Carbapenemase and virulence factors of Enterobacteriaceae in North Lebanon between 2008 and 2012: evolution via endemic spread of OXA-48

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Objectives: To investigate the resistance to carbapenems in Enterobacteriaceae and the underlying resistance mechanisms in North Lebanon between 2008 and 2012.

Methods: A total of 2767 Enterobacteriaceae isolates recovered from clinical samples collected in Nini Hospital (North Lebanon) were screened for a decrease in susceptibility or resistance to ertapenem (MIC > 0.25 mg/L). Enterobacteriaceae were similarly screened from 183 faecal samples obtained from non-hospitalized patients. The bacterial isolates were assigned to clonal lineages by PFGE and multilocus sequence typing. Carbapenemase genes, their genetic environment and virulence genes were characterized by molecular approaches.

Results: The rate of Enterobacteriaceae exhibiting a decrease in susceptibility or resistance to ertapenem increased from 0.4% in 2008–10 to 1.6% in 2012 for the clinical isolates recovered from hospitalized patients. Of these isolates, scattered among seven enterobacterial species, 88% produced OXA-48 carbapenemase. However, Escherichia coli represented 73% of the OXA-48-producing Enterobacteriaceae collected in 2012 at this hospital. During the faecal carriage study performed in non-hospitalized patients, E. coli was the only species producing OXA-48. The blaOXA-48 gene was mainly found within Tn1999.2-type transposons inserted into E. coli chromosomes or in ~50, ~62 or ~85 kb plasmids. The plasmids and chromosomal inserts were related to pOXA-48a. Molecular typing of the isolates revealed clonal diversity of E. coli and Klebsiella pneumoniae producing OXA-48. OXA-48 was observed in all major E. coli phylogroups, including D and B2, and isolates harbouring virulence genes of extra-intestinal pathogenic E. coli. Although not belonging to highly virulent capsular genotypes, the OXA-48-producing K. pneumoniae harbouring genes associated with virulence or host colonization.

Conclusions: Horizontal transfer of related plasmids has facilitated the spread of the blaOXA-48 gene into several Enterobacteriaceae species, including virulent E. coli. Their clonal diversity and the presence of faecal carriers in the community suggest an endemic spread of OXA-48.

Keywords: OXA-48, Escherichia coli, Klebsiella pneumoniae

Introduction

Carbapenems are broad-spectrum antibiotics that constitute the last-line therapeutic option available to treat infections caused by multidrug-resistant Enterobacteriaceae. However, due to the emerging resistance to carbapenems worldwide, the anti-microbial activity of these drugs is no longer guaranteed. The most concerning mechanism of resistance is the acquisition of carbapenem-hydrolysing β-lactamases. The most prevalent carbapenemases are Ambler class A enzymes such as KPC-type β-lactamases, class B enzymes (such as VIM-, IMP- and NDM-type metallo-β-lactamases) and class D enzymes (such as OXA-48). OXA-48 was initially identified from a Klebsiella pneumoniae isolate in Turkey and then spread to various Enterobacteriaceae species, especially throughout the southern Mediterranean area and in Europe. OXA-48-encoding genes are primarily found in K. pneumoniae and, to a lesser extent, in Escherichia coli and
Enterobacter spp. Their emergence is mediated by the rapid spread of broad host-range conjugative plasmids harbouring the blaOXA-48 gene located within a Tn1999-type composite transposon. The blaOXA-48 gene was essentially observed in the IncI/M plasmids pOXA-48a and derivatives (62 kb), pKpXoA-48N2 (160 kb) characterized from a K. pneumoniae strain isolated in France and pJEG011 (72 kb) characterized from a K. pneumoniae strain isolated in Australia.

In addition to often being multiresistant, K. pneumoniae is an opportunistic pathogen and can give rise to severe diseases responsible for community- and hospital-acquired infections. Its virulence trait is due to the presence of the K1 and K2 capsular serotypes, lipopolysaccharide synthesis, iron-scavenging systems and adhesins and its ability to form biofilms. E. coli organisms include commensal isolates belonging to the E. coli phylogroups A and B1 and virulent isolates, including intestinal pathogenic E. coli and extra-intestinal pathogenic E. coli (ExPEC). The latter belong to the B2 and D E. coli phylogroups and have acquired pathogenicity islands encoding virulence factors. Although the association of virulence with resistance was observed in an OXA-48-producing E. coli isolate, the virulence traits of carbapenemase-producing bacteria remain largely unknown.

The aim of this work was to investigate the evolution of the resistance to carbapenems in Enterobacteriaceae isolated in North Lebanon between 2008 and 2012, the underlying resistance mechanisms and the genetic background and virulence factors of these bacteria.

Materials and methods

Bacteria

A total of 2767 Enterobacteriaceae strains isolated from clinical samples in Nini Hospital, Lebanon, between January 2008 and December 2012 were screened for reduced susceptibility or resistance to ertapenem (MIC >0.25 mg/L). Nini Hospital has 125 general-care beds and 12 intensive-care beds. The annual number of hospital admissions is ~18000 and the hospital serves an area population of ~1000000. Twenty-four non-redundant clinical strains were recovered from hospitalized patients. None of the patients included in the study had travelled recently.

In parallel, Enterobacteriaceae exhibiting decreased susceptibility or resistance to ertapenem (MIC >0.25 mg/L) were collected from 183 faecal samples obtained in December 2012 from healthy children who had not been admitted to hospital and had not taken antibiotics in the previous 6 months. Non-redundant intestinal Enterobacteriaceae were recovered by spreading the corresponding faecal samples on MacConkey agar plates (bioMérieux, Marcy-l’Étoile, France) supplemented with ertapenem (0.5 mg/L) or ceftazidime (4 mg/L).

The strains were identified using the MALDI-TOF mass spectrometry system VITEK MS (bioMérieux). Rifampicin-resistant E. coli C600 was used for mating-out assays and E. coli DH5α for electroporation.

Determination of genetic relatedness and the E. coli phylogroup

PFGE was performed using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s recommendations. Pulsotypes were interpreted according to criteria previously defined by Tenover et al. Allelic profiles and sequence types (STs) were assigned using the multilocus sequence typing (MLST) scheme described by Diancourt et al. (http://www.pasteur.fr/recherche/genopole/PF8/mlst/kpneumoniae.html) and Achtman (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Antibiogram and MIC determination

Antibiotic susceptibility was assessed by the disc diffusion method and the determination of MICs by a broth microdilution method according to the guidelines of EUCAST (http://www.eucast.org/). Antimicrobial agents were obtained from Sigma Chemical Co. (St Louis, MO, USA) or directly from manufacturing companies. Carbapenemase production was detected by a modified Hodge test using combined disc tests of ertapenem with and without clavulanic acid and the Mastdiscs 1D inhibitor combination disc method (Mast Diagnostics, Bootle, UK). Extended-spectrum β-lactamase (ESBL) production was detected by both the combination disc test and the double-disc synergy test according to the guidelines of EUCAST (http://www.eucast.org/).

Molecular identification of β-lactamases and additional associated resistance genes

β-Lactamase-encoding genes (blaTEM, blaCTX-M, blaPC, blaNDM, blaVIM and blaOXA-48) were detected by PCR amplification (Table S1, available as Supplementary data at JAC Online). The genetic environment of the blaOXA-48 gene was further investigated by PCR and sequencing using primers specific for the insertion sequence IS1999 and the blaOXA-48 gene to map the composite transposon Tn1999 and its derivatives Tn1999.2, Tn1999.3 and Tn1999.4.

Analysis of plasmids and chromosome

Transferability of the blaOXA-48 gene was studied by a mating-out assay. When these experiments failed, plasmid DNA was extracted with Nucleobond Xtra ( Macherey-Nagel, Germany) and transferred by electroporation into a bacterial recipient. Selection was performed on agar plates supplemented with ticarcillin (32 mg/L) and rifampicin (300 mg/L) for the mating-out assay and ticarcillin (32 mg/L) for electroporation. The plasmid content of the bacteria and the size of plasmids were determined using plasmid DNA extracted by the method of Kado and Liu with the plasmids Rsa I (39 kb), TP114 (61 kb), pCF04 (85 kb) and pCF14 (180 kb) as standards.

PCR-based replicon typing (PBRT) was used to identify plasmid incompatibility groups in transconjugants or transformants. The repA, traU and parA genes of the pOXA-48a plasmid were detected by PCR, as previously described.

Chromosome analysis

The location of the blaOXA-48 gene was investigated by PFGE using the endonuclease 1-CeuI and hybridizations with probes specific for the 16S rRNA gene, the blaOXA-48 gene and IS1999, as previously described. The chromosomal insertion of the blaOXA-48 gene was further mapped by PCR using pOXA-48a-containing DNA fragments purified from PFGE gels and primers specific for pOXA-48a, as previously described.

Virulence factor-encoding genes and pathogenicity islands

K. pneumoniae virulence genes (allS, cf29a, Kfu, magA, mrkD, rmpA, uge, ureA, wadG, wzy and wxx) and the polyketide synthetase (pks) genomic island were screened by PCR, as previously described. Similarly, virulence factors encoded by the E. coli isolates were detected by PCR targeting the genes encoding adhesins (papC, papA alleles, sfa and hra), siderophores (fya, Iron and ear), cyclophilins (pks, cnp and hlyA), malX and pathogenicity islands (I136, I156, I1-CFT073, I1-CFT073, I136, I156, I156, I136, I1-CFT073 and I1-CFT073), as previously described.
**Biofilm formation**

Biofilm production was assessed using the microtitre plate assay, as described by the modified protocol of O'Toole and Kolter. The F plasmid-bearing*Escherichia coli* TG1 strain was used as a positive control.

**Results**

**Decrease in susceptibility to ertapenem in hospitalized patients**

In total, 2767 Enterobacteriaceae isolates were recovered from clinical samples at Nini Hospital, Tripoli, Lebanon, between 2008 and 2012. These isolates mainly belonged to the species *E. coli* (n=2284, 82.6%), *K. pneumoniae* (n=230, 8.3%), *Enterobacter cloaca* (n=10, 0.3%), *Enterobacter aerogenes* (n=6, 0.2%) and *Proteus mirabilis* (n=106, 3.8%). Twenty-four non-redundant isolates exhibiting resistance or reduced susceptibility to ertapenem (MIC >0.25 mg/L) were recovered between 2008 and 2012. The isolates belonged to seven bacterial species and were predominantly collected from urine (n=14) and pus (n=6) (Table 1). The rate of isolates exhibiting reduced susceptibility or resistance to ertapenem increased from 0.4% (n=6/1385) in 2008–10 to 0.9% (n=6/650) in 2011 and 1.6% (n=12/732) in 2012.

**Intestinal carriage in the community**

In parallel, the foecal carriage of Enterobacteriaceae exhibiting resistance or reduced susceptibility to ertapenem (MIC >0.25 mg/L) was determined.

Table 1. Phenotypic and genotypic characteristics of OXA-48-producing isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>STa (E. coli phylogroup)</th>
<th>Isolation site (year)</th>
<th>transposon</th>
<th>parA b</th>
<th>repA b</th>
<th>traU b</th>
<th>genetic support</th>
<th>Other β-lactamases</th>
<th>ETP</th>
<th>IPM</th>
<th>MEM</th>
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<td>chromd</td>
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<td>NTf</td>
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<td>+</td>
<td>+</td>
<td>62 kb</td>
<td>CTX-M-15, TEM-1</td>
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<td>chromd</td>
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<td>chromd</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>chromd</td>
<td>–</td>
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<td>0.5</td>
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<td>–</td>
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<td>chromd</td>
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<td>2.0</td>
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<td>0.25</td>
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<td>Tn1999.2</td>
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<td>+</td>
<td>62 kb</td>
<td>CTX-M-15, TEM-1</td>
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<td>0.25</td>
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<td>128</td>
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<td>+</td>
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<td>+</td>
<td>62 kb</td>
<td>SHV-1</td>
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<td>62 kb</td>
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<td>62 kb</td>
<td>SHV-1, TEM-1</td>
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</table>

**Notes:**

- ETP, ertapenem; IPM, imipenem; MEM, meropenem; CTX, cefotaxime; CAZ, ceftazidime.
- STs for *E. coli* and *K. pneumoniae* isolates.
- PCR for genes specific for the pOXA-48 backbone.
- Incomplete structure of Tn1999.2.
- Faecal carriage in children having no known contact with the hospital.
- Non-typeable Tn1999-type environment.
- ~85 kb plasmid encoding OXA-48.
- ~50 kb plasmid encoding OXA-48.
>0.25 mg/L) was screened in 2012 from samples obtained from 183 healthy children in the community. The children were enrolled in two schools with different socio-economic levels. School 1 (n = 120) was a welfare school catering for children of poor families. School 2 (n = 87) was a private institution, with pupils that were drawn from upper-class families. Three E. coli isolates exhibiting resistance to ertapenem (n = 3/183, 1.5%) were recovered from children (9, 11 and 12 years old) enrolled in three different classes at school 1.

**Phenotypic and molecular identification of β-lactamases**

PCR and sequencing confirmed the presence of the blaOXA-48 Gene in 88% of the clinical isolates (n = 21/24) and in the three faecal E. coli isolates from healthy children (Table 1). No other gene encoding carbapenemase was detected in the isolates, including those devoid of the blaOXA-48 gene (Enterobacter spp., n = 3/24). In addition, the ESBL synergy test was positive for eight OXA-48-producing isolates. PCR and sequencing showed the presence of the ESBL-encoding genes blaCTX-M-15 and blaCTX-M-24 in five and three isolates, respectively (Table 1).

**MICs of β-lactams for OXA-48-producing bacteria**

The OXA-48-producing isolates were homogeneously resistant to penicillins and their combinations with β-lactamase inhibitors. The MIC of temocillin, which has been proposed as a screening test for OXA-48 detection, was >32 mg/L for all isolates except E. aerogenes EA2. The isolates exhibited various levels of resistance to carbapenems (Table 1). The MICs of oxyimino cephalosporins were in the resistance range for isolates producing both OXA-48 and an ESBL. The MIC values of cefotaxime and ceftazidime were in the susceptible range (0.06–2 mg/L) for the isolates devoid of an ESBL, except for three K. pneumoniae isolates (KP9, KP26 and KP112) and E. cloacae ECL8.

**Plasmidic genetic support of the blaOXA-48 gene**

Plasmids from natural isolates and their transconjugants or trans- infectious bacteria were extracted and hybridized with a probe specific for the blaOXA-48 gene. The results showed that the blaOXA-48 gene was predominantly located on ~62 kb conjugative plasmids (Table 1). Further analysis of these plasmids from the transconjugants revealed similar restriction profiles (data not shown). However, the blaOXA-48 gene was also harboured by plasmids of ~50 and ~85 kb in Citrobacter freundii CF29 and K. pneumoniae KP112 (Figure 1 and Table 1). Resistance to non-β-lactam antibiotics was not observed in the transconjugants and transformants, except for that containing the ~85 kb plasmid, which exhibited resistance to sulphonamides and spectinomycin. No plasmids could be assigned to an incompatibility group by the PBRT method. We detected the repA, traU and parA genes of the pOXA-48a plasmid in all of the transformants or transconjugants (Table 1). These results suggested that the plasmids had an IncL/M-pOXA-48a-like backbone, including the ~50 and ~85 kb plasmids.

**Chromosomal genetic support of the blaOXA-48 gene**

Despite many attempts, no transconjugants or transformants were obtained from six clinical and two faecal E. coli isolates. The insertion of the blaOXA-48 gene into the bacterial chromosome was therefore investigated by PFGE after I-CeuI nucleate treatment and hybridization. Different DNA fragments (size range ~75 to ~0.88 Mb) hybridized with the blaOXA-48 probe (Figure 2). These results show that the insertion of the blaOXA-48 gene into the E. coli chromosome is not a rare event (33%, n = 8/24) and may occur at different sites. The parA gene of pOXA-48a (but not repA and traU) was detected by PCR and sequenced in the blaOXA-48-harbouring DNA fragments, which were eluted from PFGE. These results suggest that the strains contain a large fragment inserted into the chromosome and that the DNA fragments harbouring the blaOXA-48 gene originated in a pOXA-48a-type plasmid.

**Genetic environment of the blaOXA-48 gene**

To characterize the genetic environment of the blaOXA-48 gene in our strains, we mapped Tn1999-type transposons harbouring the blaOXA-48 gene by PCR and sequencing. The blaOXA-48 gene was predominantly associated with Tn1999.2 (63%, n = 15/24). In addition, seven blaOXA-48 genes (29%, n = 7/24) were located in incomplete Tn1999.2 elements. These incomplete Tn1999.2...
elements were observed only in the isolates containing a chromosome-mediated \( \text{bla}_{\text{OXA-48}} \) gene.

**Genetic background and virulence factors of \( \text{K. pneumoniae} \) and \( \text{E. coli} \)**

The genetic background of \( \text{K. pneumoniae} \) and \( \text{E. coli} \) isolates producing OXA-48 was investigated by PFGE and MLST analyses (Table 2). Most of the OXA-48-producing \( \text{K. pneumoniae} \) and \( \text{E. coli} \) isolates were different according to Tenover's criteria for interpreting PFGE patterns (data not shown). Only \( \text{K. pneumoniae} \) isolates KP9, KP26 and KP112 were closely related. Two \( \text{E. coli} \) isolates presented identical pulsotypes (EC254 and EC265) and one was closely related (EC49).

Four STs were represented among the six \( \text{K. pneumoniae} \) isolates (Table 1). No \( \text{K. pneumoniae} \) isolate exhibited the virulent capsular serotype K1 or K2. Among the genes encoding adhesins, only \( \text{mrkD} \) was detected. However, all isolates harboured the genes \( \text{wabG} \) and \( \text{uge} \), which favour host colonization and virulence (Table 2). The ferric iron uptake system encoded by \( \text{kfu} \) was detected in four isolates. All strains produced biofilms; four isolates in particular showed biofilm production that was not significantly different from that observed for the reference strain TG1 (Table 2; ANOVA, \( P > 0.05 \)).

Seven STs were represented among the \( \text{E. coli} \) isolates (Table 1), belonging predominantly to the commensal phylogroups A (n = 6) and B1 (n = 1). Five isolates were assigned to phylogroup D or B2. These latter isolates harboured virulence genes and pathogenicity islands previously observed in ExPEC strains (Table 3). Strains EC8 and EC15, which belonged to phylogroup B2, contained a higher number of pathogenicity islands (five or more) and virulence genes (six or more) than the \( \text{E. coli} \) isolates of phylogroup D.

**Discussion**

In this study, we showed that the rate of Enterobacteriaceae exhibiting a decrease in susceptibility to ertapenem in Nini Hospital, North Lebanon, increased from 0.4% in 2008–10 to 1.6% in 2012. This rise was associated with the emergence of carbapenemase OXA-48, which had been previously reported in Lebanon in 2008–10. \( \text{E. coli} \) was the predominant species producing OXA-48, in contrast to previous studies, which generally cite \( \text{K. pneumoniae} \) as the main OXA-48-producing Enterobacteriaceae. \( \text{E. coli} \) isolates represented 10% of the OXA-48 clinical producers in 2008–11 and 73% in 2012. In addition, intestinal carriage of OXA-48-producing \( \text{E. coli} \) was observed in the community and was marked by a diversity of
strains, suggesting that OXA-48 has become endemic in North Lebanon.

OXA-48 was observed in all major phylogroups of E. coli. Most of the isolates belonged to phylogroup A, which predominantly comprises commensal strains. However, D and B2 OXA-48-producing E. coli strains harboured virulence genes of extra-intestinal pathogenic E. coli. No highly virulent OXA-48-producing K. pneumoniae isolate was found. However, OXA-48-producing K. pneumoniae isolates produced a significant amount of biofilm and harboured genes previously associated with virulence and favouring host colonization.

A recent study showed that pOXA-48a is highly transferable in vitro. Our findings showed that the emergence of OXA-48 carbapenemase was facilitated by the horizontal transfer of plasmids related to pOXA-48a-like plasmids. A recent study reported the presence of the blaOXA-48 gene in the bacterial chromosome of a sporadic E. coli isolate. In this study, the blaOXA-48 gene was located in the chromosome in 33% of the OXA-48-producing Enterobacteriaceae and in 67% of the OXA-48-producing E. coli. A chromosomal location of the blaOXA-48 gene is therefore not rare and is observed in unrelated E. coli isolates. The Tn1992 element was predominant in the plasmids and the E. coli chromosomes harbouring the blaOXA-48 gene. However, chromosome-mediated Tn1992 was truncated, most likely as a result of its insertion into the E. coli chromosome. Furthermore, the parA gene, which is located ~13 kb from the blaOXA-48 gene on pOXA-48a, was observed in a chromosomal DNA fragment harbouring the blaOXA-48 gene, suggesting that Tn1992.2 is most likely not the only DNA fragment inserted. These results suggest that multiple acquisitions of the blaOXA-48 gene in E. coli chromosomes involve a Tn1992.2-containing fragment of a pOXA-48a-like plasmid.

In conclusion, the distribution and diversity of Enterobacteriaceae producing OXA-48 carbapenemase demonstrate that this resistance mechanism is becoming widespread in hospitals and in the community in North Lebanon. The occurrence of the blaOXA-48 gene is worrying due to the spread of both highly diffusing plasmids and their presence in E. coli producers. This situation resembles the ecological success story of CTX-M-type ESBLs, the worldwide emergence of which is most likely due to their association with E. coli.

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Transparency declarations
None to declare.
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