Detection of ESBLs and carbapenemases among Enterobacteriaceae isolated from diabetic foot infections in Ouargla, Algeria

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Abstract

Introduction: The emergence and rapid spread of Enterobacteriaceae carrying extended spectrum beta-lactamases (ESBLs) and carbapenemases represent a great threat to clinical treatment due to their multi-drug resistance. This study investigated ESBLs and carbapenemases encoding genes in Enterobacteriaceae collected from diabetic foot infections (DFIs) in Ouargla, southern Algeria.

Methodology: A total of 70 Enterobacteriaceae strains were recovered from 76 patients with DFI between February 2017 and April 2018. Antimicrobial susceptibility testing was performed using the disc diffusion method, and the presence of bla genes was detected using polymerase chain reaction (PCR) and DNA sequencing. The genetic transfer of the plasmids was carried out by conjugation using the broth mating method.

Results: The most common isolate was Proteus mirabilis, followed by Escherichia coli, Morganella morganii and Klebsiella pneumoniae. The prevalence of ESBL and carbapenemase-producing Enterobacteriaceae was 11.42% and 2.85% respectively. Plasmid-mediated AmpC was detected in 5.71% isolates. Conjugation experiments showed the transferability of blaCTX-M-2.

Conclusions: Our findings support the view that various pathogens found in DFIs differ from one part of the country to another. This study reports the first description of metallo-β-lactamase NDM-5 producing Klebsiella pneumoniae clinical isolate in Algeria.

Key words: Enterobacteriaceae; NDM-5; ESBL; Diabetic Foot Infections; Ouargla.


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Introduction

Diabetes mellitus is associated with several complications and diabetic foot infections (DFIs) are one of the most feared complications. These infections are one of the leading causes of morbidity [1,2]. Several epidemiological studies from subtropical countries have demonstrated that the most common organisms isolated from DFIs were aerobic Gram negative bacilli (GNB) including Enterobacteriaceae [3]. Antibiotic resistance especially extended-spectrum beta-lactamase producers (ESBLPs) constitute a major problem associated with treatment failure of DFIs [4,5]. Genes encoding ESBLs are common among Enterobacteriaceae [5]. In addition, there is an emergence of carbapenemases encoding genes worldwide. Metallo β-lactamases (MBLs) can hydrolyze almost all β-lactams, including extended-spectrum cephalosporins and carbapenems [6].

There is a paucity of data on ESBL and carbapenemase-producing Enterobacteriaceae carrying bla genes from DFIs in Algeria. In addition, no study on antibiotic-resistant strains in DFIs in the southern part of the country has been reported. Therefore, this study was conducted to describe ESBLs and carbapenemases producing Enterobacteriaceae isolated from DFIs in Ouargla, southern Algeria.

Methodology

Sample size and bacterial isolates

A total of 103 clinical samples were collected from patients with DFIs from February 2017 to April 2018 at the Mohamed Boudiaf and Maison du diabète hospitals in Ouargla, southern Algeria.

All Enterobacteriaceae species were identified both by conventional techniques and by using the API 20E Gallery (BioMérieux, Marcy l’Etoile, France) and the Vitek2 automated system (BioMérieux, Marcy l’Etoile, France).
Antimicrobial drug susceptibility testing and ESBL detection

Antimicrobial drug susceptibility was determined by a disc-diffusion method on Mueller–Hinton (MH) agar plates (Bio-Rad, Marnes-la-Coquette, France), according to the recommendations of the Antibiogram Committee of the French Society for Microbiology [7]. The following antimicrobial agents were tested: amoxicillin (AMC 20 µg), amoxicillin/clavulanic acid (AMC 20/10 µg), cephalixin (CL 30 µg), cefoxitin (FOX 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), aztreonam (ATM 30 µg), ertapenem (ETP 10 µg), imipenem (IPM 10 µg), piperacillin/tazobactam (PZM 30/10 µg), gentamicin (CN 10 µg), nalidixic acid (NA 30 µg), trimethoprim-sulfamethoxazole association (SXT) trimethoprim (1.25 µg), sulfamethoxazole (23.75 µg). ESBL genotyping was performed using the following β-lactamase genes: *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA-1</sub>, *bla*<sub>PER</sub>, *bla*<sub>VEB</sub>, *bla*<sub>AMP</sub>, and the carbapenem-hydrolyzing β-lactamase genes (*bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>GES</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>) as described previously [8,9]. The β-lactamase producing strains *E. coli* U2A1790 (CTX-M-1), *E. coli* U2A1799 (CTX-M-9), *Salmonella* sp. U2A2145 (CTX-M-2), *Salmonella* sp. U2A1446 (TEM-1 and SHV-12), *Pseudomonas aeruginosa* U2A1125 (PER), *Acinetobacter baumannii* U2A2026 (VEB) and *E. coli* U2A2446 (OXA-1) were used for all β-lactamase detection methods as positive controls. *E.coli* K12J5 strain was used as the negative control.

Sequencing of resistance genes

All the amplified products were sequenced to validate their identities. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130x1 sequencer (Applied Biosystems, Foster City, CA, USA), with the same primers that were used for PCR amplification. The nucleotide and deduced protein sequences were analyzed with the tools available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Conjugation experiments and plasmid analysis

Conjugation assays were performed by a broth mating method using the azide-resistant (AzR) mutant of *E. coli* K12J5 as the recipient strain. Transconjugants were selected on MH agar containing azide (250 mg/L) and ceftazidime (2 mg/L) (Bio-Rad, Marnes-la-Coquette, France) and incubated for 18–24 h at 37 °C. The putative transconjugants were tested for susceptibility to all antibiotics, as indicated previously, to identify transferable antibiotic resistance determinants. Minimal inhibitory concentrations of β-lactams (ceftazidime) were determined by E-test for ESBL carrying strains and their transconjugants. Plasmid DNA was extracted from both donor and transconjugants using a Plasmid Mini-Prep Kit (Qiagen Ltd, West Sussex, UK) according to the manufacturer’s instructions. The sizes of the plasmids were estimated by electrophoresis on 0.7% agarose using the plasmids from *E.coli* V517 as the standard markers.

Results

Study population

Out of 76 patients, 56 (74%) were male and 20 (26%) were female. The majority had type 2 diabetes and the age was 30-81 years.

Bacterial isolates

A total of 112 bacterial isolates were obtained from 82 positive cultures; GNB (n = 74; 66%) were isolated from diabetic foot infections more frequently than Gram-positive cocci (GPC) (n = 38; 34%). In addition, 70 Enterobacteriaceae strains were isolated from DFIs and were identified as *Proteus mirabilis* (n = 31), *Escherichia coli* (n = 13),
Morganella morganii (n = 11), Klebsiella pneumoniae (n = 9), Proteus vulgaris (n = 3), Enterobacter cloacae (n = 2) and Citrobacter freundii (n = 1).

Antimicrobial drug susceptibility patterns

Enterobacteriaceae isolates were screened for the production of ESBL. Among the 70 strains, 11.42% were ESBL producers (n = 8) including Klebsiella pneumoniae (50%, n = 4), Escherichia coli (37.5%, n = 3), and Enterobacter cloacae (12.5%, n = 1).

The results of antibiotic susceptibility indicated that all isolates that were ESBL producers were resistant to amoxicillin, amoxicillin-clavulanic acid, cephalexin, cefotaxime, ceftazidime, cefepime and aztreonam. 62.5% of the isolates exhibited resistance to cefotaxim. Two Klebsiella pneumoniae were carbapenem-resistant (ertapenem and imipenem).

Resistance to non-β-lactam including trimethoprim-sulfamethoxazole, gentamicin, nalidixic acid and ciprofloxacin was also recorded at 100%, trimethoprim-sulfamethoxazole, gentamicin, nalidixic acid and ciprofloxacin was also recorded at 100%, 87.5%, 75%, and 75% respectively.

Molecular detection of bla-genes and DNA sequencing

Ten isolates of Enterobacteriaceae were positive for bla genes. The strains contained a diversity of β-lactamases namely CTX-M-1, CTX-M-2, SHV and TEM enzymes. However, blaCTX-M-9, blaoxa-1, blaper and blavEB were not detected. The combination blaCTX-M-1 + blaTEM was the most common (5/10) and the genotype blatem + blashv was identified in 4 strains. The blavim and blandm genes were amplified from two Klebsiella pneumoniae isolates. These two strains were found to coproduce CTX-M-1 and CTX-M-2 ESBLs in combination with other beta-lactamases (TEM and SHV). blaimp, blakPC, blages, and blaoxa-48 were not detected in any isolate. Plasmid-mediated AmpC gene bladm was detected in three isolates (1 Proteus mirabilis, 1 Morganella morganii and 1 E.coli) and blact was found in two E.coli.

DNA sequencing analysis demonstrated that one isolate carried the CTX-M-28 subgroup. The subgroup CTX-M-15 and CTX-M-55 were carried by two and one isolate respectively, blatem and blashv sequences indicated that the isolates harboured the TEM-1 (n = 1), SHV-9 (n = 1). The subgroup for bladm was DHA-13 (one isolate); sequencing revealed the presence of blandm in Klebsiella pneumoniae. All the combinations of bla genes that were identified are listed in Table 1.

Results of conjugation experiments

Conjugation experiments were carried out for two isolates (S36 and S61). Transfer of an ESBL phenotype was successful in only one isolate (TC61), the transconjugant was resistant to amoxicillin, amoxicillin-clavulanic acid, cephalexin, ceftazidime, cefepime, aztreonam, and gentamicin; the minimal inhibitory concentration (MIC) of ceftazidime was 4 µg/mL. Donor strain S61 transferred the blaCTX-M-2 gene to the recipient strain on a 7.2 kb plasmid.

Discussion

Our study was carried out over a period of 14 months, from February 2017 to April 2018, on 76 patients with DFI.

### Table 1. Phenotypic and genotypic features of BL-PE isolates collected from DFIs.

<table>
<thead>
<tr>
<th>Code</th>
<th>Isolate</th>
<th>Ward</th>
<th>Gender / age (years)</th>
<th>Resistance phenotype</th>
<th>β-Lactamases genes</th>
<th>Carbapenems resistance gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>S7</td>
<td>Enterobacter cloacae</td>
<td>General surgery</td>
<td>M/46</td>
<td>AML,AMC,CL,FEPC,CTX,FOX,CAZ,AT,M-CN,SXT,NA</td>
<td>blactX-1</td>
<td></td>
</tr>
<tr>
<td>S10</td>
<td>Proteus mirabilis</td>
<td>General surgery</td>
<td>M/50</td>
<td>AML,AMC,CL,FOX,AN</td>
<td>blapiHA</td>
<td></td>
</tr>
<tr>
<td>S22</td>
<td>Escherichia coli</td>
<td>General surgery</td>
<td>M/46</td>
<td>AML,AMC,CL,FEPC,CTX,CAZ,AT,M-CN,SXT,NA,CIP</td>
<td>blactX-M3,blactX-M2,blatem</td>
<td></td>
</tr>
<tr>
<td>S25</td>
<td>Escherichia coli</td>
<td>General surgery</td>
<td>M/46</td>
<td>AML,AMC,CL,FEPC,CTX,FOX,CAZ,AT,M-CN,SXT,NA,CIP</td>
<td>blactX-M3,blactX-M2,blatem</td>
<td></td>
</tr>
<tr>
<td>S36</td>
<td>Klebsiella pneumoniae</td>
<td>MD</td>
<td>M/46</td>
<td>AML,AMC,CL,FEPC,CTX,FOX,CAZ,AT,M-ERT,IMP,CMN,SXT,NA,CIP</td>
<td>blactX-M15,blactX-M2,blashv,blatem</td>
<td>blavim, blandm</td>
</tr>
<tr>
<td>S42</td>
<td>Morganella morganii</td>
<td>MD</td>
<td>M/61</td>
<td>AML,AMC,CL,FOX,SXT</td>
<td>blapiHA</td>
<td></td>
</tr>
<tr>
<td>S51</td>
<td>Klebsiella pneumoniae</td>
<td>MD</td>
<td>M/73</td>
<td>AML,AMC,CL,FEPC,CTX,CAZ,AT,M-CN,SXT,NA,CIP</td>
<td>blactX-M15,blashv-9,blatem</td>
<td></td>
</tr>
<tr>
<td>S55</td>
<td>Escherichia coli</td>
<td>ICU</td>
<td>M/80</td>
<td>AML,AMC,CL,FEPC,CTX,FOX,CAZ,AT,M-CN,SXT</td>
<td>blatem-1,blapiHA</td>
<td></td>
</tr>
<tr>
<td>S59</td>
<td>Klebsiella pneumoniae</td>
<td>MD</td>
<td>M/66</td>
<td>AML,AMC,CL,FEPC,CTX,CAZ,AT,M-CN,SXT,CIP</td>
<td>blactX-M15,blctX-M15,blashv,blatem</td>
<td></td>
</tr>
<tr>
<td>S61</td>
<td>Klebsiella pneumoniae</td>
<td>MD</td>
<td>M/66</td>
<td>AML,AMC,CL,FEPC,CTX,FOX,CAZ,AT,M-ERT,IMP,CMN,SXT,NA,CIP</td>
<td>blactX-M15,blactX-M2,blashv,blatem</td>
<td>blavim, blandm-5</td>
</tr>
</tbody>
</table>

BL-PE: bêta-lactamase-producing Enterobacteriaceae; M: male; MD: Maison du diabète; ICU: intensive care unit.
Our results report that DFIs were higher in males (74%) than in females (26%). The male predominance could be attributed to factors such as strenuous outdoor work and poor compliance to foot care practices [10–13].

The majority of the patients were elderly, although the age range was 30-81 years. Thus, predominance of elderly patients can be explained based on the fact that diabetic foot ulcer is a chronic complication of diabetes [10] and diabetic complications directly correspond to the span of diabetes [14]. The majority of the patients suffered from type 2 diabetes, which is similar to reports by other studies [10,12,13].

In our study, GNB were more prevalent (66%), and Enterobacteriaceae dominated among all isolates. These findings correlated with those of Djahmi et al. and Bouharkat et al. [13,15]. Studies in warm countries [Middle-East and North Africa (MENA) and south-east Asia] have found a higher prevalence of aerobic GNB [10,16–18]. In contrast, studies from the western countries reported GPB to be the most frequently isolated pathogens [19,20]. This discrepancy in the prevalence of GNB in DFIs between eastern and western countries remains largely unknown. Self-treatment with antimicrobials, sanitary habits leading to suboptimal hand hygiene and foot sweating caused by hot climate are proposed to be responsible for increased prevalence of GNB in the developing countries compared to the west [21].

Predominance of GNB, especially Enterobacteriaceae in our study could be attributed to late presentation of the patient with DFI, walking barefoot, use of inappropriate footwear and delay in medical care.

A total of 70 Enterobacteriaceae strains were isolated from DFIs, Proteus mirabilis was the most prevalent (44.28%) in our study. Among Enterobacteriaceae, this predominance is observed in several studies [11,22], including a study in Algeria [15]. Other studies reported that E.coli was the most commonly isolated among Enterobacteriaceae [13,23]. Hence, bacterial predominance in DFIs is not conclusive and different from region to region. There are several reports of bacterial predominance in the literature.

Over the last few years, the emergence of antibiotic-resistant bacterial strains poses a major risk of therapeutic inadequacy leading to therapeutic failure of DFI [24]. Enterobacteriaceae have been responsible for several types of infections in communities and hospitals. The spread of ESBL-producing bacteria restraints the activity of a wide number of antibiotics, leading to serious therapeutic difficulties to treat both community and hospital infections [22]. Furthermore, DFIs are the most frequent complication requiring hospitalization [25].

Extensive studies on infection with ESBL and carbapenemase-producing organisms in patients with DFI in Algeria are scarce. In this study, we focused for the first time on Enterobacteriaceae isolated from DFIs in the region of Ouargla (Southern Algeria). 11.42% of Enterobacteriaceae were ESBL producers, and two isolates (2.85%) were carbapenemase producers. These findings are similar to the results reported by Jouhar et al. and Belfquih et al. [18,26].

Recently, studies reported the presence of ESBL-producing Enterobacteriaceae to be 33% and 27% respectively [10,27], and the rates of ESBL-producing microorganisms have been rising steadily over the last few years. In our study, out of eight ESBL-producing isolates, 50% were identified as Klebsiella pneumoniae and 37.5 % as E.coli strains that were isolated from DFIs. Saltoglu et al. have reported 38% of E.coli and Klebsiella isolates to be ESBL producers in DFIs [28]. Indeed, ESBL-producing E. coli and Klebsiella were reported to have increased [29]. Another study by Saseedharan et al. reported that among the Enterobacteriaceae, 43% were ESBL producers (33% of E.coli isolates and 61% of K. pneumoniae) [30].

Infections due to ESBL-producing bacteria increased the hospitalization rate of DFI patients [31]. Hence, routine screening of ESBL-producing Enterobacteriaceae should be emphasised.

In the case of the non-β-lactam drugs, ESBL-producing Enterobacteriaceae strains were found to be 100% resistant to trimethoprim-sulfamethoxazole, while resistance to gentamicin, nalidixic acid and ciprofloxacin were 87.5%, 75% and 75% respectively. This could be attributed to the unregulated use of these antibiotics. Additionally, the different frequencies of antibiotics prescriptions could explain the different rates of antibiotics resistance in bacteria isolated from DFIs [32].

The dissemination of ESBL-producing Enterobacteriaceae worldwide is due to the increased use of third-generation cephalosporins [3]. Our results revealed that blactxM was the most common gene (87.5%), followed by blatem (75 %) and finally blasiv (50%). These results agree with those reported by previous studies in Egypt and India [33,34]. In addition, CTX-M type β-lactamase has been reported in earlier studies, which also supports our findings [35]. There are reports of increased prevalence of blactxM among ESBLs producers in neighbouring countries such as...
Morocco (80%) [36], Tunisia (83%) [37] and Lybia (85.9%) [38]. In fact, in the last decade, blaCTX-M has explosively disseminated and become the most common resistance gene in β-lactams around the world, indeed CTX-M is endemic [39].

Among the isolates, 4 (5.71%) were identified as AmpC producers, two cefoxitin-resistant ESBL-producing E.coli and two cefoxitin-resistant non ESBL producing Proteus mirabilis and Morganella morganii carried plasmid-mediated AmpC (blaCTX and blaDHA). The prevalence of isolates harboured plasmid-mediated AmpC from hospitalized patients in Algiers and Tlemcen were 2.4% and 1% respectively [40,41]. In Algeria CMY-2 and DHA-1 were previously reported in clinical isolates of Enterobacteriaceae by Messai et al., Iabadene et al.and Nedjai et al. [40,42,43]. In addition, CMY-4 was detected in clinical isolates in Algeria [44]. However, we have detected for the first time blaCIT in clinical isolates and DHA-13 enzyme in Algeria; CTX-M-2 was co-produced with CIT, and DHA cephalosporinase was found associated with TEM-1 in two E.coli strains recovered from two hospitalized patients with DFI.

The emergence of carbapenemases among Enterobacteriaceae is a major public health problem globally [45]. This situation has become apparent as a threat to public health in Algeria [46]; few studies have reported carbapenemase producers among clinical Enterobacteriaceae isolated in Algeria [47,48], and one study from southern Algeria reported faecal carriage in hospitalized patients [49]. Our study reports the detection of MBL-producing K. pneumoniae isolates in southern Algeria; the two K. pneumoniae co-harboured blaNDM, blAVIM and ESBL genes. The authors have previously reported that the Indian subcontinent, the Balkans regions, and the Middle East constitute the main reservoir for NDM-producing isolates [50,51]. Robin et al. described the first CPE in Algeria; the VIM-19 variant was reported in five Enterobacteriacae strains (E. coli [n = 2], K.pneumoniae [n = 2], and Providencia stuartii [n = 1]) [52] and during the same study period two strains E.coli and Klebsiella pneumoniae producing VIM-19 were recovered from an Algerian patient [53].

Here, we report an NDM-5 producing Klebsiella pneumoniae isolated from a patient with DFI. The NDM-5 is a variant of NDM-1; the first description of NDM-5 producing E.coli in the United Kingdom was from a patient returning from a recent hospitalization in India [54]. The NDM-5 enzyme confers high resistance to carbapenems and an expanded-spectrum cephalosporins relative to NDM-1 [54]. Several studies from India have reported NDM-producing Enterobacteriaceae in patients with diabetic foot ulcers [30,55]. Recently, the Middle East has been considered an additional and important reservoir for blaNDM [47]. Numerous recent studies from this area reported NDM-5 producing Escherichia coli and Klebsiella pneumoniae [48,56].

We have reported the first NDM-5-producing K. pneumoniae clinical isolate in Algeria, from DFI. The blaNDM-5 gene was associated with blaCTX-M-15, blaCTX-M-2, blaTEM, blaSHV and blaVIM. This strain was also resistant to trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin and gentamicin. This strain was isolated from a patient who had a history of recent travel to hajj and acquired diabetic foot ulcer there. The present data and other reports [56,57] show that the screening for such isolates may be effective in limiting the spread of these resistant microorganisms in the hospital setting, as well as in the community. The development of rapid, cheap and easy-to-handle diagnostic techniques is therefore, essential for the identification of carbapenemase producers [58].

In the present study, drug susceptibility testing showed that the transconjugant strain was resistant to β-lactams (amoxicillin, amoxicillin/clavulanic acid, cephalexin, ceftazidime, cefepime and aztreonam). In addition, the transconjugant exhibited co-resistance to gentamicin. Conjugation experiments and plasmid analysis demonstrated that plasmid-encoded ESBL gene blaCTX-M-2 was transferred to the recipient strain. Resistance genes such as aminoglycosides, tetracyclines, sulfonamides and trimethoprim were often carried by plasmids carrying the blaCTX-M genes [36]. The spread of ESBL genes is usually due to transferrable plasmids which constitute one of the most important mechanisms driving the emergence of antibiotic resistance.

Conclusions

In conclusion, the microbiological profile of DFI should be taken into consideration when choosing empiric antimicrobial treatment. Our findings established that the pattern of resistance in the south of Algeria has a different molecular profile in comparison with other regions of the country and it is necessary to prevent further dissemination of ESBLs and MBLs producing strains. Thus preventive strategies to control the dissemination of such strains are urgently required. This study can help physicians to better define the empiric treatment of DFIs caused by Enterobacteriaceae. However, more studies from other regions of the country are needed. In addition, it is
important to spread awareness among patients with diabetes to seek timely medical care.

References


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