Detection of an IncA/C plasmid encoding VIM-4 and CMY-4 β-lactamases in *Klebsiella oxytoca* and *Citrobacter koseri* from an inpatient cardiac rehabilitation unit

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SUMMARY

A 62-year-old patient was transferred to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri after undergoing a heart transplantation at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia. On 1 August 2013 and during hospitalization in the rehabilitation unit, *Klebsiella oxytoca* and *Citrobacter koseri* clinical isolates were simultaneously recovered from the patient's preputial swab. Both the *K. oxytoca* and *C. koseri* strains were carbapenem-resistant by MicroScan System (Beckman Coulter). Carbapenem-resistant *K. pneumoniae* had previously been reported in the same rehabilitation facility.

The aim of the study was to identify the carbapenem resistance mechanisms among the enterobacterial species recovered. Phenotypic screening tests useful to detect the β-lactamases/carbapenemases were performed. Carbapenem MICs were obtained by Etest. AmpC and MBL encoding genes were identified by PCR and sequencing. Conjugation assays and plasmid characterization were performed.

Both of the *K. oxytoca* and *C. koseri* isolates were multi-drug resistant, showing resistance to amoxicillin-clavulanic acid, three generation cephalosporins, ertapenem (K. oxytoca MIC, >32 mg/L; C. koseri MIC, 4 mg/L), imipenem (*K. oxytoca* MIC, 4 mg/L; *C. koseri* MIC, 12 mg/L), thrimethoprim-sulphamethoxazole and gentamicin. Susceptibility was retained to fluoroquinolones, colistin and tigecycline. Molecular characterization confirmed the co-presence of bla_{CMY-4} and bla_{VIM-4} determinants in a 150 Kb transferable plasmid of IncA/C group.

This case is the first detection in Italy of the *K. oxytoca* and *C. koseri* clinical isolates co-producing the CMY-4 and VIM-4 enzymes.

KEY WORDS: Metallo-β-lactamases, Cephalosporinases, Multi-drug resistant *Enterobacteriaceae*, Rehabilitation unit.

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INTRODUCTION

Klebsiella oxytoca and *K. pneumoniae* are opportunistic pathogens increasingly implicated in clusters of community and nosocomial outbreaks, particularly in specific medical units (Watson *et al.,* 2005; Migliavacca *et al.,* 2013).

The acquisition of an extended-spectrum β-lactamase (ESβL) is the most common mechanism of resistance to broad-spectrum cephalosporins in *K. oxytoca* (Romero *et al.,* 2007; Sturm *et al.,* 2010), while acquired AmpC cephalosporinases are less frequently detected in this species (Yamasaki *et al.,* 2010). Since AmpC β-lactamase production is frequently accompanied by multi-drug resistance, therapeutic options became limited. In addition, failure to identify AmpC β-lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality (Tsakris *et al.,* 2011).

Citrobacter koseri, an environmental Gram-neg-

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ative bacterium, is occasionally found as a coloniser of the human gastrointestinal tract as part of the normal flora.
Although the potential virulence of the species is considered low, it is sporadically implicated in serious nosocomial infections.
The antimicrobial treatment of infection caused by *C. koseri* has been changing, due to several reports of isolates carrying ESβL and other resistance encoding genes (Doran, 1999). Anyway, the isolation of *C. koseri* and/or *K. oxytoca* strains showing resistance to carbapenems remains very infrequent in Italy (Giani *et al.,* 2013). To date, we have only two reports on the presence of $bla_{\text{VIM-1}}$ or $bla_{\text{KPC-2}}$ genes in *C. koseri* clinical isolates in the Mediterranean area (Castanheira *et al.,* 2009; Mavroidi *et al.,* 2011).
Here we report the detection of *K. oxytoca* and *C. koseri* strains co-producing a VIM-4 metallo-β lactamase (MBL) and an acquired CMY-4 Amp-C enzyme from a single patient. The objective of this study was to evaluate the localization of $bla_{\text{VIM-4}}$ and $bla_{\text{CMY-4}}$ resistance genes and to assess their spreading potential.

MATERIALS AND METHODS

On 18 July 2013, a 62-year-old male patient was admitted to the cardiac rehabilitation unit of the I.R.C.C.S. Fondazione S. Maugeri in Pavia (Northern Italy) with a diagnosis of cardiac complications, septic shock, pneumonia and preputial edema.
The man had been previously admitted to hospital in February 2013, at the Acute Care Hospital I.R.C.C.S. S. Matteo of Pavia, were he underwent a heart transplantation.
On 1 August 2013 *Candida glabrata*, *Pseudomonas aeruginosa*, *K. oxytoca* and *C. koseri* were also isolated from both preputial swab and urine samples of the patient. The patient was then treated with colistin in monotherapy (EV 1.000.000 U 4/*die*). After the antibiotic therapy, both the samples resulted negative for the three bacterial species previously identified.
Species identification and susceptibility testing were carried out using the MicroScan AutoSCAN4 automated-system (Beckman Coulter).
Ertapenem (ETP), meropenem (MER) and imipenem (IPM) MICs were determined by Etest (bioMérieux); the results were interpreted according to EUCAST 2014 criteria (The European Committee on Antimicrobial Susceptibility Testing, Version 3.1, 2014). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were routinely included during testing for quality assurance.
The *K. oxytoca* and *C. koseri* strains were screened and than phenotypically confirmed for carbapenemase production by the Modified Hodge test (MHT) - using both ETP and IPM - and the KPC/MBL Confirm kit (Rosco Diagnostic).
Phenotypic ESBL and AmpC detection were performed with both the double disk synergy test (DD) (Jarlier, 1988), using piperacillin-tazobactam (TZP), cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ) and aztreonam (ATM), and with the ESBL + AmpC Screen kit (Rosco Diagnostic). The β-lactamase preliminary identification was performed by Isoelectricfocusing (IEF), as described elsewhere (Pagani *et al.,* 2002).
Crude sonic extracts from *E. coli* harbouring TEM-1 (pI, 5.4), SHV-2 (pI,, 7.6) and SHV-12 (pI,8.2) were used as Isoelectric point (pI,) markers.
Conjugal transfer of resistance determinants was performed in liquid medium using the *E. coli* K12 strain J62 (pro^-, his^-, trp^-, lac^-, Sm^r) and J53 (*met*, pro^-, Rif^r) as recipients. The initial donor/recipient ratio was 0.01.
The transconjugants were selected on McConkey agar containing CTX (8 mg/L) plus streptomycin (1000 mg/L) or rifampin (100 mg/L). Species identification and susceptibility testing of the obtained *E. coli* transconjugants were carried out by MicroScan AutoSCAN4 automated-system.
Transconjugants MICs against MER, ETP and IPM were determined by Etest (bioMérieux).
The presence of bla_{VIM}, bla_{IMP} and bla_{AmpC} genes was assessed by multiplex PCR analysis using the primers and the conditions described elsewhere (Rossolini *et al.,* 2008; Pérez-Pérez *et al.,* 2002; Hujer *et al.,* 2006; Koeleman *et al.,* 2001). PCR products were purified using the kit Quantum Prep PCR Kleen Spin Columns (BioRad) and subjected to double-strand sequencing (Macrogen Inc., Seoul, South Korea). The nucleotide sequences were analyzed according to

the BLAST program (http:/www.ncbi.nlm.nih.gov/blast/).
Plasmid DNA was extracted and purified by PureLink™ HiPure Plasmid Filter Purification Kits (Life technologies™).
Plasmids were subsequently typed according to their incompatibility group using the PBRT kit-PCR based replicon typing scheme, as described previously (Carattoli *et al.,* 2006).
The sizes of the plasmids were estimated using the S1 nuclease PFGE method (Barton *et al.,* 1995).

RESULTS

The antimicrobial susceptibility results by MicroScan System showed that both of *K. oxytoca* and *C. koseri* isolates were characterized by multi-drug resistance, retaining susceptibility only to fluoroquinolones, colistin and tigecycline, and showing resistance to amoxicillin-clavulanic acid (AMC), third generation cephalosporins (3GC), cefoxitin, trimethoprim-sulfamethoxazole and gentamicin (according to the 2014 EUCAST breakpoints).
The *C. koseri* isolate resulted resistant to ETP (MIC, >1 mg/L), IPM (MIC, >8 mg/L) and MER (MIC, >8 mg/L) by MicroScan System, while *K. oxytoca* was only ETP (MIC, >1 mg/L) and MER (MIC, >8 mg/L) resistant, showing a IPM MIC, 8 mg/L, using the same tool.
These values were not always coherent with those of the Etest for *K. oxytoca,* ETP MIC being >32 mg/L; IPM MIC, 6 mg/L but MER MIC, 0,75 mg/L, lower than expected. The values for *C. koseri* were consistent only in the case of the ETP MIC >32 mg/L; with IPM MIC, 1 mg/L,

TABLE 1 - *In vitro activity of selected antimicrobial agents tested against Citrobacter koseri, Klebsiella oxytoca and their transconjugants.*

Antimicrobial Agents	*[a]MIC mg/L*				
	Citrobacter koseri	*J53/J62R C. koseri*	*Klebsiella oxytoca*	*J53/J62R K. oxytoca*	*E. coli J53/J62*
Amikacin	≤8	≤8	16	≤8	≤8
Amoxicillin/clav	>8/4	>8/4	>8/4	>8/4	≤2/1
Ampicillin	>8	>8	>8	>8	≤2
Cefepime	4	4	8	4	≤1
Cefotaxime	>16	>16	>16	16	≤1
Cefpodoxime	>1	>1	>1	>1	≤1
Ceftazidime	>8	>8	>8	>8	≤1
Ciprofloxacin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cloramphenicol	>8	>8	>8	>8	≤8
Colistin	≤2	≤2	≤2	≤2	≤2
Ertapenem	>1	>1	>1	1	≤0.5
Fosfomicin	≤32	≤32	≤32	≤32	≤32
Gentamicin	>4	>4	>4	>4	≤2
Imipenem	>8	≤2	8	≤2	≤2
Levofloxacin	≤1	≤1	≤1	≤1	≤1
Meropenem	>8	≤2	>8	≤2	≤2
Moxifloxacin	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Piperacillin/tazobactam	>16	>16	>16	16	≤4
Piperacillin	-	>16	>16	16	≤4
Tetracyclin	>8	>8	>8	>8	≤4
Tigecycline	≤1	≤1	≤1	≤1	≤1
Tobramycin	>4	>4	>4	>4	≤2
Trimethoprim/sulfamethoxazole	>4/76	>4/76	>4/76	>4/76	≤2/38

MIC: Minimum Inhibitory Concentration; [a]According to European Committee on Antimicrobial Susceptibility Testing (EUCAST 2014) criteria: MIC values were determined using NBC46 card, AutoScan4 System (Beckman Coulter).

and a MER MIC lower than the expected, being equal to 0,5 mg/L.
MHT carbapenemase screening test showed positive results for both the isolates studied.
KPC/MBL Confirm kit and ESBL + AmpC Screen kit tests showed synergistic effect with both dipicolinic and boronic acid; this is typical of MBL and AmpC producers respectively.
The isolates were then further studied for β-lactamase production by biochemical and molecular assays.
Analytical IEF performed using crude enzymatic extracts from the clinical isolates and nitrocefin as chromogenic substrate, showed the presence of a unique β-lactamase band with pI 9.2.
Both of the *K. oxytoca* and *C. koseri* donor strains were able to transfer the resistance plasmid to *E. coli* K12 strain J62 (*pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$, *Sm*^{r}) and J53 (*met, pro-, Rif*^{r}) as recipients.
Transfer of CTX resistance was observed at a frequency of approximately 10^{-3} transconjugants per recipient from both *K. oxytoca* and *C. koseri*.
Compared to the recipient *E. coli* J53 and J62 strains, the transconjugants exhibited a decreased susceptibility to several β-lactams (including carbapenems), trimethoprim-sulphamethoxazole and aminoglycosides. The resistance phenotypes of transconjugants were similar to those of the donors *K. oxytoca* and *C. koseri* for the other antimicrobial agents tested (Table 1).
PCR analysis performed on both donors and transconjugant strains yielded positive results for the co-presence of the bla_{VIM} and bla_{CMY} determinants in all the isolates. Amplicons sequencing revealed that *K. oxytoca*, *C. koseri* and *E. coli* transconjugants carried both bla_{VIM-4} and bla_{CMY-4} genes.
Plasmid analysis showed that the above resistance determinants were located in a 150 kb conjugative plasmid belonging to the IncA/C incompatibility group. The IncP and IncN incompatibility groups were also observed in *K. oxytoca*.
The IncA/C multi-resistance plasmid, from the *E. coli* transconjugant, was characterized. PCR results showed that the plasmid contained two distinct resistant *loci* carrying the VIM-4 and CMY-4 β-lactamase genes, with bla_{VIM-4} found as the first gene cassette of a class 1 integron.

DISCUSSION

Although VIM-type carbapenemases have already been described as widely spread in *K. pneumoniae* in Italian rehabilitation hospitals (Nucleo E. *et al.,* 2013), to our knowledge this is the first report on the detection of MDR *K. oxytoca* and *C. koseri* clinical isolates co-producing VIM-4 and CMY-4 enzymes in Italy. The above clinical strains were both recovered from the preputial sample of a patient admitted to the cardiac rehabilitation unit of I.R.C.C.S. Fondazione S. Maugeri of Pavia.
The susceptibility profiles of the studied strains were coherent with carbapenemase production (MER MIC ≥0,5 mg/L), and phenotypic tests used for screening/confirmation of carbapenemase production yielded positive results. The co-presence of a CMY-type enzyme was suggested by IEF results (pI 9.2) and confirmed by PCR and sequencing.
CMY-4 enzyme differs from CMY-2 by one substitution (Arg for Trp at position 221) and from CMY-3 by two substitutions (Glu for Gly at position 42 and Ser for Asn at position 363). The deduced amino acid sequence is 98-99% identical to CMY-3 and to those of the plasmid-mediated AmpC-type β-lactamases originated from *C. freundii*.
The *K. oxytoca* and *C. koseri* MDR strains retained complete susceptibility to colistin. The administration of colistin monotherapy led to a positive outcome, with a complete resolution of the infection.
The bla_{VIM-4} and bla_{CMY-4} resistance genes were co-transferred to *E. coli* during conjugation. The high transfer frequency highlights the plasmid potential of diffusion and dissemination among susceptible isolates, also of different species.
The variability of plasmids mediating antimicrobial resistance in *Enterobacteriaceae* is high. There are plasmid families that are largely prevalent and also plasmids prevalently associated with specific resistance genes. The IncFII, IncA/C, IncL/M and IncI1 plasmids showed the highest occurrence among typed resistance plasmids. These plasmids can be considered "epidemic", being detected in different countries, and in bacteria of different origins and sources. The occurrence of these plasmid types

seems closely linked to the selective pressure exerted by antimicrobial use, incrementing their prevalence compared to that observed in bacterial populations that are not preselected for antimicrobial resistance.

Incompatibility group IncA/C plasmids are large, low copy, plasmids that have been described in the literature for over 40 years. However, they have only recently been intensively studied at the genomic level because of their association with the emergence of multi-drug resistance in enteric pathogens of humans and animals. These plasmids are unique among other enterobacterial plasmids in many aspects, including their modular structure and gene content.

Circulation of IncA/C plasmids in Gram-negative pathogens is now common, and these plasmids bring with them the ability to encode resistance to broad arrays of antimicrobial agents (Johnson *et al.,* 2012).

IncA/C plasmids carrying both the *bla*$_{VIM-4}$ and *bla*$_{CMY-4}$ genes were already identified in Italy in clinical isolates of *K. pneumoniae* and *E. cloacae* (Luzzaro *et al.,* 2004). The scaffolds of these plasmids were similar to those of the IncA/C plasmids carrying *bla*$_{CMY-4}$ or *bla*$_{CMY-2}$ from *Salmonella enterica* isolated in United States and the United Kingdom, but the carbapenemase gene was not present on these *Salmonella* plasmids and likely represents a novel acquisition for the IncA/C plasmids (Carattoli, 2009).

The emergence of carbapenemases in *K. oxytoca* and *C. koseri* poses major clinical problems. The coexistence in the same bacterial cell of a plasmid carrying epidemiologically important emerging resistance genes is worrisome, since it could predict the generation and the spread of pan-resistant bacteria and the consequent treatment option limitations that can lead to significant morbidity and mortality (Luzzaro *et al.,* 2004).

This report demonstrates that the problem of MBL-producing pathogens no longer entails Gram-negative non-fermenters alone but also involves enterobacteria. Moreover, this case study confirms the need for continuous monitoring for other β-lactamases genes (i.e. AmpC cephalosporinases) that can co-exist in carbapenemase producers, and underline the importance of an active surveillance on the trends of ESBL and acquired AmpC enzymes among species which can cause infections in immuno-compromised hosts (Zàrate et al., 2008). Surveillance may be of value in the difficult battle against life-threatening bacterial infections.

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