Enterobacteriaceae isolates carrying the New Delhi metallo-β-lactamase gene in Yemen

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Ten carbapenem-resistant Enterobacteriaceae (eight Klebsiella pneumoniae isolates and two Enterobacter cloacae) isolates from Yemen were investigated using in vitro antimicrobial susceptibility testing, phenotypic carbapenemase detection, multilocus sequence typing (MLST) and replicon typing. Carbapenemase, extended-spectrum β-lactamase (ESBL) and plasmid-mediated quinolone resistance determinant genes were identified using PCR and sequencing. All of the 10 carbapenem-resistant Enterobacteriaceae were resistant to β-lactams, tobramycin, ciprofloxacin and cotrimoxazole. Imipenem, doripenem and ertapenem MICs ranged from 2 to >32 mg l⁻¹ and ertapenem MICs ranged from 6 to >32 mg l⁻¹. All of the K. pneumoniae isolates showed ESBL activity in phenotypic tests. Genes encoding blaNDM were detected in all strains. All K. pneumoniae strains produced CTX-M-15 ESBL and SHV β-lactamases. TEM-1 β-lactamase was detected in seven isolates. Nine isolates were qnr positive including QnrB1, QnrA1 and QnrS1, and six isolates produced AAC-6’-Ib-cr. MLST identified five different sequence types (STs): ST1399, ST147, ST29, ST405 and ST340. Replicon typing showed the presence of IncFI1K plasmids in four transformants. To the best of our knowledge, this is the first report of NDM-1-producing Enterobacteriaceae isolates in Yemen.

INTRODUCTION

The emergence and spread of antimicrobial resistance conferred by the production of β-lactamases among Gram-negative bacteria are a serious problem in the hospital environment as well as in the community. Due to the emergence of resistance by extended-spectrum β-lactamas (ESBLs), carbapenems are often used for the treatment of infections caused by Gram-negative bacteria. However, extensive use of carbapenems is probably the most significant reason for the increasing resistance to this class of β-lactams by production of carbapenemases (Nordmann et al., 2011).

Ambler class A (mostly blaKPC), class B [metallo-β-lactamas (MBLs); mostly blaVIM and blaNDM and, to a lesser extent, blaIMP] and class D (oxacillinases; mostly blaOXA-48) are the major carbapenemases reported worldwide in Enterobacteriaceae (Nordmann et al., 2011).

Abbreviations: ESBL, extended-spectrum β-lactamase; MBL, metallo-β-lactamase; MLST, multilocus sequence typing; ST, sequence type.

MBL expression in Gram-negative bacteria confers resistance to penicillins, cephalosporins and carbapenems. MBLs are not inhibited by the presence of commercially available β-lactamase inhibitors, and susceptibility to monobactams appears to be preserved in the absence of concomitant expression of other resistance mechanisms, such as ESBL production (Patel & Bonomo, 2013).

NDM-1 was first identified in 2008 in Sweden in a patient previously hospitalized in India. The patient was colonized with one Klebsiella pneumoniae strain and one Escherichia coli strain carrying blaNDM-1 on transferable plasmids. NDM-1 confers resistance to all β-lactams (including carbapenems) except aztreonam (Yong et al., 2009). To date, 11 minor variants of NDM-1 (NDM-2 to NDM-12) have been identified (http://www.lahey.org/studies/).

Since the first description in 2008, NDM-1-producing organisms have been reported in hospitalized patients in Europe, Australia and North America, and in patients returning from South Asian countries, especially India and Pakistan (Kumarasamy et al., 2010). International travel
and the use of multiple healthcare systems have contributed to the rapid spread of blaNDM-1, with potentially serious consequences (Muir & Weinbren, 2010).

In the Arabian Peninsula, NDM-1 has been reported in Oman, the United Arab Emirates, Kuwait, Saudi Arabia and Qatar (Poirel et al., 2011a; Ghazawi et al., 2012; Jamal et al., 2012; Al-Agamy et al., 2013; Shibli et al., 2013; Zowawi et al., 2014) but not in Yemen. Here, to the best of our knowledge, we describe the first NDM-producing Enterobacteriaceae strains isolated in Yemen.

**METHODS**

**Bacterial isolates.** During February 2013, eight *K. pneumoniae* isolates and two *Enterobacter cloacae* isolates with a reduced susceptibility to carbapenems were collected in Al-Thawra Hospital, Saudi German Hospital and the AlAilaq private medical laboratory in Sana’a, Yemen. They were identified using the API 20E identification system (bioMérieux) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (IVD MALDI Biotyper; Bruker Biospin SAS).

*Escherichia coli* ATCC 25922 was used for quality control. Azide-resistant *Escherichia coli* J53 was used as the recipient strain in conjugation experiments and *Escherichia coli* ElectroMAX DH10B competent cells (Invitrogen) for electrotransformation.

The following carbapenemase-producing isolates were used as controls and were kindly provided by P. Courvalin (Antibacterial Agents Unit Institut Pasteur Paris, France; U2A2730: *Escherichia coli* OXA-48; U2A1977: *K. pneumoniae* NDM-1; U2A2242: *Enterobacter cloacae* KPC, U2A2257: *Pseudomonas aeruginosa* IMP-1; U2A2016: *K. pneumoniae* VIM), R. Bonnet (Laboratoire de Bactériologie CHRU Clermont-Ferrand, France; *Proteus mirabilis* OXA-23, *Acinetobacter baumannii* OXA-24) and L. Poirel (Service de Bactériologie-Virologie CHU Bicêtre, Le Kremlin-Bicêtre, France; *A. baumannii* OXA-58).

The following cephalosporinase-producing isolates were used as PCR controls and were kindly provided by G. Arlet (KP SLK54: *K. pneumoniae* ACC-1; KP760: *K. pneumoniae* DHA-1; KOL: *K. pneumoniae* MOX-2; 96D: *K. pneumoniae* MIR-1; Bhe CD93: *K. pneumoniae* CMY-4; 1731: Klebsiella oxytoca FOX-3).

**Antimicrobial susceptibility testing.** Antibiotic susceptibility was determined on Mueller–Hinton agar using the standard disk-diffusion procedure as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014), for the following antibiotics: ticarcillin, ticarcillin–clavulanic acid, piperacillin, piperacillin-tazobactam, amoxicillin–clavulanic acid, cefazidime, cefotaxime, cefepime, ceftoxitin, imipenem, doripenem, ertapenem, meropenem, aztreonam, amikacin, tobramycin, ciprofloxacin, chloramphenicol, tetracycline, ticarcycline, fosfomycin and cotrimoxazole.

Susceptibility to carbapenems were collected in Al-Thawra Hospital, Sana’a, Yemen. They were identified using the API 20E identification system (bioMérieux) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (IVD MALDI Biotyper; Bruker Biospin SAS).

**Detection of antibiotic resistance genes.** Carbapenemase-encoding genes *blaKPC*, *blaVIM*, *blaIMP*, *blaNDM*, *blaOXA-23*–*like*, *blaOXA-24*–*like*, *blaOXA-58*–*like* and *blaGES* were screened using multiplex PCRs (Table 1). Amplicon sizes were, respectively, 86, 254, 238, 187, 146, 120, 166 and 214 bp. PCR was performed in 50 μl reaction mixtures containing 2.5 U *Taq* DNA polymerase (Qiagen), 2 mM MgCl₂ and 0.25 μM each primer. The following cycling parameters were used: initial denaturation at 95 °C for 3 min, then 35 cycles of denaturation at 94 °C 40 s, annealing at 55 °C for 30 s and extension at 72 °C for 20 s, with a final extension at 72 °C for 10 min.

Plasmid-mediated AmpC-type genes *blaACC*, *blaFOX*, *blaOXO*, *blaPAH*, *blaVIM* and *blaIMP* were screened using multiplex PCRs (Table 1). Amplicons sizes were, respectively, 232, 327, 507, 459, 613 and 142 bp. PCR was performed in the same conditions as for carbapenemase genes multiplex PCR.

ESBL gene detection (*blaTEM*, *blaSHV*, *blaCTX-M* and *blaGES*) was screened with PCR as described previously (Poirel et al., 2000; Kermas et al., 2012) (Table 1).

Screening of *qnrA, qnrB, qnrS, qnrC, qnrD* and *qepA* genes was carried out with a multiplex real-time PCR assay as described previously (Guillard et al., 2011). Pyrosequencing was used for detection of the *aac(6’)-Ib-cr* determinant (Guillard et al., 2010).

All PCR products were sequenced, and the sequencing results were compared with reported sequences available in GenBank.

**Multilocus sequence typing (MLST).** MLST was performed on the *K. pneumoniae* isolates using seven conserved housekeeping genes (*gapa, infB, ndh, pgi, phoE, tetB* and *tonB*) (Diancourt et al., 2005). A detailed protocol of the MLST procedure, including allelic type and sequence type (ST) assignment methods, is available in MLST databases from the Pasteur Institute, Paris, France (http://www.pasteur.fr/recherche/genopole/PB8/mlst/Kpneumoniae.html).

**Transfer of the carbapenem resistance determinant by conjugation.** Azide-resistant *Escherichia coli* J53 was used as the recipient strain for broth and filter mating assays (Guillard et al., 2014). Transconjugants were selected using a brain–heart infusion (BHI) agar plate supplemented with 1 μg ertapenem ml⁻¹ and 100 μg sodium azide ml⁻¹.

When the resistance plasmid transfer failed in mating experiments, transformation was used. Plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer’s protocol. *Escherichia coli* ElectroMAX DH10B competent cells (Invitrogen) were transformed with plasmid DNA by electroporation (MicroPulser electroporator; Bio-Rad Laboratories) according to the manufacturer’s instructions. Transformants were selected on BHI agar containing 1 μg ertapenem ml⁻¹.

**PCR-based replicon typing.** Plasmid incompatibility groups were determined using PCR-based replicon typing (Carattoli et al., 2005). Four multiplex PCRs were used for the detection of A/C, T, FI1As, W, N, FIB, L/M, I1-1Y, X, H12, FIA and Y replicons. Replicons P, R, U, F, FIC, H11, B/O and K were detected by simplex PCR (Carattoli et al., 2005; García-Fernandez et al., 2009). Replicons H11K, H12K, NewXXX (also named ZK), LVPK and Amet were detected using the PCR method described by D. Decré and G. Arlet (G. Arlet, personal communication). The *repA, traU* and *parA* genes were detected by PCR as described by Poirel et al. (2012).
RESULTS

Detection of NDM-1-producing isolates and antibiotic susceptibility

During February 2013, 10 Enterobacteriaceae isolates (eight K. pneumoniae and two Enterobacter cloacae isolates) from 10 patients with a reduced susceptibility to carbapenems (tested by the disk-diffusion technique) were included in this study. Specimen sources were urine (n=3), pus (n=3), blood (n=1), sputum (n=1), vagina (n=1) and ascites fluid (n=1). The clinical characteristics of the 10 patients are shown in Table 2.

With disc susceptibility testing, all the Enterobacteriaceae isolates were resistant or intermediate to β-lactams (including carbapenems, extended-spectrum cephalosporins, penicillins and aztreonam), tobramycin, ciprofloxacin and cotrimoxazole. In vitro susceptibility was found for tigecycline (100%), fosfomycin (90%), chloramphenicol (60%), imipenem (40%), tetracycline (30%) and amikacin (20%).

Imipenem, doripenem and meropenem MICs ranged from 2 to >32 mg l⁻¹ and ertapenem MICs ranged from 6 to >32 mg l⁻¹ for the 10 isolates (Table 2).

A modified Hodge test, MBL Etest and imipenem-EDTA synergy test were positive for the 10 isolates. All K. pneumoniae isolates showed ESBL activity in phenotypic tests.

PCR detection of resistance genes

PCR detected the presence of blaNDM genes in all strains. In addition, all K. pneumoniae strains produced CTX-M-15 ESBL, and TEM-1 β-lactamase was detected in seven strains: one Enterobacter cloacae and six K. pneumoniae isolates (Table 2). All K. pneumoniae isolates expressed SHV β-lactamases: SHV-1 (two isolates), SHV-11 (four
isolates), SHV-76 (one isolate) and one isolate (K. pneumoniae 27) produced a new SHV variant, SHV-184 (http://www.lahey.org/studies). These SHV variants were not considered as ESBL.

None of the isolates produced VIM-, IMP- and KPC-type carbapenemases, oxacillinase-type carbapenemases, GES-type ESBLs or plasmid-mediated AmpC-type β-lactamases.

All isolates except K. pneumoniae 1176 were qnr positive: five QnrB1 (18, 18B, 4866, 4161 and 4166), two QnrA1 (3141 and 4644) and two QnrS1 (27 and 10877) (Table 2). In addition, six isolates produced AAC-6'-Ib-cr and four isolates produced AAC-6'-Ib.

**MLST**

MLST analysis of the eight K. pneumoniae isolates identified five different STs: ST1399, ST147, ST29, ST405 and ST340 (Table 2).

**Plasmid studies**

No transconjugants were obtained from any of the donors. After electroporation, five transformants expressed bla$_{\text{NDM-1}}$, three transformants co-expressed bla$_{\text{NDM-1}}$, bla$_{\text{CTX-M-15}}$ and bla$_{\text{TEM-1}}$, and two transformant co-expressed bla$_{\text{NDM-1}}$ and bla$_{\text{CTX-M-15}}$. In addition, two transformants expressed qnrS determinant (Table 2).

No replicons type could be determined in three of the 10 parental strains tested. An F replicon was detected in five isolates, a Lvpk replicon in two isolates and an A/C replicon in two isolates. In addition to an F replicon, Enterobacter cloacae 18 carried the repA, traU and parA genes (Table 2).

Among transformants expressing only NDM-1, the FII1K replicon was detected in four transformants from two Enterobacter cloacae and two K. pneumoniae donors. The rest of the transformants were untypable (Table 2).

**DISCUSSION**

Strains of Enterobacteriaceae harbouring bla$_{\text{NDM-1}}$ have been identified worldwide, including in the Arabian Peninsula. However, to the best of our knowledge, no bla$_{\text{NDM-1}}$ genes have been reported in Enterobacteriaceae in Yemen so far. In a previous study in a neonatology ward in Yemen, no resistance to imipenem was recorded among K. pneumoniae isolates from Yemen. CTX-M-15 is predominant in Saudi Arabia (Agamy et al., 2009), Kuwait (Rotimi et al., 2008), the United Arab Emirates (Alfaresi et al., 2011) and Oman (Poirel et al., 2011a). This is the first report of CTX-M-15 in Yemen. CTX-M-15 is predominant in Saudi Arabia (Agamy et al., 2009), Kuwait (Rotimi et al., 2008), the United Arab Emirates (Alfaresi et al., 2011) and Oman (Poirel et al., 2011a). This high prevalence may be due to immigrants from countries with a high rate of CTX-M-15-positive Enterobacteriaceae (Ensor et al., 2009).

In concordance with previous reports, all the NDМ-1-producing isolates were resistant to multiple antibiotics, including aminoglycosides, fluoroquinolones and cephalosporins, except for tigecycline and colistin. In our study, NDМ-1 was co-expressed with CTX-M-15, SHV and TEM-1 β-lactamases. This is the first report of CTX-M-15 in Yemen. CTX-M-15 is predominant in Saudi Arabia (Agamy et al., 2009), Kuwait (Rotimi et al., 2008), the United Arab Emirates (Alfaresi et al., 2011) and Oman (Poirel et al., 2011a). This high prevalence may be due to immigrants from countries with a high rate of CTX-M-15-positive Enterobacteriaceae (Ensor et al., 2009).
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*ATH, Al-Thawra Hospital; APL, Ailaqi private laboratory; SGH: Saudi German Hospital.
†MEM, meropenem; DOR, doripenem; IMP, imipenem; ERT, ertapenem; ATM, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; ND, not determined; UT, untypeable.
‡PMQR, plasmid-mediated quinolone resistance determinant.
The association of qnrB genes with blaNDM-1 observed in our study was similar to previous reports from Egypt (Abdelaziz et al., 2013), Morocco (Poirel et al., 2011c), Australia (Shoma et al., 2014), Singapore (Teo et al., 2014), India (Khan & Nordmann; 2012), Belgium (Bogaerts et al., 2011) and France (Arpin et al., 2012). We also observed the previously described associations between qnrS and blaNDM-1 genes (Peirano et al., 2011; Islam et al., 2012) and blaNDM-1 and qnrA genes in Bangladesh (Islam et al., 2012).

This study was not based on systematic surveillance of carbapenem resistance and so the sample size was small. However, our findings can form the basis for further investigations of carbapenem-resistant Enterobacteriaceae in Yemen. Awareness of these strains is essential in order to prevent their selection and spread.

Given the reported propensity of this resistance mechanism to disseminate, it seems inevitable that further isolation of NDM-producing organisms will continue to occur in this region. It is therefore imperative that clinicians and laboratories remain vigilant, both in detecting these organisms and in instituting appropriate infection control measures to prevent NDM-producing Enterobacteriaceae from causing further epidemics.

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pneumoniae sequence type 340 strains in four patients at a South Korean tertiary care hospital.


