9	0.5	2	0.5	OXA-48	Kp2
10	1	4	2	NDM-1	ST15
11	1	4	2	NDM-1	ST15
12	1	4	2	NDM-1	ST15
13	1	4	1	OXA-48	ND

IMP: imipenem; ERT: ertapenem; MER: meropenem.

Table 3. Antibiotic susceptibility of carbapenemase-producing strains.

Strains	IMP	ERT	TZP	AMC	CTX	CRO	CAZ	CPO	ATM	FOS	COS	C	GENT	AN	CIP	SXT
1	S	R	R	R	R	R	S	R	S	S	S	I	R	R	S	R
2	S	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R
3	S	R	R	R	R	R	R	R	R	S	S	R	R	S	R	R
4	R	R	R	R	R	R	R	R	R	S	S	S	R	I	R	R
5	I	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R
6	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R
7	R	R	R	R	R	R	R	R	R	S	S	I	I	S	R	R
8	S	R	R	R	R	R	R	R	R	S	S	S	R	I	R	R
9	S	R	R	R	R	R	R	R	R	S	S	S	R	S	R	R
10	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R
11	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R
12	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R
13	S	R	R	R	R	R	R	R	R	S	S	R	R	S	R	R

IMP: imipenem 10 ug ; ERT: ertapenèm 10 ug ; TZP: piperacilline/tazobactam 75/10 ug ; AMC: amoxicillin/clavulanic acid 30 ug ; CTX: cefotaxime 30 ug ; CRO: ceftriaxone 30 ug ; CAZ: ceftazidime 30 ug ; CPO: cefpirome 30 ug ; ATM: aztreonam 30 ug ; FOS: fosfomycin 50 ug; COS: colistin 50 ug ; C: chloramphenicol 30 ug ; GENT: gentamicin 30 ug ; AN: amikacin 30ug ; CIP: ciprofloxacin 5 ug ; SXT: sulfametoxazole/trimetoprim 1,25/23,75 ug.

increased prevalence of carbapenem-resistance of *Enterobacteriaceae* members in hospital environment constitutes a serious threat for hospitalized patients and to control their nosocomial infection.

The rate of carbapenemase producing *Enterobacteriaceae* in our study is 2.8%. Most published studies can't be compared with our results since these were reported epidemic outbreaks of infection due to carbapenem-resistance *Klebsiella* spp. and *E.coli* strains. According to data from the European Antimicrobial Resistance Surveillance Network (EARS. Net, formerly EARSS), the rates of carbapenemase producing *K. pneumoniae* in 2009 reached 43.5% in Greece, 17% in Cyprus, 1.3% in Italy, and 1.2% in Belgium, whereas less than 1% was reported from 23 other European countries (6). Investigation of our isolates genotypes demonstrated that 3 *K. pneumoniae* strains host a plasmid which carries a gene coding for an NDM-1-type β metallo enzyme. These strains were isolated from patients who had not visited India or Pakistan as the case with most other reported studies (19,20).

This study showed a high frequency (10 /13) of OXA-48 producing strains among *Klebsiella* and *Enterobacter* spp. In Morocco, the first *K. pneumoniae* strain testing positive for OXA-48 was isolated in our laboratory in 2009 (5). Other OXA-48 *Enterobacter cloacae* strains have since then been isolated, especially in France, from patients of Moroccan origin (6). It appears from the molecular epidemiology investigation that our carbapenem resistant isolates belong to various clones, which suggests an endemic spreading of these strains in our country.

Enterobacteriaceae isolates which carry genes coding for carbapenems are not always resistant to every carbapenem *in vitro*, although many ertapenem resistant strains may remain susceptible to other carbapenems *in vitro* (21).
In this study, most carbapenemase producing strains showed susceptibility to imipenem and/or meropenem (**Table 2**). However, therapeutic efficacy against infections with carbapenemase producing bacteria which are showing a susceptible carbapenem MIC is controversial. Treatment failure has been reported by some authors due to infection with susceptible carbapenem MIC *Klebsiella* producing carbapenemase (22, 23). Others studies found no significant difference in mortality when imipenem MIC of *Klebsiella pneumoniae* is ≤4µg/ml in septicemia cases with or without carbapenemase production, but a significant increased mortality has been observed when MIC was >4µg/ml (24,25).

Conclusion

Carbapenemase producing *K. pneumoniae* and *Enterobacter cloacae* strains constitute a high proportion of bacteria isolates in Cheikh Zaid International University hospital. Early detection of *K. pneumoniae* (NDM-1) is epidemiologically and therapeutically important task in clinical laboratory. In addition, it is highly important to prevent the spread of carbapenemase producing strains among hospitalized patients by using extensive infection control measurement.

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