Molecular characterization of carbapenem-resistant Gram-negative bacilli clinical isolates in Algeria

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Objectives: The aims of this study were to investigate the occurrence of carbapenem-resistant Gram-negative bacilli (GNB) isolated from inpatients and outpatients in Algeria between July and September 2015, and to screen their resistance mechanisms and genetic relatedness.

Materials and methods: A total of 68 non-redundant isolates were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) antibiotic susceptibility testing was performed using disk diffusion and Etest methods. Carbapenemase activity was carried out using modified Carba NP test, EDTA assay, and the modified Hodge test. Molecular characterization of carbapenemases and extended-spectrum β-lactamase (ESBL) genes were detected by standard PCR and sequencing. Genotyping of carbapenem-resistant isolates was performed by multilocus sequence typing (MLST) analysis.

Results: Of the 68 GNB isolates, 13 (19%) showed reduced susceptibility to carbapenems, including, four Klebsiella pneumoniae, one Escherichia coli, six Acinetobacter baumannii, and two Pseudomonas aeruginosa. blaOXA-48 gene was detected in the five Enterobacteriaceae isolates, and blaOXA-23 was identified in all A. baumannii isolates. oprD mutations were revealed in the two P. aeruginosa isolates. A total of 11 out of the 13 carbapenem-resistant GNB were detected in inpatients, and the two remaining strains were isolated from outpatients. Molecular typing showed the presence of four sequence types (STs) among the OXA-48-producing K. pneumoniae isolates: ST101, ST147, ST163, and ST2017. ST533 was identified for the OXA-48 producing E. coli isolate. All of the A. baumannii and P. aeruginosa were assigned to the international clonal lineages ST2 and ST654, respectively.

Conclusion: This study reports the first detection of the epidemic multidrug-resistant lineage, K. pneumoniae ST147 coproduced blaOXA-48 and ESBL genes in Algeria and represents the first description of OXA-48-producing E. coli ST533 and K. pneumoniae ST163 and ST2017. In addition, this study describes for the first time the emergence of OXA-48-producing E. coli and K. pneumoniae in the community in Algeria, leading to major problems for managing microbial infections.

Keywords: Gram-negative bacilli, carbapenem resistance, epidemic lineages, Algeria

Introduction

Antibiotic resistance has increased dramatically over the last few years, and the incidence of carbapenemase-producing Gram-negative bacilli (GNB) is rising in many countries around the world.1 Carbapenems are considered as one of the last resorts for treating multidrug-resistant GNB,2–4 including ESBL-producing Enterobacteriaceae.5

Carbapenem resistance has been described in non-fermentative GNB (Acinetobacter baumannii and Pseudomonas aeruginosa) and in Enterobacteriaceae,2,6 mainly in Klebsiella pneumoniae compared to Escherichia coli or other Enterobacterial species.2,3

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These GNB are major nosocomial pathogens and are the most common bacteria responsible for a wide range of community-acquired and nosocomial infections such as pneumonia, sepsis, peritonitis, meningitis, and urinary tract infections. Therefore, acquired carbapenemase-encoding genes constitute a real clinical concern for antimicrobial management, notably that resistance to carbapenems may be coupled with resistance to other classes of antibiotics, such as aminoglycosides and fluoroquinolones.

Carbapenem resistance in GNB can be mediated by carbapenem-hydrolyzing β-lactamases, including class A β-lactamases (KPC, IMI, and GES), class B metallo-β-lactamases (NDM, IMP, and VIM), and class D β-lactamases (OXA-48, OXA-23, OXA-24, and OXA-58). OXA-β-lactamases were first described in P. aeruginosa; those carbapenemases have subsequently been reported in many other GNB, including A. baumannii and Enterobacteriaceae. The OXA-48 enzyme is the most frequently detected carbapenemase in Enterobacteriaceae. This carbapenemase is not inhibited by clavulanic acid or ethylene diamine tetra-acetic acid (EDTA). Its spectrum of activity is substantial against penicillins, but it has low activity against third-generation cephalosporin, aztreonam, and carbapenems. The decreased permeability due to the loss or alteration of oprD porin in the outer membrane has been mainly described as linked to carbapenem resistance, especially in P. aeruginosa strains. Resistance mechanisms to imipenem in P. aeruginosa can also be related to the production of carbapenemases or to the presence of an efflux pump.

In Algeria, the spread of carbapenem resistance in hospitalized patients has been mainly reported among A. baumannii, followed by P. aeruginosa, and very few cases have been reported among Enterobacteriaceae. However, until now, no information regarding the dissemination of carbapenem resistance among Algerian outpatients has been reported.

The objectives of this study were to investigate the occurrence of carbapenem-resistant GNB isolated from hospitalized and external patients in Algeria and to screen extended-spectrum β-lactamase (ESBL)-encoding genes and carbapenem resistance mechanisms in resistant isolates. The clonal relatedness of strains was assessed using multilocus sequencing typing (MLST).

**Materials and methods**

**Bacterial isolates and species identification**

A total of 68 non-redundant GNB isolated from hospitalized (42.6%) and community (57.3%) patients were recovered from the clinical microbiology laboratory at an Algerian hospital in Annaba city in North East Algeria, between July and September 2015. It was notable that patients from the community included in this study had not had a previous hospitalization or antibiotic treatment. The 68 clinical specimens were collected from 68 different patients.

Strains were identified using the API 20E and the API 20NE strips (bioMérieux, Marcy-l’Étoile, France), for the Enterobacteriaceae and the Non-Enterobacteriaceae isolates, respectively, and confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (MicroflexTm; Bruker Daltonic, Bremen, Germany) with flex control and biotyper 3.0 software (Bruker Daltonic).

**Antibacterial susceptibility testing**

Antimicrobial drug susceptibility was determined by the standard disc diffusion method on Mueller–Hinton agar as recommended by the Antiibogram Committee of the French Society for Microbiology/European Committee for Antimicrobial Susceptibility Testing (CA-SFM/EUCAST). The antimicrobial agents used were ticarcillin, ticarcillin/clavulanic acid, ceftriaxone, ceftazidime, cefepime, aztreonam, ertapenem, imipenem, gentamicin, amikacin, ciprofloxacin, fosfomycin, and trimethoprim–sulfamethoxazole (all from I2A, Montpellier, France). The minimum inhibitory concentrations (MICs) were determined using Etest strips (bioMérieux) for colistin in all strains and for imipenem in all carbapenem-intermediate or resistant isolates. Interpretations were made according to CA-SFM/EUCAST breakpoints.

**Phenotypic screening of carbapenemases**

Carbapenemase production was screened phenotypically for all strains exhibiting resistance or intermediate resistance to carbapenems using the modified Carba NP (MCNP) test, EDTA assay and the modified Hodge test (MHT) as previously described.

**Molecular mechanisms of antibiotic resistance**

DNA extraction from all isolates showing non-susceptibility to carbapenems was performed using EZ1 DNA extraction kits (Qiagen NV, Venlo, the Netherlands) with the EZ1 Advanced XL biorobot according to the manufacturer’s instructions.

Real-time PCR and standard PCR were performed to screen for the presence of carbapenem hydrolyzing enzyme-encoding genes: \(bla_{OXA-23}\), \(bla_{OXA-24}\), \(bla_{OXA-58}\), \(bla_{OXA-48}\), \(bla_{KPC}\).
Molecular typing by MLST

Molecular typing of carbapenem-resistant isolates was carried out by MLST, as described on the E. coli MLST Database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) for E. coli, for P. aeruginosa (https://pubmlst.org/paeruginosa/), and according to the Pasteur schemes available at Institute Pasteur’s MLST Web site (bigdb.web.pasteur.fr) for K. pneumoniae and A. baumannii.

Statistical analysis

Chi-square tests and Fisher’s exact tests were performed using two-sided comparisons to establish the statistical difference in levels of antibiotic resistance between hospitalized and external patients. Statistical significance was set at P<0.05.

Results

Bacterial strain identification

Identification of GNB using conventional microbiological tests and MALDI-TOF MS showed that Enterobacteriaceae represent 69.1% of the strains (n=47), including E. coli (57.4%; n=27) and K. pneumoniae (42.5%; n=20). Nonfermentative isolates represent 30.9% of the strains (n=21), including A. baumannii (57.1%; n=12) and P. aeruginosa (42.8%; n=9). The strains were isolated from various clinical specimens, including urine (51.5%), wounds (26.5%), bloodstream infections (20.6%), and cerebrospinal fluid (1.5%).

Antimicrobial susceptibility

Antimicrobial resistance patterns according to species and patient types are shown in Table 1. The results showed that most of the isolates were resistant to ticarcillin and ticarcillin-clavulanic acid, and a high resistance level to ciprofloxacin and trimethoprim/sulfamethoxazole was observed. Cefalosporins and gentamicin exhibited high activity against E. coli strains compared to other GNB isolates. Amikacin and colistin were found to be the most active on the isolates tested. Carbapenem activity showed that one E. coli (3.7%) and four K. pneumoniae isolates (20%) exhibited resistance to ertapenem, with two of them also being intermediate to imipenem with MICs of 3 µg/mL. Six A. baumannii and two P. aeruginosa isolates were resistant to imipenem with MICs >32 µg/mL. On the other hand, we found that strains isolated from hospitalized patients were significantly more resistant than those of external origin (P<0.05) for most of the antibiotics tested (Table 1).

Phenotypic screening of carbapenemases

Phenotypic detection of carbapenemases showed that EDTA-antibiotic disc synergy test was negative for all carbapenem-resistant strains, which indicates the absence of metallo-β-lactamase production. The MCNP test and modified Hodge assay were found positive for all isolates resistant to carbapenem including K. pneumoniae (n=4), E. coli (n=1), and A. baumannii (n=6) except for the imipenem-resistant P. aeruginosa isolates (n=2).

Molecular mechanisms of antibiotic resistance

Molecular characterization of acquired carbapenemase-encoding genes showed the presence of the blaOXA-48 gene in the four K. pneumoniae isolates and in one E. coli isolate resistant to carbapenem. All imipenem-resistant A. baumannii carried the blaOXA-23 gene. OprD gene mutations were detected in the two imipenem-resistant P. aeruginosa isolates, using as reference that of the PA01 reference strain. OprD gene mutations had several polymorphism types (deletion, substitution, and insertion) leading to the premature stop codon “TGA” on the OprD protein for the P. aeruginosa strain S1, and the premature stop codon TAG for the P. aeruginosa strain S2 (Table 2). The first mutations in the oprD genes are the substitution of T to C in the nucleotide number 243 for the strain (S1), and G to A in the nucleotide number 220 for the strain (S2). None of the isolates were positive for blaOXA-24*, blaOXA-58*, blaIMP*, blaKPC*, blaVIM*, or blaNDM genes. All OXA-48-producing Enterobacteriaceae isolates from hospital origin showed co-resistance to most classes of the antimicrobial agents tested compared to isolates from external origin. On the other hand, we found that 18.2% of carbapenem-resistant isolates (one K. pneumoniae and one E. coli) were identified...
in outpatient strains (Table 2). The molecular analysis of β-lactamases encoding genes for carbapenem-resistant isolates is presented in Table 2.

**Molecular typing by MLST**

Genotyping of carbapenem-resistant Enterobacteriaceae isolates revealed that the OXA-48-producing *E. coli* isolate belonged to sequence type (ST)533, and the four OXA-48-producing *K. pneumoniae* isolates belonged to four distinct STs, including ST163, ST2017, and the international high-risk resistant lineages ST101 and ST147. MLST results for carbapenem-resistant non-fermentative isolates showed that all of *A. baumannii* and *P. aeruginosa* strains were assigned to the international clonal lineages ST2 and ST654, respectively (Table 2).

**Discussion**

Increasing drug resistance causes a real problem in therapeutic choices, especially with the rise of carbapenem resistance among GNB isolates, which has emerged and been reported in many countries. In Algeria, carbapenem-resistant Enterobacteriaceae have rarely been reported compared to non-fermentative pathogens. In our epidemiological survey, we observed an increased frequency of infection with OXA-48 carbapenemase-producing *E. coli* (3.7%) and *K. pneumoniae* (20%) isolates. The first description of OXA-48-producing *E. coli* in Algeria was reported in 2014 with the prevalence of 1.1% among the collection of 448 isolates. In addition, Agabou et al reported in 2016 the detection of an OXA-48-producing *E. coli* strain isolated from a patient in the Constantine region. OXA-48-producing *E. coli* strains were also reported in numerous countries, including France, Belgium, Germany, Italy, Spain, India, Turkey, Lebanon, and Senegal. OXA-48-producing *K. pneumoniae* was first reported in Turkey in 2001. It has since been extensively identified in Turkey and many regions of the world including neighboring countries such as Morocco, Tunisia, and Libya, and in Mediterranean countries (France, Spain, Italy, Slovenia, Greece, Egypt, and Lebanon).

The occurrence of OXA-48-producing *K. pneumoniae* has also been reported in Senegal, Saudi Arabia, India, Ireland, Switzerland, and Russia. Recently, in Algeria, a few clinical cases of OXA-48-producing *K. pneumoniae* have been described: in Algiers (one isolated case, in 2014), in Constantine (three isolated cases, in 2015), in Guelma (two isolated cases, in 2014), in Ouargla (one isolated case recovered between 2014 and 2015), in Batna (seven isolated strains collected between 2014 and 2015), and two strains isolated from...
<table>
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<tr>
<th>GNB clinical isolates</th>
<th>Sources</th>
<th>Ward</th>
<th>Sample source</th>
<th>Resistance phenotype</th>
<th>IMP MIC (µg/mL)</th>
<th>Phenotypic detection of carbapenemase</th>
<th>β-Lactamases genes</th>
<th>Carbapenems resistance genes</th>
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<td>OXA-48</td>
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<td>3</td>
<td>+ + + +</td>
<td>MHT, SHV-11</td>
<td>OXA-48</td>
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<td>+ + +</td>
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<td>MHT</td>
<td>OXA-48</td>
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<td>Pseudomonas aeruginosa (S1) Hospital Urology Urine</td>
<td>TIC, TCC, CAZ, FEP, ATM, IMP, GN, CIP, FO, FOF</td>
<td>&gt;32</td>
<td>+ + + +</td>
<td>MHT</td>
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<td>+ + + +</td>
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<td>+ + + +</td>
<td>MHT</td>
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<tr>
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<td>MHT</td>
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<td>MHT</td>
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<td>MHT</td>
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<td>MHT</td>
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<td>+ + + +</td>
<td>MHT</td>
<td>OXA-23</td>
<td>2</td>
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Notes: P1: several mutation types leading to the premature stop codon TGA in oprD, resulting in a truncated polypeptide of 83 amino acid residues; P2: several mutation types leading to the premature stop codon TAG in oprD, resulting in a truncated polypeptide of 414 amino acid residues.

Abbreviations: TIC, ticarcillin; TCC, ticarcillin/clavulanic acid; ETP, ertapenem; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; ATM, aztreonam; IPM, imipenem; GN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; FOF, fosfomycin; SXT, trimethoprim/sulfamethoxazole; ICU, intensive care unit; MIC, minimum inhibitory concentration; MCNP, modified Carba NP; MHT, modified Hodge test; EDTA, ethylene diamine tetra-acetic acid; CSF, cerebrospinal fluid.
collection of 55 isolates collected between 2013 and 2015 from hospitalized patients in the cities of Annaba (one isolate) and Skikda (one isolate). However, it is important to note that the Enterobacteriaceae family- and also non-fermentative bacilli-producing carbapenemase have always been associated with hospitalized patients. The increased prevalence of carbapenemase producers in the hospital setting occurred as a result of many factors, including antibiotic selection pressure, the horizontal transfer of plasmids containing carbapenemase genes between different Enterobacteriaceae species, and the spread of clones harboring these genes among inpatients through hand contact with health care personnel. However, it is worth noting that in this study, 18.2% of carbapenemases detected (two out of the 11) were identified from outpatient strains (one in *E. coli* and one in *K. pneumoniae* isolates). Given that the outpatients included in our investigation had not previously used antibiotics, this suggests that resistance could be acquired via contact with animals or transmitted to humans through the food chain. Therefore, our document represents the first report describing *E. coli* and *K. pneumoniae* isolates harboring the *bla* _OXA-48_ carbapenemase gene from the community in Algeria. Notwithstanding, OXA-48 carbapenemase producers of community origin have been reported in Morocco and Switzerland. The emergence of carbapenemase-producing strains in the community represents a major epidemiological and therapeutic problem. Furthermore, the emergence of carbapenem-resistant Enterobacteriaceae producing OXA-48 of animal origin and from environmental samples has been reported in Algeria and in other countries.

In this study, the identification of OXA-48-producing *E. coli* and *K. pneumoniae* from external patients highlights the wide dissemination of the *bla* _OXA-48_ gene in Algeria. On the other hand, it should be noted that these two OXA-48 carbapenemase producers from the community were resistant to ertapenem but susceptible to imipenem. Indeed, comparing the resistance pattern of carbapenemase-producing strains of hospital origin with those of external origin, we found that external origin strains were more sensitive and also were negative for ESBL production (Table 2). That is why we propose to use the ertapenem antibiotic for detecting OXA-48 carbapenemase, particularly in non-multidrug resistant strains.

In our investigation, we report the first identification of the epidemic multidrug-resistant lineage *K. pneumoniae* ST147, co-producing ESBL and the *bla* _OXA-48_ gene from a hospital setting in Algeria. Also, the present study represents the second report of the internationally successful lineage ST101-producing OXA-48 in Algeria. The first identification of this ST was very recently reported in an outbreak of OXA-48-producing *K. pneumoniae* in Batna University Hospital, Algeria. Interestingly, these epidemic strains carrying *bla* _OXA-48_ beta-lactamase have been involved in several outbreaks in North African countries and also in some European regions. We note that strains belonging to these lineages often harbor resistance determinants for different classes of antibiotics, such as β-lactam, quinolone, and carbapenem. In addition, our report describes the first detection of OXA-48 carbapenemase-producing *E. coli* ST353 and *K. pneumoniae* ST163 and ST2017 in Algeria. *E. coli* ST353 and *K. pneumoniae* ST163 of clinical origin have been described in Korea and China, respectively. However, *E. coli* ST353 had previously been detected in the gut of chickens in China and in uropathogenic strains isolated from dogs in Switzerland. To date, there are no reports describing *K. pneumoniae* ST2017. For GNB non-fermentative isolates, carbapenemase-encoding genes were identified only among *A. baumannii* isolates. The resistance of *A. baumannii* to carbapenem is mostly mediated by OXA-23 carbapenemase. This oxacillinase has been extensively reported in numerous countries in the world, including Algeria, and was identified in all *A. baumannii* isolates resistant to imipenem included in this study. OXA-23 carbapenemase has thus become the dominant and endemic gene of carbapenem-resistant *A. baumannii* in Algerian hospitals. As reported, *A. baumannii* OXA-23 producers are generally multiresistant, similar to those found in our survey. MLST analysis showed that all *A. baumannii* OXA-23 producers belonged to the international clone ST2, which has a wide geographical distribution. In addition, ST2 was the predominant clone detected in various clinical and environmental specimens, in Algeria and in other countries.

In this study, the molecular investigation of the carbapenem resistance mechanism in *P. aeruginosa* isolates revealed the chromosomic mutations in the *oprD* gene. The alteration of the *oprD* gene was the main mechanism for imipenem resistance in *P. aeruginosa* clinical strains, as previously described in Algeria and in other countries. On the other hand, we found that imipenem-resistant *P. aeruginosa* isolates displayed multidrug resistance profiles and also harboring of the ESBL gene. To our knowledge, ESBL genes have never been detected in imipenem-resistant *P. aeruginosa* strains in Algeria. Moreover, these isolates were assigned to the successful international clone ST654, usually associated with a multidrug-resistant phenotype, and this clone has been previously reported in Algeria, among imipenem-resistant *P. aeruginosa* clinical and surface strains that had mutational inactivation of *oprD* gene. In addition, the clone ST654 associated with KPC and VIM-producing *P. aeruginosa* has
been described in Argentina and in the United Kingdom, respectively.37

**Conclusion**

This study represents the first report of epidemic multidrug-resistant lineage *K. pneumoniae* ST147 co-producing *bla* <sub>OXA-48</sub> and ESBL genes in Algeria and describes the first detection of OXA-48-producing *E. coli* ST533 and *P. aeruginosa* ST163 and ST2017. Also, this study represents the first description of ESBL-producing imipenem-resistant *P. aeruginosa* strains in Algeria. In addition, this study showed that carbapenem-resistant GNB are propagated in this country. *E. coli* and *K. pneumoniae* isolates harboring *bla* <sub>OXA-48</sub> carbapenemase are emerging in the community, which is very worrying. Therefore, we highlight the need to establish monitoring systems in order to prevent a much wider dissemination of carbapenemase producers in Algeria.

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**Disclosure**

The authors report no conflicts of interest in this work.

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