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Original Article

OXA-48-producing *Enterobacteriaceae* in North of Morocco: Data from regional hospital of Tangier

Younes Mahrach[1], Nadira Mourabit[2], Mohamed Bakkali[3], Abdelhay Arakrak[3], Amine Laglaoui[3]

[1] *Higher Institute of Nursing Professions and Technical Health of Tangier, Morocco*
[2] *Laboratory of Research and Development in Engineering Sciences, Faculty of Sciences and Techniques of Al-Hoceima, Abdelmalek Essaadi University, Tetouan, Morocco*
[3] *Biotechnology and Biomolecule Engineering Research Laboratory, Faculty of Sciences and Techniques of Tangier, Abdelmalek Essaadi University, Tetouan, Morocco*

Abstract

Introduction: This is a three-year retro-prospective study aimed at assessing the prevalence of carbapenem-resistant Enterobacteriaceae and to study three-year retrospective study aimed at determining the prevalence of carbapenem-resistant Enterobacteriaceae and analyzing the characteristics of OXA-48 producers.

Methodology: Six thousand one hundred eighteen bacteriological samples were assessed at Mohamed V Regional Hospital microbiology laboratory in Tangier, Morocco.

Results: Of the 1,228 identified *Enterobacteriaceae*, *Escherichia coli* was the most isolated bacteria (68%). Fifty-eight *Enterobacteriaceae* were resistant to carbapenem (4.7%). *Klebsiella pneumoniae, Escherichia coli,* and *Enterobacter cloacae* were the most common carbapenemase-producing isolates (43.1%, 27.5%, and 20.7%, respectively). All isolates were resistant to ertapenem, 62% to imipenem, whereas the lowest rate of resistance to carbapenems was against meropenem (51%). Among carbapenemase-producing isolates, 13.8% also produced extended-spectrum beta-lactamase. Most carbapenemase-producing *Enterobacteriaceae* isolates harbored the gene bla_{OXA-48} (84.5%).

Conclusions: Our findings highlight the alarming situation of the probable misuse of antibiotics. Awareness-raising sessions about the appropriate use of antibiotics and improving hospital hygiene are highly needed.

Key words: Carbapebemases; Morocco; OXA-48; antimicrobial resistance.

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Introduction

Carbapenems are a group of antibiotics considered the last resource for treating infections caused by multidrug-resistant bacteria, especially *Enterobacteriaceae,* producing extended-spectrum β-lactamases (ESBLs). The inappropriate use of these antibiotics has led to the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) [1], which has become a significant public health problem worldwide. The situation is more worrying when the resistance is mediated by carbapenemase production, which is the most powerful carbapenemase carbapenemase production, the most powerful and potent mechanism of resistance to carbapenems [2]. These resistance genes can spread by horizontal and vertical gene transfer.

As indicated by Ambler, Oxacillinases, or class D ß-lactamases with carbapenemase properties, have been reported among different species of gram-negative bacteria, including *Enterobacteriaceae* [3,4]. OXA-48 is the most common variant of oxacillinase [5]. Resistance to oxacillinases was frequently linked to other antibiotic resistance. The blaOXA-48 gene encoding for these enzymes has often been shown to be associated with genes encoding ESBLs. This increases the hydrolysis spectrum of β-lactams [6].

Since its first identification in *Klebsiella pneumoniae* isolates from Turkey in 2001 [6], OXA-48 has been widely spread worldwide [2,4]. In Morocco, the first case of OXA-48 was described in 2010 by Benouda *et al.* [7]. Other carbapenemase-producing *Enterobacteriaceae* have been later documented in Morocco [8]. OXA-48 was the most common carbapenemase in Morocco [9], and was reported in several settings (hospitals, community, and environment) [8,10,11]. These studies presented an overview of OXA-48 carbapenemase resistance in Rabat, Casablanca, Fez, and Marrakech. However, our knowledge is still limited in the North Morocco. The current study aimed to determine for the first time in the North of Morocco the prevalence of CRE and to study

the characteristics of OXA-48 producers over three years.

Methodology

Study design and sample population

This is a three-year retro-prospective study carried out between January 2014 and January 2017 at the microbiology laboratory of Mohamed V Regional Hospital in Tangier, Morocco. A total of 6,118 bacteriological samples were included. The strains were isolated from different biological liquids (pus and urine) of hospitalized patients or those followed in outpatient clinics as part of the microbiological diagnosis requested by their attending physicians.

Information on sex, age, ward, specimen type, infection type, and other information was collected from vouchers of medical examination and laboratory records.

In this study, all *Enterobacteriaceae* were isolated, but only CRE was assessed to determine resistance mediated by carbapenemase production, specifically OXA-48 producers. The isolated strains were not duplicated.

Culture and identification of isolates

Bacterial culture was carried out on Cystine Lactose Electrolyte Deficient (CLED), Eosin Methylene Blue (EMB), and MacConkey media. *Enterobacteriaceae* were identified using conventional methods (Gram staining, growth in selective agar media, and the API 20E gallery) [12]. The positivity criteria were 10^5 CFU according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2015 [13].

Antibiotic susceptibility tests

Antibiotic resistance was tested using the Kirby-Bauer agar diffusion method [14] on Mueller Hinton medium, according to the recommendations of EUCAST 2015 [13].

The antibiotics tested were: Amoxicillin (30 µg) Amoxicillin/clavulanic acid (20/10 µg), Piperacillin/tazobactam (30/6 µg), Aztreonam (30 µg), Cefepime (30 µg), Cefoxitin (30 µg), Cefixime (5 µg), Cefotaxime (5 µg), Ceftriaxone (30 µg), Ceftazidim (10 µg), Norfloxacin (5 µg), Ciprofloxacin (5 µg), Gentamycine (10 µg), Tobramycin (10 µg), Amikacin (30 µg), Ertapenem (10 µg), Imipenem (10 µg), Meropenem (10 µg), Trimethoprim/Sulfamethoxazole (1.25 µg /23.75 µg), Tetracycline (30 µg), Tigecycline (15 µg), Fosfomycin (50 µg) and Colistin (50 µg). A Cloxacillin disk (5 µg) was also used to detect hyperproduction of cephalosporinases with porin loss. The *E. coli* strain ATCC 25922 was used as an internal quality control [13].

Phenotypic Tests

Carbapenem-resistant strains were screened by resistance to Ertapenem, Imipenem, or Meropenem. Some phenotypic tests were used to determine carbapenemase classes, including specific and non-specific ones. The modified Hodge test and Carba NP were used to detect carbapenemases production. Phenyl boronic acid (PBA) and cloxacillin were used to detect class A, and EDTA-disk synergy to detect class B [15]. Strains that phenotypically produce carbapenemases (positive modified Hodge test at least) were assessed for molecular confirmation and determining the carbapenemase classes.

Detection of carbapenemase-encoding genes

All 58 phenotypically carbapenemase-producing *Enterobacteriaceae* were genotypically tested. Chromosomal DNA was extracted as previously described. Plasmid DNA was extracted using a commercial mini-prep kit (Bioline Isolate plasmid Mini Kit Cat No. Bio-52026) according to the manufacturer's instructions.

The polymerase chain reaction (PCR) technique was used to amplify two genes encoding class A enzymes (KPC-1, NMC-1), two genes encoding class B enzymes, metallo-beta-Lactamase (MBL) groups (VIM-1, IMP-1), and one gene encoding class D enzyme (OXA-48) (Table 1).

PCR amplification was carried out in a total volume of 50 µL, including 2 µL of DNA, 0.5 pmol/µL of each

Table 1. Primers for carbapenemase genes amplification.

Enzyme	Primers	Size (pb)	T°C	Ref
KPC-1	F : ATGTCACTGTATCGCCGTCT R : TTTTCAGAGCCTTACTGCCC	893	55.4 55.4	[2]
IMP-1	F : TCGTTTGAAGAAGTTAACGG R : ATGTAAGTTTCAAGAGTGATGC	568	49.3 49.3	[28]
VIM-1	F : GGTGTTTGGTCGCATATCGCAA R : ATTCAGCCAGATCGGCATCGGC	520	55.6 57.5	[28]
OXA-48	F : TTGGTGGCATCGATTATCGG R : GAGCACTTCTTTTGTGATGGC	744	55.3 55.0	[2]

primer, 25 mM $MgCl_2$, 2.5 mM of each dNTP, 1X Taq DNA buffer, 1 unit of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany).

Amplification was performed via 2720 thermal cycler-applied biosystems. The amplification conditions were as follows: 94 °C for 2 minutes, followed by 35 cycles of 94 °C for 30 seconds, annealing temperature for 45 seconds (Table 1), and 72 °C for 1 minute. Finally, 72 °C for 7 minutes.

PCR products were detected by 1% agarose gel electrophoresis.

Results

Isolated strains

Overall, 6,118 samples were analyzed. 1,808 samples met the positivity criteria 10^5 CFU (29.5%). 1,228 *Enterobacteriaceae* were identified (67.9%), with *E. coli* being the most isolated bacteria (835; 69.4%) (Table 2).

Based on phenotypic tests, 58 (4.7%) *Enterobacteriaceae* resistant to carbapenems were identified, of which 49 were OXA-48 carbapenemase-producing *Enterobacteriaceae*. Among these 58 isolates, *Klebsiella pneumonia* was the most identified bacteria (25; 43.1%) (Table 3).

These carbapenemase-producing strains were isolated from 54 inpatients and four outpatients. The male/female sex ratio was 0.9. The average age of patients was 52 (15-80), with 40 (69%) being older than 60.

More than half (30; 52%) of carbapenemase-producing *Enterobacteriaceae* strains were isolated from pus, while 48% (28) were from urine.

Antibiotic susceptibility profile

Resistance to beta-lactams

Among the 58 carbapenemase-producing isolates, 13.8% (8) produced also ESBL (Figure 1). Antibiogram showed that all isolates were resistant to amoxicillin and amoxicillin-clavulanic acid. The majority of the strains were resistant to piperacillin-tazobactam (88%), cefoxitin (C2G) (78%), Ceftazidime and Ceftriaxone (C3G) (81%), and Cefotaxime (C3G) (84%). While 76% and 69% were resistant to cefepimes (C4G) and aztreonam, respectively.

Regarding carbapenems, all isolates were resistant to ertapenem. Almost two-thirds (62%) of the isolates showed resistance to imipenem, whereas the lowest rate

Figure 1. Synergy tests (champagne corks appearance on the specific ESBL antibiogram) between the AMC disc and the C3G discs (CTX and CAZ), the C4G disc (FEP), and the aztreonam (ATM) in a carbapenemase-producing Enterobacteriaceae.

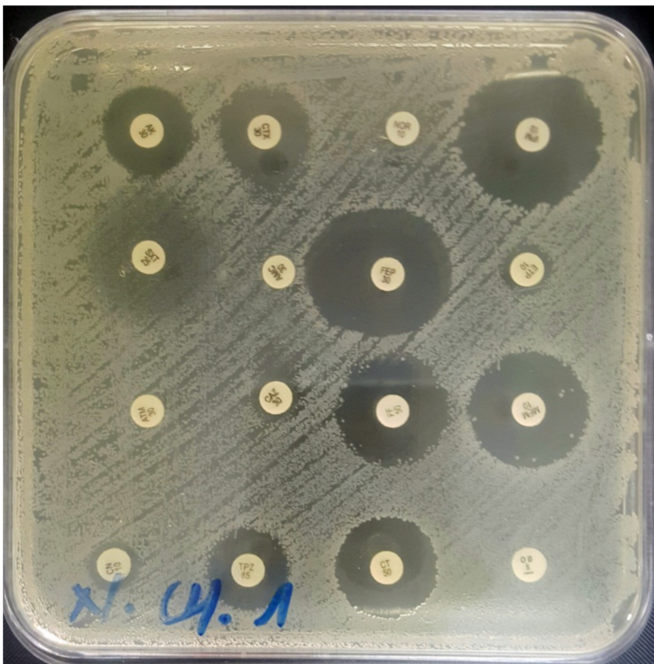


Table 2. The isolated strains.

Bacteria	n	(%)
Escherichia coli	835	69.4
Klebsiella pneumoniae	248	20.1
Enterobacter cloacae	92	7.4
Proteus mirabilis	18	1.4
Serratia marcescens	11	0.8
Klebsiella oxytoca	5	0.4
Providencia spp	5	0.4
Others	14	1.1

Table 3. Distribution of carbapenemase-producing strains.

Bacteria	n	(%)
Klebsiella pneumoniae	25	43.1
Escherichia coli	16	27.6
Enterobacter cloacae	12	20.7
Proteus. mirabilis	2	3.4
Klebsiella. oxytoca	2	3.4
Serratia. marcescens	1	1.7

Table 4. The resistance pattern of the isolated carbapenemase-producing *Enterobacteriaceae* to other families of antibiotics.

Antibiotics family	Resistance %
Aminoglycosides	
Gentamycin	83
Tobramycin	79
Amikacin	65
Cyclins	
Tetracycline	77
Tigecycline	43
Fluoroquinolones	
Ciprofloxacin	93
Norfloxacin	93
Levofloxacin	81
Trimethoprim-sulfamethoxazole	
Trimethoprim-sulfamethoxazole	81
Fosfomycin	
Fosfomycin	34
Polymyxins	
Colistin	0

Table 5. Antibiotic resistance profile according to isolated bacteria.

Variables (n)	GN	TOB	AK	TET	TIG	NOR	LEV	SXT	FOS	CS	CIP
E. coli (16)	10	09	06	12	05	15	09	12	04	0	15
K. pneumonia (25)	23	22	20	20	11	24	22	22	08	0	24
K. oxytoca (2)	1	1	1	1	0	2	2	1	0	0	02
E. cloacae (12)	11	11	08	10	08	11	11	10	08	0	11
P. mirabilis (2)	2	2	2	1	1	2	2	2	0	0	02
S. marcescens (1)	1	1	1	1	0	1	1	1	0	0	01
Total	48	46	38	45	25	54	47	47	20	0	54

Ak: Amikacin; CS: Colistin; FOS: Fosfomycin; GN: Gentamycin; LEV: Levofloxacin NOR: Norfloxacin; SXT: Trimethoprim / Sulfamethoxazole; TET: Tetracyclin TIG: Tigecycline; TOB: Tobramycin; CIP: Ciprofloxacin ; *E. coli: Escherichia coli; K. pneumonia: Klebsiella pneumonia; K. oxytoca: Klebsiella oxytoca; E. cloacae: Enterobacter cloacae; P. mirabilis: Proteus mirabilis; S. marcescens : Serratia marcescens.*

of resistance to carbapenems was against meropenem with 51%.

Resistance to other antibiotics

Ciprofloxacin and Norfloxacin had the highest resistance rate (93%), followed by Gentamycin (83%), trimethoprim/sulfamethoxazole, and Levofloxacin (81%) (Table 4).

The resistance patterns of the isolated bacteria are summarized in Table 5.

Evolution of antibiotic resistance over time

The evolution of resistance over time varied according to the antibiotic types. Since 2014, no strain has been sensitive to Amoxicillin, Amoxicillin combined with Clavulanic acid or Ertapenem. For other antibiotics, the resistance increased remarkably. However, this resistance increase affected Fosfomycin, Amikacin, and Tigecycline less over the three years (Figure 2).

Phenotypic detection of carbapenemases

Modified Hodge test

The modified Hodge test analyzed all 58 *Enterobacteriaceae* resistant to carbapenems to detect carbapenemase production. All the strains were positive (Figure 3).

Figure 3. Results of the Modified Hodge Tests. Isolates were considered positive when the inhibition halo was distorted.

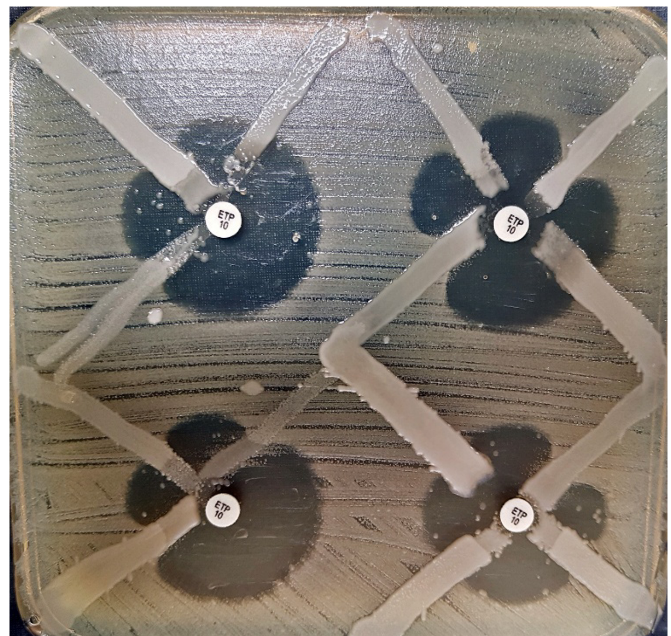
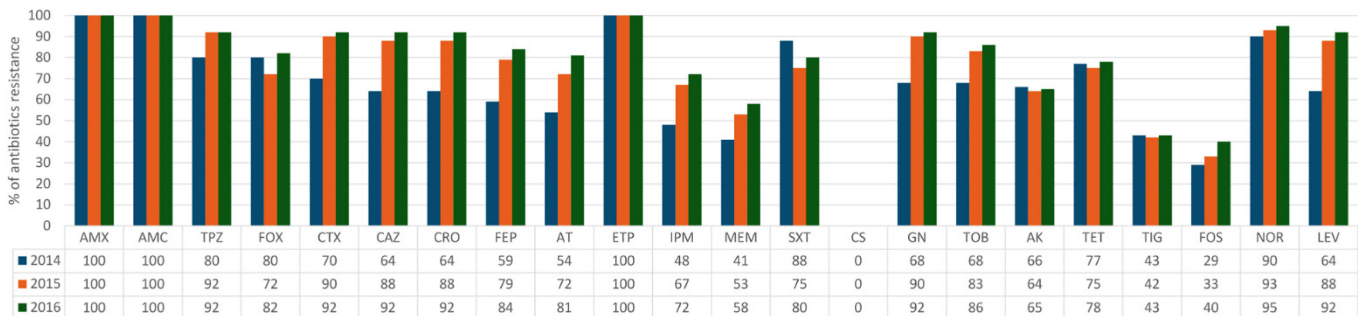


Figure 2. The evolution of antibiotic resistance according to three years of study.



Ak: Amikacin; AMX: Amoxicillin; AMC: Amoxicillin/clavulanic acid; ATM: Aztrenam; CAZ: Ceftazidime; CRO: Ceftriaxone; CTX: Cefotaxime; FEP: Cefepime; FOX: Cefoxitin; CS: Colistin; ETP: Ertapenem; FOS: Fosfomycin; GN: Gentamycin; IPM: Imipenem; MEM: Meropenem; NOR: Norfloxacin; SXT: Trimethoprim / Sulfamethoxazole; TET: Tetracyclin TIG: Tigecycline; TOB: Tobramycin; TPZ: Piperacillin/tazobactam

Synergy tests with EDTA, PBA, and cloxacillin, and Carba NP Biochemical Test

The synergy test with EDTA was positive for six isolates (Figure 4). As such, 10.3% of the isolates were considered as MBL-producing bacteria. The synergy test with PBA and cloxacillin was positive for two isolates showing that 3.4% were carbapenemase class A. These tests were negative for all the remaining isolates.

Carba NP Biochemical test was positive for 84% (49/58) of our CRE.

Prevalence of Carbapenemase-encoding genes

Among the 58 carbapenemase-producing isolates, 49 (84.5%) harbored the *bla*OXA-48 gene, and nine isolates (15.5%) had other genes. Of these 9 cases, 7 (12%) were producers of Ambler class B beta-lactamase carbapenemase, with 4 (7%) producing VIM-1 and 3 (5%) producing IMP-1, and 2 (3.5%) producers of KPC-1 carbapenemase of class A. Figure 5 shows the PCR-amplified OXA-48 gene.

Discussion

This work was conducted in the North of Morocco to study carbapenemase-producing *Enterobacteriaceae*, especially OXA-48 producers.

In the present report, we have identified 1,228 *Enterobacteriaceae*, which represented 68% of all the collected bacteria. The prevalence of CRE in Morocco is not well established. Only a few studies have been published and conducted in a limited duration. The present study isolated and identified 4.7% of CRE (58/1228). This prevalence is two times inferior to that of Loqman *et al.* in 2018 at Mohamed VI University Hospital, Marrakech, Morocco (8.17%) [9]. However, our frequency remains higher than that reported by El Wartiti *et al.* study (4.7% *versus* 2.8%), conducted over 19 months in a University Hospital in Rabat, Morocco [16]. This later included only 463 initial *Enterobacteriaceae* strains. Our prevalence is also higher than the rate recorded by Essayagh *et al. study* (4.7% *versus* 1.42%) over six months among the strains of *K. pneumoniae* isolated from the military hospital in Rabat [17]. The prevalence of CRE in the United States was generally around 1.4–4.2% [18,19]. Larger multicenter studies are needed to determine the prevalence of CRE in our country.

Most of our isolates were from patients with prolonged hospital stays in different surgical services who were likely to have nosocomial infections caused by carbapenemase-producing *Enterobacteriaceae.* The average age of these patients was 52, and 69% of those harboring these strains were older than 60. This indicates that this type of infection affects most vulnerable and/or immunocompromised individuals, which is well documented in the literature [11,20].

Our sex ratio was 0.9, showing that both sexes were almost equally affected. The distribution of isolates in samples showed that infections caused by carbapenemase-producing *Enterobacteriaceae* have no particular tendency site, as 52% of carbapenemase-producing *Enterobacteriaceae* strains were isolated from pus, and 48% were from urine.

The Mediterranean area is known for the increased prevalence and variability of carbapenemases due to the great diversity and population mixing in this world region. The carbapenemase types differ across countries, partially according to the population exchange relationship between the areas and the possible reservoirs of each carbapenemase [21]. We showed that *K. pneumoniae* was the most prevalent carbapenemase-producing *Enterobacteriaceae* (43%), which aligns with Chabah *et al. study* [22]. The

Figure 4. Test with imipenem and imipenem combined with EDTA showing the difference between the levels of inhibition of the bacteria.

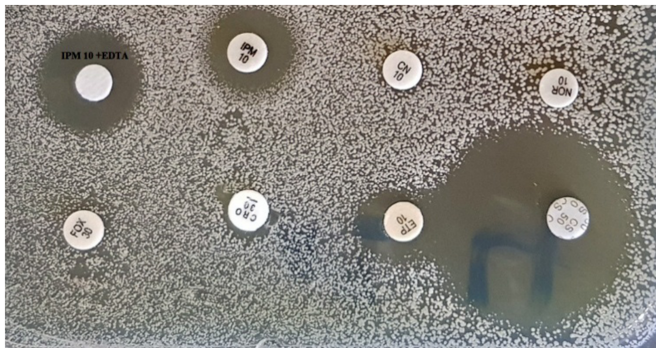
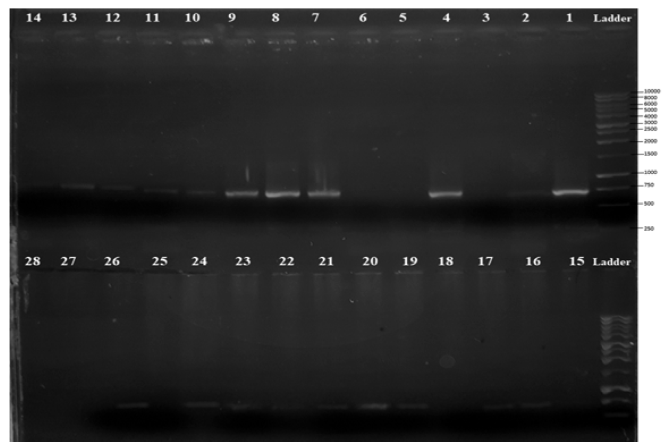


Figure 5. Agarose gel electrophoresis showing PCR-amplified OXA-48 gene (744 bp).



Lane Ladder: DNA ladder; lane 28: negative control; lanes (1, 2, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 24 and 26): isolates positive for OXA-48 gene.

distribution of carbapenemase enzyme among *Enterobacteriaceae* proves that it is not linked to a type of bacteria. Still, it is generalized with a predominance of *K. pneumoniae*, as reported in other studies [8,23,24].

Based on our findings, OXA-48-producing strains were most frequently isolated from our hospital (84.5%). This follows Moroccan-published studies [10,22,25] reporting that this enzyme was the most common carbapenemase in our country. OXA-48 has been considered a source of nosocomial infections in many countries [22]. Other carbapenemase genes, such as *KPC*, *IMP*, *VIM*, and *NDM-1*, have also been identified in Morocco, with a low frequency compared to OXA-48 [26].

A study by Poirel *et al.* showed that African countries, the Middle East, Turkey, and India, represent the most common reservoirs for OXA-48 type carbapenemases. Moreover, OXA-48 has become well-documented in European countries, with some reported hospital outbreaks [27]. Our results support national studies and those from Mediterranean countries about the resistance of *Enterobacteriaceae* to carbapenems dominated by OXA-48.

The majority of our isolated strains (93.1%) were from patients with prolonged hospital stays, while 6.9% were isolated from outpatients. Patients carrying these strains have a hospitalization history, which suggests that the strains were of hospital origin but circulated in town. This is a worrying situation given that all the detected resistance genes were mediated by plasmids, which increases the risk of spreading resistance by crossing with other bacteria in the city that are known to be sensitive.

Our strains were isolated from infected patients (54 inpatients and four outpatients). According to Girlich *et al.,* who conducted a study at the Cheikh Zayed Hospital in Rabat in 2013, 10/77 rectal swabs were carbapenemase-producing *Enterobacteriaceae* with a carrying rate of 12.8% [8]. However, a study by Gijón *et al.* conducted on hospitalized and non-hospitalized patients highlighted the fecal carriage of carbapenemase-producing *Enterobacteriaceae* in non-infected patients with no contact with infected or colonized patients [28]. Hence, a systematic search for carbapenemase-producing *Enterobacteriaceae* is highly needed in all newly hospitalized patients.

Our results showed that resistance to non-beta-lactamase antibiotics varies over the years, with a general tendency towards increasing resistance, according to the antibiotic family. Resistance to fluoroquinolones and aminoglycosides represented the highest resistance rate. Furthermore, we found a lower Tigecycline and Fosfomycin resistance rate (34%). This could be related to the non-commercialization of these antibiotics in our country. Thus, the strains maintained their sensitivity to these antibiotics.

Colistin is considered one of the last options to treat infections caused by carbapenemase-producing *Enterobacteriaceae* and other multidrug-resistant bacteria. The increase in the use of colistin is associated with the appearance of resistant isolates due to the loss of lipopolysaccharide production [29], and this use was linked to higher nephrotoxicity (0-53%) [30]. In our work, we found that all strains were sensitive to colistin.

Available evidence suggests that resistance through the production of carbapenemases, particularly by *Enterobacteriaceae,* is dramatically increasing worldwide [31]. These resistant strains have limited treatment options. Medical treatments are based on the in vitro activity of some antibiotics, which classifies strains as sensitive, intermediate, or even resistant according to EUCAST 2015 recommendations [32]. However, antibiotic treatment showing *in vitro* sensitivity does not necessarily lead to therapeutic success. Hence, developing active antibiotics against these carbapenem-producing bacteria is highly required [31].

Detection, rapid identification, and adequate treatment are needed to control the spread of multi-resistant bacteria, especially carbapenemase-producing *Enterobacteriaceae*.

Despite the importance of phenotypic methods, the detection of carbapenemase-producing *Enterobacteriaceae* by these techniques remains insufficient, as the resistance level to carbapenems can sometimes fall within the sensitivity threshold. The subjective interpretation may also affect phenotypic results, especially in perplexing and/or borderline cases.

Molecular methods such as PCR amplification of the target gene (followed or not by sequencing and microarray technology) are widely used to confirm and determine the type of carbapenemases. These methods are sensitive and specific and reduce the detection time, which allows a successful intervention [33]. However, these molecular techniques are limited by their relatively high costs and the requirement for trained microbiologists. Another disadvantage is the predefined nature of the resistance genes that could be detected. Hence, these methods are not able to detect new carbapenemase gene types. We also used PCR amplification, which revealed the presence of at least one gene encoding carbapenemases. However, we could not confirm our findings by sequencing.

Our study showed some limitations. Interpretation of susceptibility depends on the recommended thresholds used when testing. Moreover, the coexistence of several carbapenem-resistance genes, particularly the genes responsible for carbapenemase production, is another difficulty for interpretation. On the other hand, our strains may harbor other carbapenem-resistance genes that we did not study due to financial issues. Finally, limiting our study to *Enterobacteriaceae* and the lack of data about hospital stays and detailed patient histories represent other limitations for our work.

A systematic early screening of all hospitalized patients is needed to detect potential resistance and colonization. Our findings should be communicated to the local and regional authorities so that they can take the appropriate actions to limit the risk.

Conclusions

In our study, 58 carbapenemase-producing *Enterobacteriaceae* were detected, including 49 OXA-48 producers. The most common strains were *K. pneumoniae, E. coli, and E. cloacae*. Other *Enterobacteriaceae* were also detected with a less frequent rate, which proves the wide spread of carbapenemases among different *Enterobacteriaceae*. All isolates were resistant to ertapenem, while the lowest rate of resistance to carbapenems was against meropenem. The isolated carbapenemase-producing *Enterobacteriaceae* exhibited high resistance to many antibiotics except for tigecycline, fosfomycin, and colistin. *OXA-48*, *VIM-1*, *IMP-1,* and *KPC-1* genes were identified with a predominance of *OXA-48*.

The majority of carbapenemase-producing *Enterobacteriaceae* are isolated from patients with prolonged hospital stays in different surgical services, which indicates that most of these strains are of nosocomial origin. Hence, combining phenotypic and molecular methods is important for the rapid detection of carbapenem-resistant bacteria to prescribe a suitable treatment and establish the appropriate precautionary measures.

The spread of multidrug-resistant strains will seriously limit therapeutic options shortly. To overcome this situation, raising awareness about the appropriate use of antibiotics and searching for new therapeutic alternatives would be necessary. Appropriate monitoring or even the establishment of a national program to overcome carbapenemase-producing *Enterobacteriaceae* is warranted to avoid the emergence of uncontrolled bacterial generation.

Authors' contributions

YM drafted the work and conceptualized it, collecting, acquiring, analyzing, and interpreting data. NM contributed to the conception of the work, data collection and interpretation, and phenotypic study. AA and MB revised the manuscript critically for important intellectual content. AL supervised the work and revised the manuscript. All authors approved the publication of the version.

Corresponding author

Younes Mahrach, PhD
Higher Institute of Nursing Professions and Technical Health of Tangier
90 000 Tetouan, Morocco.
Tel: +212 667 993 521
Email: younesuniver@gmail.com

Conflict of interests

No conflict of interests is declared.

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