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Short Communication

Characterization of OXA-48-producing *Klebsiella oxytoca* isolates from a hospital outbreak in Tunisia

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ABSTRACT

Objective: There is very limited information about OXA-48-producing *Klebsiella oxytoca*. The aim of this study was to describe the phenotypic and molecular characterization of OXA-48-producing *K. oxytoca* isolates that caused an outbreak in a hospital in Tunisia.
Methods: Nineteen OXA-48-producing *K. oxytoca* were isolated from 2013 to 2016 in the University Hospital Farhat Hached, Sousse, Tunisia. Antibiotic susceptibility testing was performed by broth microdilution. Carbapenemase activity was investigated using the modified carbapenem inactivation method (mCIM). Phenotypic tests were also carried out to detect extended-spectrum β-lactamases. PCR was used to test for the presence of carbapenemase genes (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC} and bla_{OXA-48}). Genetic relatedness among isolates was investigated using rep-PCR. Whole genome sequencing (WGS) was performed in three representative isolates.
Results: mCIM was positive in all isolates. None of the isolates presented an ESBL phenotype. All strains were susceptible to cefoxitin, ceftazidime, cefepime, aztreonam, imipenem, meropenem, fluoroquinolones, aminoglycosides and colistin, and resistant to piperacillin-tazobactam, ertapenem, ticarcillin and ampicillin-sulbactam. All isolates presented the bla_{OXA-48} gene located in a ca. 63 kb IncL plasmid, which carried no additional resistance genes. They belonged to the new ST220.
Conclusion: Isolates from this study did not co-express an ESBL, which could complicate their detection in clinical laboratories. As OXA-48 has been mostly reported in *K. pneumoniae* there is a risk that the production of this enzyme is not suspected in the less common species *K. oxytoca*. These difficulties could play an important role in the hidden spread of this enzyme.

1. Introduction

In recent years, the spread of carbapenemase-producing Enterobacteria (CPE) has been identified worldwide. OXA-48 type β-lactamases represent one of the most prevalent carbapenem-hydrolysing enzymes in CPE. OXA-48 is responsible for resistance to penicillins and reduced susceptibility to carbapenems, but shows low-level hydrolytic activity against broad-spectrum cephalosporins. However, in many cases, isolates producing this and related enzymes also express other resistance mechanisms, including extended-spectrum β-lactamases (ESBL) or plasmid-mediated AmpC-type enzymes [1,2].

Efficient dissemination of OXA-48 has been linked to transfer of a conjugative IncL plasmid ca. 62 kb in size. The bla_{OXA-48} gene is flanked by two IS*1999* elements forming the functional composite

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transposon Tn*1999* without other antibiotic resistance gene. Several Tn1999 variants have been described [3,4].

Since OXA-48 carbapenemases were first described in Turkey [5], they have been reported extensively in outbreaks and case reports, particularly of *Klebsiella pneumoniae*. OXA-48 and related enzymes have also been described in other species of Enterobacteria, but reports have been uncommon in *Klebsiella oxytoca* [1], an important opportunistic pathogen causing serious infections in hospitalized patients [6].

This study describes the phenotypic and molecular characterization of OXA-48-producing *K. oxytoca* isolates causing an outbreak in a Tunisian hospital affecting patients from several medical units.

2. Materials and methods

2.1. Bacterial isolates and susceptibility testing

Strains were collected from patients admitted to the University Hospital Farhat Hached, Sousse, Tunisia, from 2013 to 2016. One strain per patient was evaluated. Isolates were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (MALDI Biotyper A System; Bruker Daltonics, Madrid, Spain). Antimicrobial susceptibility testing was initially performed using a semi-automated system (MicroScan WalkAway, Beckman Coulter, Madrid, Spain) with microdilution panels NC54. MICs of ceftazidime-avibactam, ceftolozane-tazobactam and colistin were determined using Sensititre panels (Thermofisher Scientific, Madrid, Spain). MICs of meropenem, imipenem and ertapenem were also determined by Etest (bioMérieux, Madrid, Spain) and confirmed by the reference broth microdilution method. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Phenotypic detection of extended-spectrum β-lactamases and AmpC-type β-lactamase production was performed using disks (Oxoid, Thermofisher Scientific, Madrid, Spain) of cefotaxime (30 μg), ceftazidime (30 μg) and cefepime (30 μg) alone or combined with clavulanic acid (10 μg) on both Mueller Hinton (MH) agar and MH agar supplemented with 200 mg/L of cloxacillin. Carbapenemase activity was investigated by the modified carbapenem inactivation method (mCIM) using meropenem disks [7].

2.2. Molecular characterization, whole genome sequencing (WGS), resistome and core genome multilocus sequence typing (cgMLST) analysis

Multiplex PCR was performed in all 19 isolates to detect the carbapenemase genes bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC} and bla_{OXA-48} using specific primers [8]. Genetic relatedness among *K. oxytoca* isolates was investigated by repetitive element palindromic PCR (rep-PCR) [9].

WGS was performed in three representative isolates TR_232, TR_20 and TR_229, obtained in 2013, 2014 and 2015. DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen®, Hilden, Germany). Genomic DNA paired-end libraries were generated using the Nextera XT DNA sample preparation kit (Illumina Inc, San Diego, CA, USA). The libraries were sequenced using the Illumina NextSeq 500 sequencer system with 2 × 150-bp paired-end reads (Illumina Inc). The quality of the high-throughput sequence data was assessed by FastQC and short reads were subsequently assembled de novo into contigs as described [6]. Automatic de novo annotation of draft genomes was done using Prokka version 1.12-beta [10]. Genomes of isolates TR_232, TR_229 and TR_20 have been submitted to the European Nucleotide Archive under accession numbers ERS5500988, ERS5500989 and ERS5500990, respectively.

Antimicrobial resistance genes were analysed using ResFinder tool (CGE server: https://cge.cbs.dtu.dk) with an ID threshold of 98% except for β-lactamase variants, which were determined with 100% identity. Additionally, SRST2 [11] was used to detect resistance genes and alleles with ARGannot database [12]. To reconstruct the plasmids carrying the carbapenemases genes, an in-house script (Plasmid ID, https://github.com/BU-ISCIII/plasmidID) was used.

Major outer membrane protein (OMP) porins, OmpK35 and OmpK36, were examined and compared with those from *K. oxytoca* 10-5243 (GCA_000247855.1).

A cgMLST was applied as described [6] to compare *K. oxytoca* isolated in Tunisia with all whole genome *K. oxytoca* strains available in NCBI from different geographical locations (Spain, Japan and USA).

Table 1
Clinical information about patients.

Patient	Age	Gender	Service	Sample	Disease/clinical condition	Year
TR_4	61 years	Male	Emergency	Urine	Urinary tract infection	2014
TR_9	30 days	Male	Neonatology	Exudate	Diaphragmatic hernia	2014
TR_10	81 years	Male	Otolaryngology	Exudate	Diabetes	2014
TR_11	57 years	Female	Surgery	Exudate	Sepsis	2014
TR_13	63 years	Female	Infectious diseases	Puncture	Hepatic abscesses	2014
TR_15	6 months	Male	Paediatrics	Urine	Sepsis, urine source	2014
TR_19	1 year	Female	Paediatrics	Urine	Sepsis, urine source	2014
TR_20	10 years	Female	Paediatrics	Blood culture	Sepsis, urine source	2014
TR_22	47 years	Male	Haematology	Sputum	Pneumonia	2014
TR_23	7 days	Female	Neonatology	Urine	Neonatal sepsis	2014
TR_25	55 years	Female	Gynaecology	Exudate	Pyelonephritis	2014
TR_29	45 years	Male	Emergency	Urine	Urinary tract infection	2016
TR_30	2 years	Male	Paediatrics	Urine	Urinary tract infection	2016
TR_107	5 years	Female	Paediatrics	Urine	Urinary tract infection	2015
TR_175	1 day	Female	Neonatology	Urine	Neonatal sepsis	2014
TR_229	50 years	Male	Haematology	Blood culture	Sepsis, inmunocompromised patient	2015
TR_232	2 days	Male	Neonatology	Blood culture	Neonatal sepsis	2013
TR_255	53 years	Female	Urology	Urine	Urinary tract infection	2014
TR_300	7 years	Female	Paediatrics	Urine	Urinary tract infection	2014

3. Results

3.1. Bacterial isolates and susceptibility testing

Nineteen OXA-48-producing *K. oxytoca* clinical isolates were recovered from urine samples (n = 10), exudates (n = 4), blood cultures (n = 3), sputum (n = 1) and a puncture sample (n = 1). Patients were admitted to neonatology (n = 4), paediatrics (n = 6), emergency (n = 2), haematology (n = 2), surgery (n = 1), infectious diseases (n = 1), otolaryngology (n = 1), gynaecology (n = 1) and urology (n = 1). Median age was 9.4 days for neonates (21%), 4.25 years for paediatric patients (31.5%) and 56.8 years for adults (47.5%) (Table 1).

Antibiotic susceptibilities were the same in all 19 isolates. They were susceptible to cefoxitin, ceftazidime, cefepime, ceftazidime-avibactam, ceftolozane-tazobactam, aztreonam, ciprofloxacin, levofloxacin, gentamicin, tobramycin, amikacin and colistin and resistant to piperacillin-tazobactam, ticarcillin and ampicillin-sulbactam. MICs (μg/mL) of imipenem, meropenem and ertapenem by reference microdilution were 2–4 (susceptible; standard dosing or increased exposure), 0.5–1 (susceptible) and 2–4 (resistant), respectively. These results were similar to those obtained with Etest (4–6, 0.75–1 and 1.5–2 μg/mL, respectively). While MICs of meropenem with the MicroScan WalkAway systems were also ≤1 μg/mL, MICs of imipenem with this commercial system were much lower (≤1 μg/mL). The mCIM test was positive (no inhibition zone around meropenem disks) in all isolates. ESBL or AmpC-type β-lactamases were not detected by phenotypic tests in any isolate.

3.2. Molecular characterization and WGS

PCR amplification and sequencing showed the presence of bla_{OXA-48} in all isolates and the rep-PCR pattern was the same in all of them.

The three isolates studied by WGS showed an antibiotic resistance gene profile including bla_{OXA-48} and the chromosomally encoded $bla_{OXY-1.1}$ genes. Genome assemblies were analysed in a gene-by-gene approach and the allelic distance from cgMLST was visualized in a minimum spanning tree (Fig. 1). In a pairwise comparison, two pairs of Tunisian strains differed from each other by only one allele and the other pair by two alleles, and, according to the *K. oxytoca* MLST website (https://pubmlst.org/koxytoca/), they belong to the new ST220, suggesting possible local transmission of this clone in Tunisia. The bla_{OXA-48} gene was located on a ca. 63 kb IncL plasmid, almost identical to plasmid pOXA48 NZ_CP018342 (average identity >99% and coverage percentage 99.78%), which carried no additional resistance genes. It was flanked by two IS*1999* repetitive elements, one of which was truncated by an ISR element corresponding with the previously described Tn*1999.2* transposon (Fig. 2).

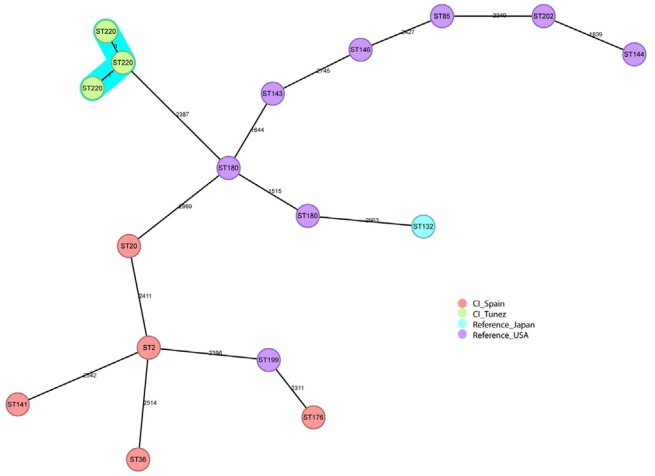

Fig. 1. Minimum spanning tree, distance based in a cgMLST scheme of 3201 genes, of three representative isolates from this outbreak and 14 *Klebsiella oxytoca* reference sequences available in NCBI. Circles are named with the ST type and colours indicate geographical origin. Strains from this study are highlighted.

Analysis of Ompk35 and OmpK36 sequences did not reveal any nonsense point mutation insertion and/or deletion causing a reading frameshift with a premature stop codon or gross disruption by an insertion sequence.

4. Discussion

The present study documents an outbreak of *K. oxytoca* producing OXA-48 affecting multiple patients and units in a hospital in Tunisia. Although most outbreaks caused by carbapenemase-producing strains have been previously described in adults, paediatric and neonatal patients were also affected in this outbreak. There is scarce information about CPE infection in children [13,14]. In addition, treatment options in paediatric patients are limited as several agents (i.e., quinolones or tetracyclines) are not usually considered because of their side effects in this population. Unfortunately, because of the retrospective nature of this study, it was difficult to perform a detailed analysis of the clinical variables (Table 1) of patients affected by the tested strains.

EUCAST and CLSI guidelines recommend reporting clinical categories of β-lactams against CPE as found, independently of the isolate producing a carbapenemase or expressing another resistance mechanism [15]. In consequence, and following those guidelines, many OXA-48 producers are, as in our case, categorized as susceptible to imipenem and meropenem. *Klebsiella oxytoca* harbours a chromosomal-encoded class A β-lactamase that confers resistance to amino- and carboxy-penicillins and when hyperproduced can also determine high-level resistance to some expanded-spectrum cephalosporins and aztreonam. The isolates from this study were susceptible to all broad-spectrum cephalosporins and did not co-express an ESBL. This is clinically relevant as expanded-spectrum cephalosporins or carbapenems have been considered as an alternative therapy for infections by OXA-48-producing enterobacteria with low MICs of those agents.

OmpK35 and OmpK36 have been implicated in reduced carbapenem susceptibility in *K. pneumoniae* and other species including *K. oxytoca* [16,17]. We did not find in our isolates any relevant mutation likely related with reduced outer membrane permeability, which is consistent with the observed phenotype.

The incL plasmid identified in this study has also been observed in many other previously described enterobacteria producing OXA-48, which reinforces the importance of this genetic element in dissemination of this enzyme.

Previous studies described outbreaks caused by OXA-48 producers involving strains that exhibited multidrug-resistant patterns because the organisms also possess other resistant mechanisms. However, isolates from this outbreak were susceptible to cephamycins, oxyimino-cephalosporins, ceftazidime-avibactam, ceftolozane-tazobactam, aztreonam, some carbapenems, fluoroquinolones, aminoglycosides, tigecycline, trimethoprim/sulfamethoxazole, fosfomycin and colistin. This peculiar resistance phenotype could be difficult to detect in other clinical laboratories. As OXA-48 has been mostly reported in *K. pneumoniae* there is a risk that the production of this enzyme is not suspected in the less common species *K. oxytoca*. These difficulties could play an important role in the hidden spread of this enzyme.

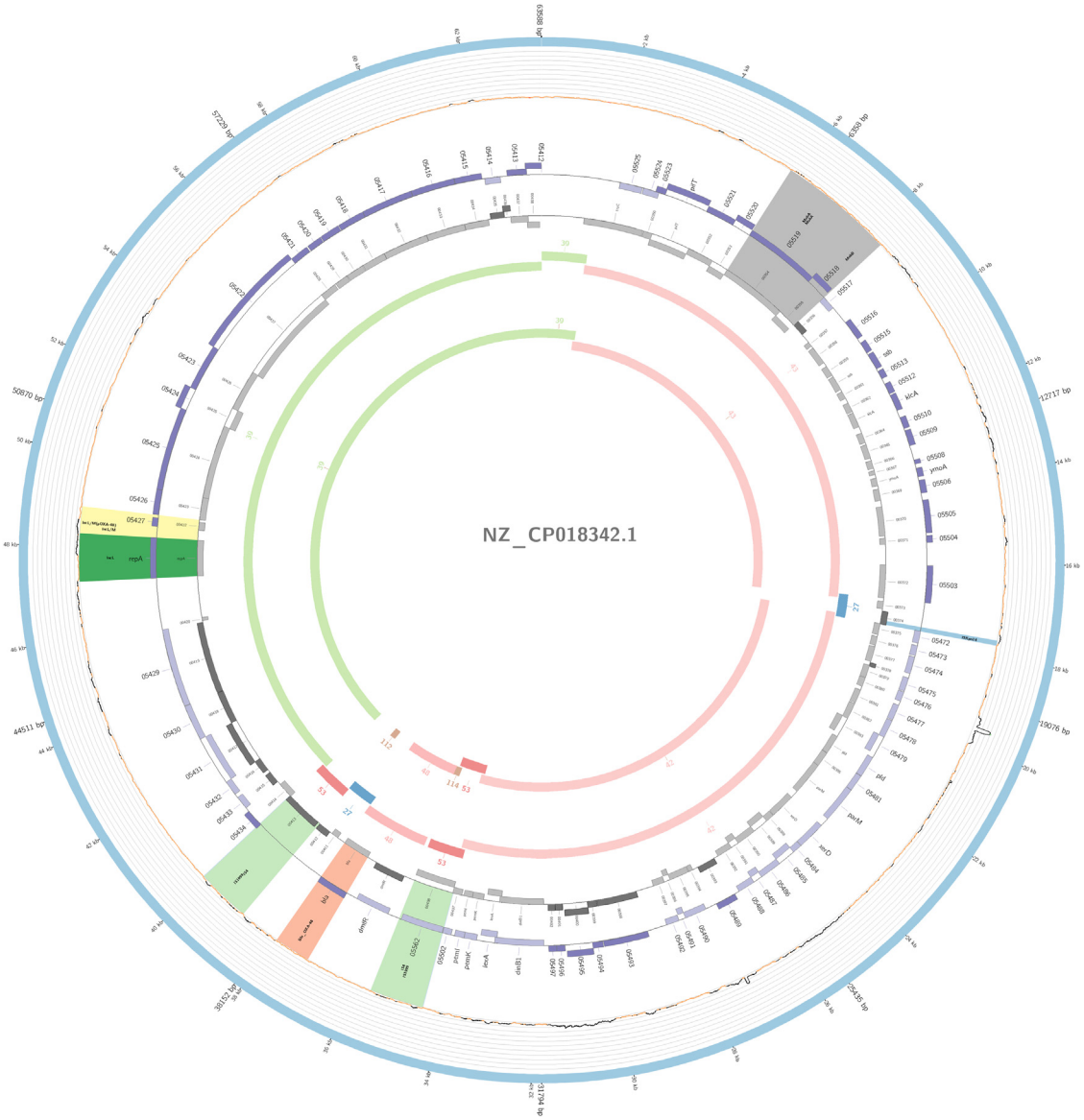

Fig. 2. Overview of the IncL plasmid harbouring *bla*$_{OXA-48}$ detected in *Klebsiella oxytoca*. The figure represents the plasmid according to the homology with a highly similar one from the database (blue outer ring). Graph represents the Illumina reads mapped against this plasmid with depth of coverage ranging from 0 (red) to 500, coloured orange when values are 1–20 and green when higher than 200 reads. Grey boxes represent cds from automatic annotation, with dark and light colour when they were found in forward or reverse strands, respectively. Coloured stripes represent a more detailed annotation that includes antibiotic resistance genes in red, transposons in light green and Rep genes in yellow or dark green. Homology between reference plasmid and assembled contigs is represented in the inner ring, with each contig coloured according to its number.

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Competing interests

None declared.

Ethical approval

Ethical approval was not required since our study was conducted on routine isolates and did not imply any intervention.

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