Prevalence of carbapenem resistance encoding genes and corresponding MIC₉₀ in enterobacteriaceae at a tertiary medical care center in Lebanon

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Abstract

Background: The aim of this study was to correlate genes involved in carbapenem resistance to MIC levels among clinical ESBL and non-ESBL producing carbapenem resistant Enterobacteriaceae (CRE) isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

Materials and Methods: *E. coli* (n=76) and *K. pneumoniae* (n=54), collected between July 2008 and July 2014, were analyzed. The MICs were determined against ertapenem (ERT), imipenem (IMP) and meropenem (MER). PCR was performed on all 130 isolates to amplify the resistance and outer membrane proteins (OMPs) encoding genes: *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{TEM-1}, *bla*_{CTX-M-15}, *ompC* and *ompF*. Sequencing was performed on selected isolates.

Results: The prevalence of *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{TEM-1} and/or *bla*_{CTX-M-15} among *E. coli* isolates were 36%, 12%, 20% and 80%, respectively, while among *K. pneumoniae* they were 37%, 28%, 28% and 72%, respectively. *K. pneumoniae* isolates positive for any of these genes had an MIC₉₀ ≥ 32 µg/ml against ERT, IMP and MER, while in *E. coli* isolates there was a variation in the MIC₉₀ values. Porin impermeabilities were due to mutations in *ompC* and *ompF* genes in *E. coli*, and loss of *ompC* and *ompF* genes in *K. pneumoniae*, and increased MIC₉₀ values. The presence of more than one carbapenem resistance encoding gene and/or ESBL encoding gene did not have an effect on the MIC₉₀ value in *K. pneumoniae* isolates, while in *E. coli* it showed higher MIC₉₀ values.

Conclusion: Levels of MIC in CRE may largely depend on the type of resistance encoding genes, and porin impermeabilities. These results may provide information for antibiotic regimen selection and epidemiological monitoring of resistance.

Keywords: CRE; MIC; PCR; Antimicrobial agents

Introduction

The dissemination of carbapenem resistant Enterobacteriaceae (CRE) globally is an alarming crisis, which attributes to a serious public health, infection control and society threat. Its fast-paced spread intensifies the clinical challenge further, requiring urgent interventions [1-5].

Several risk factors for infections with CRE have been identified including prolonged hospitalization, mechanical ventilation, exposure to broad spectrum antibiotics and previous colonization with these strains. In addition, high mortality rates of around 40% have been associated with CRE, mostly of bloodstream infections and pneumonia [4].

Carbapenem resistance can be ascribed to several enzymes encoded by resistance genes including the production of various carbapenemases: *K. pneumoniae* carbapenemase (KPC; Ambler class A), Verona integron-encoded metallo- β -lactamase (VIM), imipenemase (IMP), New Delhi metallo- β -lactamase (NDM) (all Ambler class B), and oxacillinase-48 (OXA-48; Ambler class D) [3 & 6]. Moreover, a combination of AmpC hyper-production and/or extended spectrum beta lactamase (ESBL) production (such as CTXM-15 enzyme), efflux pump and porin mutation or loss (such as lack of OMPC and/or OMPF enzymes) can also affect carbapenem resistance [4 & 7].

In the Middle East in general and in Lebanon in particular, there is a paucity of scientific reports

compared to other regions of the world, concerning CRE and underlying mechanisms of resistance. In a previous pilot study [6], we determined in a limited number of carbapenem resistant isolates the mechanism of carbapenem resistance at a tertiary medical center in Lebanon to be due to *bla*_{OXA-48} and/or *bla*_{NDM-1} and *bla*_{CTXM-15} along with outer membrane impermeabilities and to a significantly lesser effect of efflux pump activity.

This study was undertaken to screen for the prevalent carbapenem resistance (*bla*_{CTXM-15}, *bla*_{OXA-48}, *bla*_{NDM-1} and *bla*_{TEM-1}) and outer membrane porin (OMPC and OMPF) encoding genes and correlate their role singly or in combination to MIC levels among CRE producing clinical isolates of *E. coli* and *K. pneumoniae*, from Lebanon.

Materials and methods

Clinical isolates

Available phenotypically determined non-duplicate ESBL producing CRE *E. coli* (n=76) and *K. pneumoniae* (n=54) were collected at the Clinical Microbiology Laboratory of the Department of Pathology and Laboratory Medicine at the medical center, between July 2008 and July 2014. The CRE isolates were cultured on MacConkey agar (Scharlau, Spain) and stored in Brucella broth (BBL, USA), enriched with 15% glycerol for later use. The origins of each of the 130 isolates are listed in **Table 1**.

Table 1. PCR primers used for the detection resistance and outer membrane porin encoding genes

Primer	Sequence (5'-3')	Ref.
OXA-48-F OXA-48-R	5'-TTGGTGGCATCGATTATCGG-3' 5'-GAGCACTTCTTTTGTGATGGC-3'	[27]
NDM-1-F NDM-1-R	5'-GGAAACTGGCGACCAACG-3' 5'-ATGCGGGCCGTATGAGTGA-3'	[7]
CTX-M-F CTX-M-R	5'-GGTAAAAAATCACTGCGTC-3' 5'-TTACAAACCGTCGGTGACGA-3'	[28]
OT-3 OT-4	5'-ATGAGTATTCAACATTTCCG-3' 5'-CCAATGCTTAATCAGTGAGG-3'	[29]
ompF-A ompF-B	5'-CAGGTACTGCAAACGCTGC-3' 5'-GTCAACATAGGTGGACATG-3'	[30]
ompC-F ompC-R	5'-GAACTGGTAAACCAGACCCAG-3' 5'-GTTAAAGTACTGTCCCTCCTG-3'	[30]

Table 2. Positive and negative controls used for the PCR

	Isolate number	Genes
Positive controls	IMP 02	ompC and CTX-M-15
	IMP 44	ompF and blaOXA-48
	IMP 54	blaNDM-1
	IMP 14	blaTEM-1
Negative controls	CDC Strain 06-3637	-
	Water	-

Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations (MIC) for ertapenem (ERT), imipenem (IMP) and meropenem (MER) were determined by Etest (bioMérieux, France) following the manufacturer's guidelines and the results were interpreted using the CLSI standards for MIC interpretation. An MIC value ≥ 2 $\mu\text{g/ml}$, against ERT, IMP or MER is considered resistant reflecting potential CRE of these isolates [8].

DNA extraction

DNA was extracted from the 130 isolates using the QIAamp DNA Mini Kit (QIAGEN, Germany), following the manufacturer's instructions for DNA extraction from bacteria.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) for the metallo-beta-lactamases were previously done on tested isolates (data unpublished). However, due to the absence of these metallo-beta-lactamase encoding genes, only the following resistance encoding genes: *bla*_{TEM-1}, *bla*_{CTX-M-15}, *bla*_{OXA-48}, *bla*_{NDM-1}, *ompC*, and *ompF* were performed on the extracted DNA of each isolate. Primer sequences are listed in

Table 1. Positive and negative controls used for the PCR are mentioned in Table 2.

Amplification was achieved using the PCR Sprint Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR cycling conditions are listed in supplementary table 2.

PCR amplicons were electrophoresed on 1.5% agarose gel using SeaKem® LE Agarose (FMC Bio-Product, Rockland, ME, USA). A 50 base pair ladder (Fermentas Life Sciences, Burlington, Ontario, Canada) was run in parallel to the samples and served as a size marker. Amplicons were then observed using ULTRA LUM, Dual Light Transilluminator (Claremont, CA), and photographed using the Digi-Doc It program (Ultra Violet Products Ltd., Cambridge, UK).

Amplified PCR products were extracted from the gel after electrophoresis and purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Purified DNA was seen on a 1.5% agarose gel. Sequencing reactions were performed using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and purified using a DyeEx 2.0 Spin Kit (QIAGEN). Purified sequences were detected using an ABI 3500 Genetic Analyzer (Applied

Table 3. Distribution of the resistance encoding genes among *E. coli* and *K. pneumoniae*

Presence of Carbapenem Gene	Number (%) of Gene in		
	<i>E. coli</i> N= 76	<i>K. pneumoniae</i> N=54	Total N=130
<i>bla</i> _{OXA-48} +ve	27 (36%)	20 (37%)	47 (36%)
<i>bla</i> _{NDM-1} +ve	9 (12%)	15 (28%)	24 (18%)
<i>bla</i> _{CTX-M-15} +ve	61 (80%)	39 (72%)	100 (77%)
<i>bla</i> _{TEM-1} +ve	15 (20%)	15 (28%)	30 (23%)

Biosystems) and analyzed using the BioEdit Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA).

Statistical Analysis

Data statistical analysis was carried out, when applicable, using GraphPad. Chi Square with Yates' correction, one tailed statistics was carried out. The differences were considered to be statistically significant when the *p*-value obtained was less than 0.05.

Results

The prevalence of specific resistance encoding genes: *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{TEM-1} and / or *bla*_{CTX-M-15} among *E. coli* isolates were 36%, 12%, 20% and 80%, respectively, while among *K. pneumoniae* isolates were 37%, 28%, 28% and 72%, respectively (Table 3).

Upon calculating the MIC₉₀ values, the results showed that among the *E. coli* isolates positive for the resistance encoding genes, MIC₉₀ was ≥ 32 μ g/ml for ERT. IMP had MIC₉₀ of 6 μ g/ml for *bla*_{NDM-1} and *bla*_{CTX-M-15} positive isolates. As for MER, MIC₉₀ was 1.5 μ g/ml and 4 μ g/ml for *bla*_{NDM-1} and *bla*_{CTX-M-15} positive isolates, respectively. However, among *K. pneumoniae* the MIC₉₀ for the ERT, IMP and MER were all ≥ 32 μ g/ml for the isolates harboring either gene: *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{CTX-M-15} or *bla*_{TEM-1} (Table 4).

Comparing resistance encoding genes with the porin encoding genes it appeared that in *E. coli* the isolates negative for *ompC* or *ompF* for both were 4% (1/27) for *bla*_{OXA-48} positive isolates, 11% (1/9) for *bla*_{NDM-1} positive isolates, 5% (3/61) for *bla*_{CTX-M-15} positive isolates and 0% (0/15) for *bla*_{TEM-1} positive isolates. As for *K. pneumoniae*, the isolates negative for *ompC* or *ompF* or both were 35% (7/20) for

Table 4. MIC₉₀ in correlation with resistance encoding genes in the *E. coli* and *K. pneumoniae* isolates

Genes	<i>bla</i> _{OXA-48} +ve	<i>bla</i> _{NDM-1} +ve	<i>bla</i> _{CTX-M-15} +ve	<i>bla</i> _{TEM-1} +ve
<i>E. coli</i>	N = 27	N = 9	N = 61	N = 15
ERT	>32	>32	>32	>32
IMP	>32	6	6	>32
MER	>32	1.5	4	>32
<i>K. pneumoniae</i>	N = 20	N = 15	N = 39	N = 15
ERT	>32	>32	>32	>32
IMP	>32	>32	>32	>32
MER	>32	>32	>32	>32

the *bla*_{OXA-48} positive isolates, 73% (11/15) *bla*_{NDM-1}, 54% (21/39) for the *bla*_{CTX-M-15} positive isolates, and 33% (5/15) for *bla*_{TEM-1} positive isolates (**Table 5**). Furthermore, these differences in the results between *E. coli* and *K. pneumoniae* isolates were statistically significant (**Tables 5 & 6**).

The presence of more than one resistance encoding gene did not have an effect on the MIC₉₀ value in *K. pneumoniae* isolates against the tested carbapenems (results remained ≥ 32 µg/ml). However, in *E. coli* isolates the presence of resistance encoding genes *bla*_{NDM-1}+ve & *bla*_{CTX-M-15}+ve showed higher MIC₉₀ values when compared to the MIC₉₀ values of isolates positive for only one of those genes. Furthermore, when the three genes *bla*_{NDM-1}, *bla*_{OXA-48} & *bla*_{CTX-M-15} coexisted in *E. coli* isolates the MIC₉₀ values against IMP and MER increased however those against ERT decreased. However, it is important to note that since the number of isolates (N) is small (range from 2 to 14) when co-production of genes is concerned the conclusions may not be statistically significant in this regard (**Tables 3 & 4**).

Sequence analysis of the resistance-encoding genes confirmed that the PCR amplicons were positive for the gene in question. Sequence analysis of the OmpF and OmpC porins showed considerable variability for selected carbapenem-resistant *K.*

pneumoniae and *E. coli*, (both carbapenemase- and/or ESBL-producers) in relation to both of the porins when their sequences were compared with those of the reference strain *E. coli* K-12 and with sequences of other susceptible isolates of *E. coli* and *K. pneumoniae*. A number of resistant isolates showed nucleotide substitutions, deletions and insertions, sometimes of multiple nucleotides, throughout the coding region.

Discussion

In our medical center, the prevalence of CRE among *E. coli* and *K. pneumoniae* showed an increase between 2008 and 2014 from 0% in both to 1% and 4%, respectively [9]. In this study, the association between genes involved in CRE resistance and the level of MICs against carbapenems was determined to reveal a possible potential clinical application in this approach for treating patients with CRE infections.

The molecular investigation on these isolates showed that *bla*_{CTX-M-15} is the most common resistance encoding gene in both *E. coli* and *K. pneumoniae* isolates followed by *bla*_{TEM-1}, *bla*_{OXA-48} and *bla*_{NDM-1}. This is in concordance with other studies

Table 5. *E. coli* and *K. pneumoniae* number of isolates (%) with regard to *bla*_{OXA-48}, *bla*_{NDM-1}, *bla*_{CTX-M-15} and *bla*_{TEM-1} positive isolates with and without the porin encoding genes *ompC* and/or *ompF*

Genes	<i>bla</i> _{OXA-48} +ve ◇	<i>bla</i> _{NDM-1} +ve □	<i>bla</i> _{CTX-M-15} +ve ▢	<i>bla</i> _{TEM-1} +ve *
<i>E. coli</i> _Number of isolates (%)				
With porins*	26 (96%)	8 (89%)	58 (95%)	15 (100%)
Without porins**	1 (4%)	1 (11%)	3 (5%)	0 (0%)
Total	27	9	61	15
<i>K. pneumoniae</i> _Number of isolates (%)				
With porins*	13 (65%)	4 (27%)	18 (46%)	10 (67%)
Without porins**	7 (35%)	11 (73%)	21 (54%)	5 (33%)
Total	20	15	39	15

ompC*+ve and/or *ompF*+ve *ompC* -ve and/or *ompF* -ve

◇p-value= 0.0075 □p-value= 0.0057 ▢p-value= 0.0001 *p-value= 0.0250

Table 6. Comparison of MIC values among *E. coli* and *K. pneumoniae* isolates, positive for the resistance encoding genes, tested against ERT, IMP, and MER.

Resistance Encoding Genes	Carbapenems tested against	Isolates	MIC value <32	MIC value ≥32
<i>bla</i> _{OXA-48} +ve	ERT	<i>E. coli</i>	18	9
		<i>K. pneumoniae</i>	8	12
	IMP*	<i>E. coli</i>	14	3
		<i>K. pneumoniae</i>	23	4
	MER	<i>E. coli</i>	16	4
		<i>K. pneumoniae</i>	7	2
<i>bla</i> _{NDM-1} +ve	ERT*	<i>E. coli</i>	7	2
		<i>K. pneumoniae</i>	8	12
	IMP*	<i>E. coli</i>	9	0
		<i>K. pneumoniae</i>	6	9
	MER*	<i>E. coli</i>	9	0
		<i>K. pneumoniae</i>	9	6
<i>bla</i> _{CTX-M-15} +ve	ERT*	<i>E. coli</i>	46	15
		<i>K. pneumoniae</i>	17	22
	IMP*	<i>E. coli</i>	59	2
		<i>K. pneumoniae</i>	27	12
	MER*	<i>E. coli</i>	59	2
		<i>K. pneumoniae</i>	31	8
<i>bla</i> _{TEM-1} +ve	ERT	<i>E. coli</i>	8	7
		<i>K. pneumoniae</i>	4	11
	IMP*	<i>E. coli</i>	13	2
		<i>K. pneumoniae</i>	6	9
	MER	<i>E. coli</i>	12	3
		<i>K. pneumoniae</i>	8	7

*P-value < 0.05; thus statistically significant difference.

that showed that the CTXM typeESBLs are the most prevalent resistant encoding gene both worldwide and in the Middle East [7& 10]. Furthermore, and since the first report in Lebanon of *bla*_{OXA-48} in 2008 [11] and *bla*_{NDM-1} in 2012 [12], studies have shown a trend of elevated cases [6,9 &13-17].

Also concerning the geographic region of the Middle East, there are reports implying the spread of *bla*_{CTXM-1}, *bla*_{TEM-1}, *bla*_{OXA-48} and *bla*_{NDM-1} genes. In 2011, it was reported that the prevalence of *bla*_{CTXM-15} along with other ESBLs to be predominant in Egypt, Kuwait, Lebanon and the United Arab

Emirates [9]. Also in 2013, a study done in Jordan reported high incidence of CTX-M ESBL-producing *E. coli* to be found associated with fluoroquinolone-resistance and Class I integrons colonizing the intestine of Jordanian infants [10]. Plasmid that harbors OXA-48 among various Enterobacteriaceae, was first identified in Turkey in 2003, then in European countries and in the Middle East [19 &20]. As for NDM-1, it was first identified in India in 2008 and subsequently reported also from the Arabian Peninsula: United Arab Emirates, Sultanate of Oman, Kuwait and Saudi Arabia [21].

Determining the simultaneous levels of carbapenem MICs and the genes involved was partly in search of an association pertaining to treatment potential of CRE infections. This approach was not reported in any study from this region, however conducted elsewhere [22& 23]. In our study the MIC₉₀ revealed that *E. coli* isolates positive for *bla*_{OXA-48} and *bla*_{TEM-1} were resistant to the three carbapenems tested (ERT, IMP and MER), while those positive for *bla*_{NDM-1} and *bla*_{CTXM-15} showed to be resistant only to ERT and IMP, and susceptible to MER. On the other hand *K. pneumoniae* isolates positive for any one of the three resistance encoding genes, showed resistance to all three carbapenems. Thereby, it may not be advisable to treat CRE infections due to isolates harboring these genes, subsequently with carbapenems. It is suggested that doripenem is more stable against emerging resistance than the other carbapenems [4].

ERT has shown to have higher MIC levels, in both *E. coli* and *K. pneumoniae* isolates positive for any of the resistance encoding genes. This phenomenon was explained by Tangden et al., where ERT has shown to have higher MIC levels in ESBL producing isolates as compared to other carbapenems being less affected by the action of the enzymes [4]. Whereby, resistance to IMP and MER is less common. ERT resistance is indeed more frequently reported than resistance to other carbapenems, especially in ESBL-producing strains. In addition, ERT has been heavily used for UTI infections caused by ESBL-producing bacteria and proven effective for these infections, which might explain the generated resistance to this agent [4].

It seems that ERT is more affected by the presence of *bla*_{OXA-48} and the other resistance encoding genes, thus ERT may be a good indicator for their detection [12]. Metallo- β -lactamases, such as *bla*_{NDM-1}, may result in low-grade reduced susceptibility to carbapenems in some instances [4]. This was also observed in our data, and may present a challenge in the detection of the CRE, if based on only the MIC results [16].

Knowing that *K. pneumoniae* isolates had MIC values $\geq 32\mu\text{g/ml}$ against the three carbapenems regardless of the type of the resistance encoding genes, it may be concluded that the lack of the porin genes (*OMPC* and/or *OMPF*), which was significantly higher in *K. pneumoniae* than in the *E. coli* isolates, plays a major role in increasing the MIC levels. Similarly, it has been shown that carbapenem resistance levels intensify when resistance encoding genes such as *bla*_{OXA-48} are combined with permeability defects [13]. Yang et al. demonstrated similar results, whereby they confirmed that the combination of SHV-1, CTXM-3, CTXM-14, TEM-1 or OXA-11 production reduced the expression of OMPK-35/36 resulting in increased MIC, which may or may not lead to increased resistance clinically [23].

Furthermore, PCR and sequencing analysis of the *K. pneumoniae* and *E. coli* Omp encoding genes, revealed mutations associated with carbapenem resistance. On the other hand a number of *K. pneumoniae* carbapenem resistant isolates lacked the OMP encoding genes. Similarly, Little et al. reported the development of carbapenem resistance in *K. pneumoniae* due to porin disruption by insertion sequence *ISEcp1* in CTXM-15 positive isolate [24]. Therefore, the *E. coli* isolates in this study harboring any of the porin encoding genes, have shown mutations by sequence analysis. This may indicate that High MIC values detected in the TEM-1 positive isolates harboring both porin encoding genes, are mainly due to mutated porins.

The first report of a *K. pneumoniae* strain co-producing NDM-1, VIM-1 and OXA-48 carbapenemases isolated in Morocco was reported by Barguiga et al in 2012 [25]. Further, the first *K. pneumoniae* isolate co-producing OXA-48 and NDM-1 in Turkey was reported by Kilic et al [26]. The emergence of carbapenem-resistant Gram-negative bacilli in the Mediterranean region, which is in fact the cradle of western civilization representing nearly 475 million inhabitants (6.3% of world population), is of importance, since intermingling of this population

may explain the prominence of the dissemination of carbapenemase producers more rapidly in this region[27]. Thereby, these isolates showing co-production of resistance genes may be the interest of future studies .

In conclusion, levels of MIC in carbapenem resistance may largely depend on the type of carbapenemase and/or ESBL encoding genes in combination with the porin encoding genes. Such information may provide information for antibiotic regimen selection, epidemiological monitoring of resistance.

Funding: Medical Practice Plan (MPP), Faculty of Medicine, American University of Beirut

Competing interests: None declared

Ethical Approval: Not required

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