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High rate of faecal carriage of extended-spectrum β-lactamase and OXA-48 carbapenemase-producing Enterobacteriaceae at a University hospital in Morocco

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Abstract

Carbapenemase-producing Enterobacteriaceae isolates are being increasingly reported, particularly from countries surrounding the Mediterranean area. We aimed to quantify the prevalence of carbapenemase- and extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in rectal swabs from hospitalized patients in a University hospital in Morocco, and to compare the performance of three screening media: ChromID ESBL (bioMérieux), Brilliance CRE (OXOID, Thermofisher) and SUPERCARBA (home made). Genetic detection and plasmid analysis were performed by PCR and sequencing. Strain comparison was performed by multi-locus sequence typing and the Diversilab technique (bioMérieux). The prevalence of multidrug-resistant Enterobacteriaceae was high, with 33 ESBL producers (42.85%, mainly CTX-M-15) and 10 OXA-48 producers (13%), corresponding to two major clones of *K. pneumoniae* (70%) and a clone of *Enterobacter cloacae* (30%). The three screening media showed the same sensitivity for detection of carbapenemase-producing Enterobacteriaceae, whereas the SUPERCARBA medium was more specific than the two other media. The average faecal carriage of ESBL or carbapenemase-producing Enterobacteriaceae varied from 1×10^2 to $>1 \times 10^8$ CFU/g of stools. This study shows a high prevalence of multidrug-resistant Enterobacteriaceae, and particularly of OXA-48 producers. The new carbapenem-containing medium, SUPERCARBA, was as sensitive as Brilliance CRE and ChromID ESBL, and more specific for the detection of Enterobacteriaceae expressing those carbapenemases.

Keywords: Multidrug-resistant bacteria, prevalence, stools

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Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) isolates have been increasingly reported worldwide, and among them OXA-48 is mostly identified from countries surrounding the Mediterranean area [1–3].

Faecal carriage of CPE isolates has been rarely investigated, compared with carriage of extended-spectrum β-lactamases (ESBLs)-producing isolates, particularly among patients who were not selected for their relationship to outbreak cases [4,5]. The aim of this study was to evaluate the prevalence of ESBL and carbapenemase producers among intestinal microbiota of hospitalized patients at a University hospital in Morocco. This is the first study of the prevalence of OXA-48 producers in this country, where OXA-48 producers might be endemic [3].

Methods

Patients and specimens

Rectal swab specimens were randomly collected, as per survey protocol, from 77 different hospitalized patients from any medical unit of the University Hospital of Rabat (Morocco) in

March 2012. Bacteria from rectal swabs were resuspended in 1 mL of sterile 0.9% saline and immediately sent at room temperature to Bicêtre Hospital, France.

Culture screening methods

Serial dilutions of each sample (100 μL) were directly plated on to selective media designed to identify CPE: the SUPERCARBA and Brilliance CRE (OXOID, Thermo Fisher, Villebon-sur-Yvette, France) and a screening medium designed to identify extended-spectrum β-lactamases, ChromID ESBL (bioMérieux, La Balme-les-Grottes, France). Moreover, 100 μL of rectal swab sample suspension were used in an enrichment procedure consisting of an overnight culture at 37°C in 10 mL of Brain Heart Infusion broth (BHI) supplemented with 0.25 mg/L ertapenem (MSD, Paris, France) before plating on to the SUPERCARBA medium. Plates were incubated for 18 h at 37°C. Quantification was performed by counting the growing colonies and estimating the number of CFU/g of stools. Estimation of the amount of stools harboured by a swab was *c.* 100 mg.

Detection and identification of CPE- and ESBL-producing Enterobacteriaceae

Presumptive Enterobacteriaceae were identified according to the colour of the colonies on the chromogenic media (Brilliance CRE and ChromID ESBL). On SUPERCARBA, lactose-positive and oxidase-negative colonies were retained for further analysis. Presumptive CPE and ESBL-producing Enterobacteriaceae were identified by the Api 20E system (bioMérieux) and susceptibility testing was evaluated by disk diffusion according to the CLSI guidelines [6]. Non-fermenting Gram negatives growing as white or naturally pigmented colonies on ChromID ESBL and Brilliance CRE and lactose-negative and oxidase-positive colonies growing on SUPERCARBA have been excluded, as well as *Enterococci* and *Aeromonas* sp., growing as blue colonies on ChromID ESBL and Brilliance CRE. Minimum inhibitory concentrations (MICs) of imipenem, ertapenem and meropenem were determined for all Enterobacteriaceae growing on SUPERCARBA and Brilliance CRE using Etest strips (bioMérieux). Chromosomally-encoded AmpC hyperproduction was searched for by using cloxacillin-containing plates. ESBL production was confirmed by using the ESBL NDP test [7].

Molecular identification of carbapenemase and ESBL genes

PCR assays and sequencing were performed with a series of primers designed for the detection of Ambler class A (bla_{KPC}), B (bla_{VIM}, bla_{IMP} and bla_{NDM}) and D (bla_{OXA-48}) carbapenemases and ESBL genes [8].

Plasmid DNA was extracted from the stool sample swabs using the Qiagen Plasmid DNA mini kit (Qiagen, Courtaboeuf, France) and used as a template for PCR assays in the search for OXA-48-producers that would not have grown on the selective media used in this study. Plasmid DNA from carbapenemase producers was extracted using the Kieser technique [9] and repA PCR was performed with primers specific to the replicase gene as originally identified from the IncL/M-like bla_{OXA-48} -harbouring plasmid [10].

Multilocus sequencing typing (MLST) and Diversilab

MLST was performed for OXA-48-producing *K. pneumoniae* isolates, as previously described [11]. Clonal relatedness was established for OXA-48 producers by the use of DiversiLab, a semi-automated typing system based on repetitive sequence-based PCR (rep-PCR), following the manufacturer's instructions (bioMérieux).

Sensitivity and specificity

The sensitivity and specificity were calculated for each screening medium. True-positives were defined as all presumptive CPE growing on the media and genotypically confirmed to be CPE positives. False-positives were all presumptive Enterobacteriaceae growing on the media that were not confirmed to be CPE by PCR for the tested genes. Non-fermenting bacteria growing on these media are frequently recovered from stool samples. Specificity was then calculated by (i) taking into account only Enterobacteriaceae growing on those screening media and also (ii) taking into account all the non-specifically growing bacteria (including non-fermenting bacteria).

Results and Discussion

Prevalence and identification of ESBL-producing Enterobacteriaceae

Quantification showed a much higher amount of ESBL-producing Enterobacteriaceae, detected on the ChromID ESBL medium, than of carbapenem-resistant bacteria, detected on CRE/SUPERCARBA (Table 1). The occurrence of ESBL-producing Enterobacteriaceae was high (42.8%), with 33 ESBL-positives out of 77 rectal swabs (Table 1). The majority of ESBL-producing Enterobacteriaceae were *Escherichia coli* (48.5%), *Klebsiella pneumoniae* (39.4%) and *Enterobacter cloacae* (12.1%) (Table 1). CTX-M-15 and SHV-12 represented 90.9% and 9.1% of the ESBLs, respectively (Table 1).

Limited data regarding the prevalence of ESBLs in Moroccan hospitals are available. A previous study performed in 2012 in the same hospital, showed 28.6% of ESBL-producing Enterobacteriaceae in blood cultures, superficial and deep pus, and catheters [12], whereas 20.7% were recovered from October

TABLE 1. ESBL and carbapenemase-producing Enterobacteriaceae recovered from three selective media from 77 rectal swab samples

Sample	Quantification of Enterobacteriaceae (CFU/g of stools)			Occurrence of Enterobacteriaceae after enrichment procedure	MICs (μg/mL)[a]				
	ChromID ESBL	Brilliance CRE	SUPER CARBA		IPM	ETP	MEM	ESBL/AmpC	Carbapenemase
K. pneumoniae 4	$>1 \times 10^8$	1×10^4	0	Yes	0.25	0.03	0.23	CTX- M-15	–
E. coli 4	$>1 \times 10^8$	4×10^3	0	Yes	0.19	0.03	0.03	CTX- M-15	–
E. coli 6	$>1 \times 10^8$	4×10^6	0	No	0.5	0.19	0.06	SHV-12	–
K. pneumoniae 7	2×10^2	0	0	No				CTX- M-15	–
E. cloacae 8	$>1 \times 10^8$	1×10^7	0	–[b]	0.25	0.06	0.01	AmpC	–
C. freundii 8	$>1 \times 10^8$	5×10^4	1×10^3	–	0.5	0.25	0.06	AmpC	–
E. coli 8	$>1 \times 10^8$	5×10^4	0	–	0.19	0.03	0.03	CTX- M-15	–
E. coli 9	$>1 \times 10^8$	0	0	No				CTX- M-15	–
E. coli 10	4×10^7	0	0	No				CTX- M-15	–
K. pneumoniae 10	4×10^7	4×10^2	3×10^3	–	2	4	2	CTX- M-15	OXA-48
E. coli 15	$>1 \times 10^8$	0	0	No	2	4	2	CTX- M-15	–
E. cloacae 15	1×10^6	$>1 \times 10^8$	$>1 \times 10^8$	–	0.75	3	0.5	CTX- M-15	OXA-48
E. coli 22	4×10^2	0	0	No	0.75	3	0.5	CTX- M-15	–
E. coli 23	2×10^2	0	0	No				CTX- M-15	–
E. coli 26	5×10^7	1×10^5	0	0	No	0.19	0.03	CTX- M-15	–
E. coli 35	3×10^5	0	0	No				SHV-12	–
E. coli 36	3×10^5	0	0	No				CTX- M-15	–
E. coli 39	$>1 \times 10^8$	1×10^8	0	No	0.38	0.19	0.06	CTX- M-15	–
E. coli 42	1×10^4	0	0	No				SHV-12	–
43[c]	0	0	0	No		–		CTX- M-15	OXA-48
E. coli 47	5×10^3	0	0	No				CTX- M-15	–
K. pneumoniae 48	$>1 \times 10^8$	4×10^3	0	No	0.25	0.03	0.23	CTX- M-15	–
K. pneumoniae 49	7×10^6	0	0	No				CTX- M-15	–
E. coli 58	4×10^7	4×10^4	0	No	0.19	0.03	0.03	CTX- M-15	–
K. pneumoniae 58	2×10^5	0	0	No				CTX- M-15	–
E. cloacae 60	1×10^8	1×10^8	1×10^5	–	1	4	0.75	CTX- M-15	OXA-48
E. coli 73	4×10^3	0	0	No				CTX- M-15	–
K. pneumoniae 75	3×10^2	0	0	No				CTX- M-15	–
C. freundii 75	3×10^2	0	0	No				AmpC	–
E. coli 78	2×10^3	0	0	No				CTX- M-15	–
K. pneumoniae 86	$>1 \times 10^8$	$>1 \times 10^8$	$>1 \times 10^8$	–	2	3	1	CTX- M-15	OXA-48
K. pneumoniae 89	3×10^4	3×10^4	3×10^4	–	2	3	0.75	CTX- M-15	OXA-48
K. pneumoniae 90	7×10^7	7×10^7	7×10^7	–	2	4	2	CTX- M-15	OXA-48
E. cloacae 91	$>1 \times 10^8$	$>1 \times 10^8$	2×10^7	–	1	4	0.75	CTX- M-15	OXA-48
K. pneumoniae 91	$>1 \times 10^8$	$>1 \times 10^8$	2×10^7	–	2	4	1.5	CTX- M-15	OXA-48
K. pneumoniae 93	1×10^6	0	0	Yes	2	4	2	CTX- M-15	OXA-48
K. pneumoniae 95	0	0	0	Yes	2	4	1	CTX- M-15	OXA-48
E. cloacae 99	$>1 \times 10^8$	$>1 \times 10^8$	0	No	0.25	1	0.25	AmpC	–

[a]Minimum inhibitory concentrations (MICs) of carbapenems have been determined for all samples that have grown with Brilliance CRE and SUPERCARBA.
[b]–, not applicable. Positive cultures after direct plating have not been used in the enrichment procedure.
[c]This sample was positive by PCR on plasmid DNA extracts.

2006 to March 2007 in Casablanca in Enterobacteriaceae isolated from blood cultures, urine, pus and bronchial sampling [13]. The prevalence rate of ESBLs in rectal swabs of hospitalized patients obtained in our study is higher than that observed in a French hospital in 2009 (with 15.8% of ESBL-producing strains) [14].

Sensitivity and specificity of screening media for the detection of CPE

The prevalence of carbapenemase producers was quite high (13%), with ten OXA-48 producers out of the 77 rectal swabs (Table 1). Before the enrichment culture step with ertapenem, the ChromID ESBL showed a higher sensitivity (90%) than the Brilliance CRE and SUPERCARBA media (80%) for detection of OXA-48. This result was due to the fact that all OXA-48-producing isolates co-expressed an ESBL (Table 1). Nevertheless, ChromID ESBL and Brilliance CRE showed a lower specificity (68.6 and 86.6%, respectively) than the SUPERCARBA (98.5%) (Table 1) for the detection of Enterobacteriaceae. The ChromID ESBL, Brilliance CRE and SUPERCARBA media showed 100% sensitivity for detection of OXA-48 producers after the enrichment step. The enrichment procedure helped to recover two OXA-48-producing *K. pneumoniae* (isolates 93 and 95, Table 1), which were not identified by direct plating on Brilliance CRE and SUPERCARBA media (Tables 1 and 2).

Specificity

Overall, nine non-carbapenemase-producing isolates were detected on Brilliance CRE (false-positives), versus one on SUPERCARBA (Table 1). All non-carbapenemase producers growing on Brilliance CRE and SUPERCARBA were ESBL producers (CTX-M-15 and SHV-12) or AmpC-hyperproducing *E. cloacae* or *C. freundii* isolates, as previously reported (Table 1) [15,16]. The sensitivity of Brilliance CRE was similar to that previously found with reference culture strains (76.3%) [16] and this medium allowed detection of OXA-48-producing isolates in low-level carriage samples such as 4×10^2 CFU/g

TABLE 2. Sensitivity and specificity of ChromID ESBL, Brilliance CRE and SUPERCARBA media for the detection of OXA-48 producing Enterobacteriaceae

	Screening medium		
	ChromID ESBL	Brilliance CRE	SUPERCARBA
Sensitivity before enrichment (%)	90	80	80
Sensitivity after enrichment (%)	100	100	100
Specificity (%)	68.6	86.6	98.5
Specificity including the non-fermenting bacteria (%)	29.9	71.6	52.2

of stools (Table 2). The presence of non-fermenting rods (*Acinetobacter baumannii* or *Pseudomonas aeruginosa*) was recognized as non-pigmented colonies in 40/77 samples on ChromID ESBL, 17/77 samples on Brilliance CRE, and as lactose-negative colonies in 33/77 samples on SUPERCARBA (Table S1, available as a supporting information file). Brilliance CRE showed a better specificity (71.6%) than the SUPERCARBA (52.2%) when the non-fermenting bacteria were included in the calculation (Table 1). Those oxidase-positive and/or lactose-negative rods were systematically excluded from further analysis of this study but the growth of these bacteria on these selective media may interfere with results of stools that may contain low levels of CPE. Nevertheless, the enrichment procedure helped to eliminate non-fermenting rods that were first detected by direct plating on SUPERCARBA (11/33, Table S1).

The presence of traces of DNA, resulting from non-viable bacteria, or of less abundant CPE in stool samples was assessed by PCR experiments performed on plasmid DNA extracted from the 77 rectal swabs. Only one of the culture-negative samples contained OXA-48 DNA, most probably from an OXA-48 producer that had not been detected on the screening media by direct plating or after enrichment culture with ertapenem (sample 43, Table 1). This ADN-positive and culture-negative sample was excluded for the determination of sensitivity and specificity of these screening media but raises the question of true negativity for the calculation of the detection limits.

This study showed that, in the absence of non-fermenting rods in stools, CPE carriage of 4×10^2 CFU/g of stools could be detected with Brilliance CRE and SUPERCARBA media (and with ChromID ESBL for isolates co-expressing an ESBL).

A total of ten (12.8%) OXA-48-producing Enterobacteriaceae were recovered from 77 faecal samples, with a high variability in the amount of faecal carriage, ranging from 4×10^2 to $>1 \times 10^8$ CFU/g of stools (Table 1). These isolates were identified as *K. pneumoniae* (7/10) and *E. cloacae* (3/10) (Tables 1 and 3). As assessed by the result of PCR experiments, OXA-48 was the only carbapenemase identified, whereas NDM producers have already been isolated in Morocco [17]. The genetic relationship between the isolates was evaluated by multilocus sequence typing and the Diversilab technique and showed that the seven OXA-48-producing *K. pneumoniae* isolates belonged to three distinct clones with only two distinct ST types (i.e. ST 307 and ST 395) (Table 3) and the three OXA-48-producing *E. cloacae* isolates were clonally related (Table 3). As opposed to what was previously observed in this hospital [3], the isolates identified are mainly clonally related, but no relationship could be established between clones and the different wards of this hospital (Table 3). All isolates were resistant to broad-spectrum cephalosporins due to the production of the CTX-M-15 β-lactamase, and were resistant to fluoroquinolones, trimethoprim/sulphamethoxazole, gentamicin, tobramycin and chloramphenicol. Most of them were also resistant to kanamycin and tetracycline (Table 2). Plasmid analysis identified an identical plasmid as the commonly widespread IncL/M-like plasmid, harbouring the bla_{OXA-48} gene, of *c.* 62-kb in size (Table 3) [10].

Conclusion

The high prevalence of faecal carriage of OXA-48-producing Enterobacteriaceae (12.8%) among patients in this University

TABLE 3. Genetic context of the bla_{OXA-48} gene and epidemiological data

Species	Strain number	Hospitalization unit	Diversilab type/MLST	Co-resistances	Plasmid type
E. cloacae	15	Medicine	EcA	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
	60	Paediatrics	EcA	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
	91	Medicine	EcA	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
K. pneumoniae	10	Cardiology	Kp 1/ST307	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
	90	Ophtalmology	Kp 1/ST307	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
	93	ICU	Kp 1/ST307	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M
	86	Surgery	Kp 2/ST307	CIP, OFX, NOR, NA, C, SXT, TM, GM	IncL/M
	89	Ophtalmology	Kp 2/ST307	CIP, OFX, NOR, NA, C, SXT, TM, GM	IncL/M
	95	Cardiology	Kp 2/ST307	CIP, OFX, NOR, NA, C, SXT, TM, GM	IncL/M
	91	Medicine	Kp 3/ST395	CIP, OFX, NOR, NA, C, SXT, K, TM, GM, TE	IncL/M

CIP, ciprofloxacin; OFX, ofloxacin; NOR, norfloxacin; NA, nalidixic acid; C, chloramphenicol; SXT, trimethoprim-sulphametoxazole; K, kanamycin; TM, tobramycin; GM, gentamicin; TE, tetracycline.

Hospital in Morroco highlights the large dissemination of the bla_{OXA-48} gene. This prevalence is somewhat similar to, although lower than, that reported for NDM in Pakistan [18]. A high prevalence of OXA-48 producers in Morocco may in part explain their large spread in Europe [2,3,19,20]. This study further underlines the association between CTX-M-15 and OXA-48, leading to multidrug resistance [21]. The ertapenem enrichment procedure was useful for the detection of two additional samples with low carriage of OXA-48 producers. Although this enrichment step is time consuming, it could be recommended for detection of carbapenemase producers in hospitals where OXA-48 might be endemic, such as in North African countries.

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Transparency Declaration

None to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Quantification of non-fermenting bacteria recovered on three selective media from 77 rectal swab samples.

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