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In vivo acquisition and risk of inter-species spread of bla_{KPC-3}-plasmid from *Klebsiella pneumoniae* to *Serratia marcescens* in the lower respiratory tract

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Abstract

In recent years, *Serratia marcescens* has emerged as an important agent of hospital-acquired infections, such as pneumonia, urinary tract infection, septicaemia and meningitis, particularly in vulnerable patients. Compared to *Klebsiella pneumoniae* and *Escherichia coli*, *S. marcescens* is less commonly associated with bla_{KPC} genes, yet few cases of plasmid transmission at the gastrointestinal level from *K. pneumoniae* carbapenemase (KPC)-producing *Enterobacterales* to *S. marcescens* have been described. Here we report a case of *in vivo* acquisition, during a 3-month period of hospitalization in the intensive care unit, of a bla_{KPC-3} gene carried by a pKpQIL-IT plasmid, and its probable transmission at the bronchial level among different species of *Enterobacterales*, including *K. pneumoniae* and *S. marcescens*. By using whole genome sequence analyses we were able provide insight into the dynamics of carbapenem-resistance determinants acquisition in the lower respiratory tract, a novel anatomical region for such plasmid transmission events, that usually involve the gastrointestinal tract. The co-presence at the same time of both wild-type and resistant *Enterobacterales* could have been the critical factor leading to the spread of plasmids harbouring carbapenem-resistance genes, of particular importance during surveillance screenings. The possibility of such an event may have significant consequences in terms of antimicrobial treatment, with a potential limitation of therapeutic options, thereby further complicating the clinical management of high-risk critically ill patients.

Serratia marcescens has traditionally been regarded as an opportunistic pathogen, as it tends to colonize the respiratory and urinary tracts [1]. However, in recent years a growing amount of evidence has demonstrated that it has effectively adapted to hospital environments, and has emerged as an important agent of hospital-acquired infections such as pneumonia, urinary tract infections, septicaemia and meningitis, particularly in vulnerable populations [2].

Like other members of *Enterobacterales*, *S. marcescens* carries both chromosomal and plasmid-mediated resistance determinants for several antimicrobial agents, including beta-lactams and colistin. Compared to *Klebsiella pneumoniae* and *Escherichia coli*, the acquisition of carbapenem-hydrolysing activity by plasmid bla_{KPC} gene trasmission in *S. marcescens* has rarely been described. Nevertheless, when the transmission from *K. pneumoniae* carbapenemase (KPC)-producing *Enterobacterales* to *S. marcescens* was documented, it occurred at the gastrointestinal level [3–7].

However, colonization by *Enterobacterales* at the respiratory level in critically ill hospitalized patients could lead to alternative localization for transmission of resistance determinants. In the respiratory tract, having a resident flora susceptible to

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Abbreviations: CRE, carbapenem-resistant *Enterobacterales*; E. coli, *Escherichia coli*; ICU, intensive care unit; KPC, *K. pneumoniae* carbapenemase; KPC-Ec, KPC-producing *Escherichia coli*; KPC-Kp, KPC-producing *K. pneumoniae*; *K. pneumoniae*, *Klebsiella pneumoniae*; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MIC, Minimum inhibiting concentration; ML, maximum-likelihood; MSLT, Multilocus sequence typing; S. marcescens, *Serratia marcescens*; SNPs, single nucleotide polymorphisms; WGS, whole genome sequencing.
Genomic sequences obtained in the present study can be retrieved under the following accession numbers: ERS2643221–ERS2643228.
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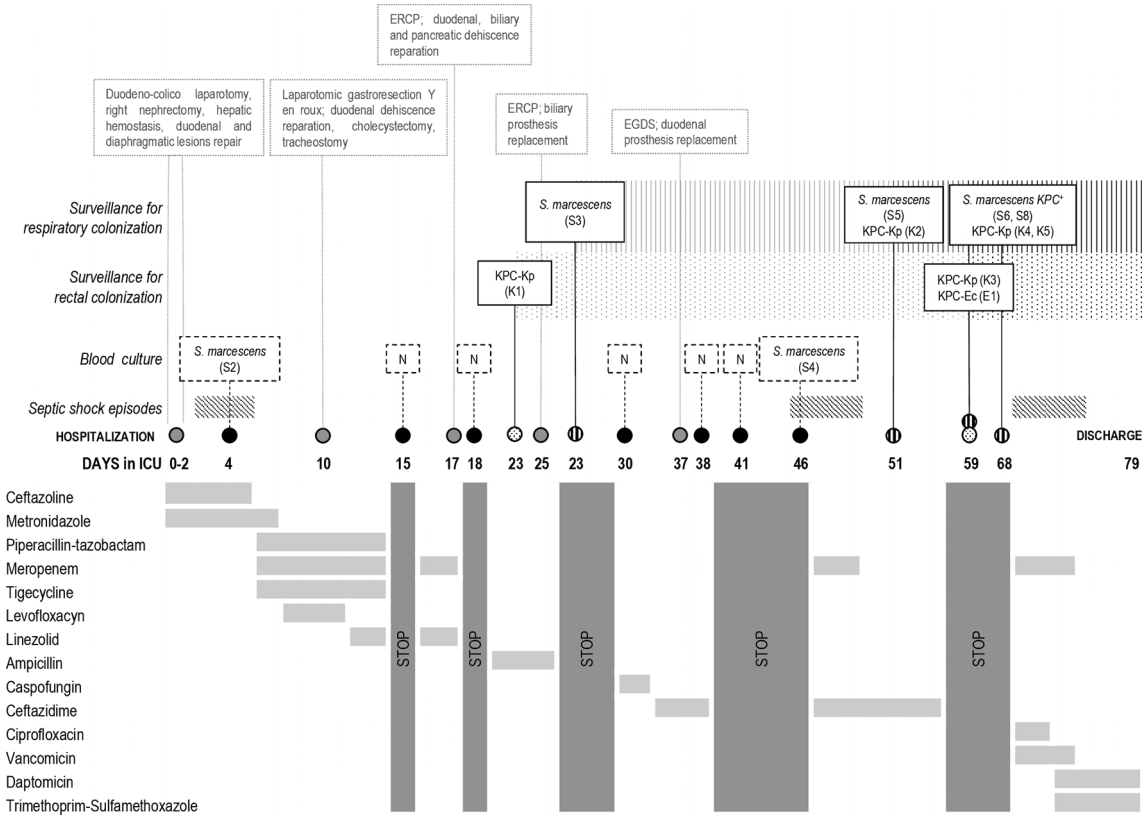

Fig. 1. Timeline clinical history of the case patient. All surgical procedures performed during hospitalization in the ICU (grey circles), along with results of microbiological surveillance (dotted or striped circles) and blood cultures (black circles), frequency of septic shock episodes and full antibiotic treatment history are reported. Periods of antibiotic treatment discontinuation are reported as 'STOP'. All relevant isolated microorganisms are reported. ERCP, endoscopic retrograde cholangiopancreatography; EGDS, oesophagogastroduodenoscopy; ICU, intensive care unit; KPC-Ec, *Klebsiella pneumoniae* carbapenemase – *Escherichia coli*; KPC-Kp, *Klebsiella pneumoniae* carbapenemase – *Klebsiella pneumoniae*; N, negative.

resistance acquisition, especially if associated with recurrent bacteraemia, as in *S. marcescens* [8], could complicate the clinical management and surveillance of critically ill patients, mainly in intensive care units (ICUs). In this regard, our case report retrospectively describes the *in vivo* acquisition of a bla_{KPC-3} gene carried by a pKpQIL-IT plasmid and its probable transmission among different species of *Enterobacterales*, including *S. marcescens*, at the bronchial level in a single patient, during a 3-month ICU hospitalization. This is a non-interventional case report, and the clinical management of the patient presented here corresponded to standard hospital clinical practice. Medical records were registered and processed by using pseudoanonymization measures, ensuring that it did not (and no longer) permit the identification of data subjects. According to the applicable relevant national legislation and local rules, specific informed consent was not mandatory.

On day 0, an adult man was admitted to the Emergency Room for haemorrhagic shock, pleural effusion and severe kidney injures due to stab wounds. Fig. 1 shows the timeline progression of the patient's clinical history with indications of most relevant collected samples, and all administered therapy. After intensive abdominal surgical procedures (duodeno-colico laparotomy, right nephrectomy, hepatic haemostasis, duodenal and diaphragmatic lesion repair), he was transferred to the ICU. Upon ICU admission, surveillance screening on selective chromogenic medium (chromID CARBA; bioMérieux) for nasal, pharyngeal (eSwab; Copan) or rectal (fecalSwab; Copan) colonization by carbapenem-resistant *Enterobacterales* (CRE) was negative, and the patient was thus started on treatment with ceftazoline and metronidazole.

On day 3, the patient was in septic shock and multi-organ failure. Microbiological cultures from the abdominal surgical wound, which appeared largely necrotic, led to the isolation of an *S. marcescens* strain (isolate S1) that was also detected in blood 1 day later (isolate S2). Identification of microorganisms was performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS with MALDI Biotyper system (Bruker Daltonics).

Susceptibility profiles of both isolates (MicroScan WalkAwayPlus automated system; Beckman Coulter) were identical. MIC values according to EUCAST v.7.1 breakpoints

(http://www.eucast.org/clinical_breakpoint/) revealed that both strains S1 and S2 had reduced susceptibility to aztreonam (MIC=16 μg ml^{-1}), ceftazidime (MIC>16 μg ml^{-1}) and piperacillin-tazobactam (MIC=64 μg ml^{-1}), but were susceptible to amikacin (MIC≤8 μg ml^{-1}), ciprofloxacin (MIC≤0.5 μg ml^{-1}), gentamicin (MIC≤2 μg ml^{-1}), ertapenem (MIC≤0.5 μg ml^{-1}), imipenem and meropenem (MIC≤1 μg ml^{-1}).

After therapy adjustment to levofloxacin, tazocin, meropenem and tigecycline, follow-up blood culture yielded no microbial growth. Yet, during the following 2 months of ICU stay, the patient experienced repeated dehiscence of intestinal anastomosis that required surgical re-interventions, along with multiple episodes of septic shock, and consequent adjustments to his antibiotic treatment (Fig. 1).

Surveillance screening for rectal and/or respiratory colonization was persistently negative until day 23, when an *S. marcescens* isolate (S3) was recovered from a respiratory sample, and a *K. pneumoniae* strain (K1) was isolated from a rectal swab. The *S. marcescens* S3 isolate showed no evidence of genetic resistance to carbapenems, and a phenotypic profile that was identical to previous abdominal (S1) and blood (S2) isolates.

On the other hand, the K1 strain was a KPC-producing *K. pneumoniae* (KPC-Kp; Table 1), as determined by GeneXpert CARBA-R assay (Cepheid). This rectal colonization by KPC-Kp persisted through the entire ICU stay, and was further complicated by the isolation of a KPC-producing *E. coli* (KPC-Ec, E1) on day 59.

At the respiratory level, simultaneous colonization by KPC-Kp (K2, K4 and K5 isolates) and *S. marcescens* (S5, S6 and S8) strains was detected after day 51. Notably, before respiratory colonization by KPC-Kp occurred, the *S. marcescens* strain recovered at this level (S3) was fully susceptible to carbapenems. By contrast, isolate S5 started to show reduced sensitivity against ertapenem, and a week later the S6 *S. marcescens* strain was also resistant to imipenem (Table 1 and Fig. 1).

No evidence of systemic dissemination of this KPC-producing *S. marcescens* was found in our patient, as in all positive blood cultures *S. marcescens* strains presented the same phenotypic

Table 1. Phenotypic and genetic features of clinical isolates found in the case patient

Isolate ID	Isolated bacteria	Days of stay in ICU	Specimen type	Carbapenems MIC (μg ml^{-1})			Whole genome sequencing	
				ERT	IMP	MER	Carbapenemase/ESBL genes	Resistance determinants
S1	*S. marcescens*	3	Surgical wound pus	≤0.5	≤1	≤1	Not performed	
S2	*S. marcescens*	4	Blood culture	≤0.5	≤1	≤1	None	*tet41*, *aac(6')-Ib-cr*
S3	*S. marcescens*	23	Bronchial aspirate	≤0.5	≤1	≤1	Not performed	
K1	*K. pneumoniae*	23	Rectal swab	>1	>8	4	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1D}, bla_{SHV-11}	*aac (6)-Ib, oqxA, oqxBgb*
S4	*S. marcescens*	46	Blood culture	≤0.5	≤1	≤1	None	*tet41*, *aac(6')-Ib-cr*
K2	*K. pneumoniae*	51	Bronchial aspirate	>1	>8	>8	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1D}, bla_{SHV-11}	*aac (6)-Ib, oqxA, oqxBgb*
S5	*S. marcescens*	51	Bronchial aspirate	>1	≤1	≤1	None	*tet41*, *aac(6')-Ib-cr*
K3	*K. pneumoniae*	59	Rectal swab	>1	>8	4	Not performed	
E1	*E. coli*	59	Rectal swab	>1	4	4	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1D}	*sul1, aadA5, dfrA17*
K4	*K. pneumoniae*	59	Bronchial aspirate	>1	>8	>8	Not performed	
S6	*S. marcescens*	59	Bronchial aspirate	>1	>8	2	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1D}	*tet41*, *aac(6')-Ib-cr*
S7	*S. marcescens*	66	Venous catheter pus	>1	>8	2	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1D}	*tet41*, *aac(6')-Ib-cr*
S8	*S. marcescens*	68	Bronchial aspirate	>1	>8	≤1	Not performed	
K5	*K. pneumoniae*	68	Bronchial aspirate	>1	>8	>8	Not performed	

ERT, ertapenem; ESBL, extended-spectrum β-lactamase; ICU, intensive care unit; IMP, imipenem; MER meropenem; MIC, minimum inhibitory concentration.

profile to the first S1 isolate, fully susceptible to carbapenems (Table 1). On day 66, a KPC *S. marcescens* strain (S7) was isolated in a venous catheter sample, but its recovery was not related to any systemic clinical manifestation during follow-up.

After 3 months from admission, the patient was discharged from the ICU in stable clinical conditions, with no ongoing septic events.

To better clarify the transmission dynamics of carbapenem resistance, the *S. marcescens* isolates from blood (S2, S4), bronchial aspirate (S5, S6) and venous catheter (S7) samples, as well as the KPC-Kp (K1, K2) and KPC-Ec (E1) strains were analysed by whole genome sequencing (WGS), performed with the Illumina MiSeq platform. Briefly, 1 ng of total DNA extracted using the DNeasy Blood and Tissue kit (Qiagen) was processed using the Nextera XT kit and the obtained library pool was loaded on an Illumina MiSeq sequencer running 250 bp paired-end reads (Illumina). The reads were assembled by the SPAdes Genome Assembler [9] and antibiotic resistance genes were identified using SRST2 [10] and ARG-ANNOT [11] databases. Multilocus sequence typing (MSLT) profiles were determined *in silico*: for *K. pneumoniae* using gene variants retrieved from BIGSdb (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) and for *E. coli* using the Wirth scheme (https://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). For each *S. marcescens* isolate the reads were aligned against the Db11 reference strain (GCA_000513215.1) and single nucleotide polymorphisms (SNPs) were called using the GATK best practice procedure [12]. For CoreSNP calling, the reference genome regions including repeats or phages, identified using MUMmer [13] and PhiSpy [14] respectively, were masked. The obtained CoreSNP alignment was then subjected to maximum-likelihood (ML) phylogenetic analysis using RaxML8 software [15], after best model identification performed using ModelTest-NG [16]. A phylogenetic reconstruction with RaxML8 software was also performed for the K1 and K2 *K. pneumoniae* isolates.

Phylogenetic analysis showed that all sequenced *S. marcescens* isolates except S2, the first collected from blood culture, clustered together (data not shown). Isolate S2 differed by 435–444 SNPs from the other strains, which instead showed a maximum distance of 10 SNPs among each other. This supported the spread of a single bacterial strain, either acquired after S2 isolation, or present as a mixed clone in the original *S. marcescens* population, not initially isolated but subsequently becoming dominant.

By contrast, both KPC-Kp from rectal (K1) and respiratory (K2) specimens were clonally related and were of ST512, while the KPC-Ec strain (E1) belonged to ST69, a well-known extra-intestinal pathogenic clone.

All *S. marcescens* isolates harboured *tet41* and aac(6')-Ib-cr resistance genes, again supporting a common genetic background. However, the S6 (brochial aspirate) and S7 (venous catheter) strains that showed phenotypic resistance to carbapenems also presented the $bla_{\text{KPC-3}}$, $bla_{\text{OXA-9}}$ and $bla_{\text{TEM-1D}}$ genetic determinants, the same as detected in KPC-Kp and KPC-Ec. Using the PlasmidFinder database (https://cge.cbs.dtu.dk/services/PlasmidFinder/), an IncFIB_pQil plasmid showing >99% identity to plasmid pKpQIL-IT (accession number JN233705) was found in all KPC-producing isolates. No plasmids were detected in carbapenem-susceptible *S. marcescens* strains.

Co-infections with various carbapenem-resistant strains of Gram-negative species represent a critical point in clinical microbiology, as movement of plasmids and other mobile genetic elements may also occur at the inter-species level, including *K. pneumoniae* and *S. marcescens* [6]. Although this is well known, the majority of these data are generally linked to transmission occurring within the gastrointestinal tract.

In our patient, the sequence of microbiological evidence suggests that the patient initially acquired a KPC-producing *K. pneumoniae* in the gastrointestinal tract, but the transmission of the resistant plasmid actually occurred in the lower respiratory tract. Indeed, the lack of such plasmidic genetic determinants ($bla_{\text{KPC-3}}$, $bla_{\text{OXA-9}}$, $bla_{\text{TEM-1D}}$) in the first bronchial *S. marcescens* isolate (S5), and the subsequent development of $bla_{\text{KPC-3}}$-related carbapenem resistance in the second bronchial isolate (S6), after co-localization with a $bla_{\text{KPC-3}}$-producing KPC-Kp harbouring a plasmid with the same incompatibility group, suggests strongly the inter-species transmission of this form of drug resistance at the respiratory level. Although not conclusively demonstrated, because we did not evaluate the transconjugant clones by conjugational transfer experiments [7], this hypothesis is further supported by the strict phylogenetic relatedness of strains S5 and S6, excluding the respiratory acquisition of a second, KPC-producing, *S. marcescens* strain during hospitalization.

Although the presence of respiratory colonization did not lead to episodes of bacteraemia by carbapenem-resistant S. *marcescens* in our patient, the recovery of a $bla_{\text{KPC-3}}$-producing strain at the level of the venous catheter, closely related phylogenetically to the bronchial population, supports the tendency of this bacterium to extend colonization also outside the lower respiratory tract. As *S. marcescens* already has intrinsic resistance to several antimicrobial agents, including some classes of beta-lactams and tetracyclines, quinolones and aminoglycosides, the possible acquisition of plasmids harbouring carbapenem-resistance genes within multiple anatomical reservoirs may further limit the therapeutic options to treat this pathogen, with potentially crucial consequences, especially in critically ill patients.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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