Successful treatment and digestive decolonization of a patient with osteitis caused by a Carbapenemase-producing Klebsiella pneumoniae isolate harboring both NDM-1 and OXA-48 enzymes

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Running title: double carbapenemase-producing *K. pneumoniae* in osteitis

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Total <3

Table: 1

Figures: 2

Summary

Objectives: Carbapenem resistance in *Klebsiella pneumoniae* (CRKP) is an increasing problem worldwide and infections caused by this bacterium can be difficult to treat. Here we reported the case of a patient from Romania hospitalized in Bulgaria after an accident trauma that came in France for the treatment of an osteitis caused by a *K. pneumoniae* carrying both bla_{NDM-1} and bla_{OXA-48}.

Method: The resistome of this extremely-drug-resistant bacterium was analyzed both with phenotypic (large antibiotic susceptibility testing) and genomic method (genome sequencing). The genetic environment of the two carbapenemases was studied.

Results: *K. pneumoniae* ST307 carrying both a bla_{NDM-1} gene and a bla_{OXA-48} gene located on two different plasmids, an Inc L/M and an IncFII. Patient was successfully treated by a combination of intravenous colistin (9 MUI, then 4.5 MUI 2 times/day), intravenous fosfomycin (4 g 3 times/day) and oral doxycycline (100 mg 2 times/day) for 3 months. Fecal microbiota transplantation was successfully conducted for a stool carriage.

Conclusion: The ST307 type is becoming endemic in hospital environment and is frequently associated with carbapenem resistance. Treatment of infection caused by multi-drug resistant bacteria are a clinical challenge and the use of old antibiotics associated with a screening and decolonization of the reservoirs can be an efficient therapeutic alternative.

1. Introduction

Carbapenemase producing *Enterobacteriaceae* (CPE) have become, in the last decade, a major concern worldwide, particularly in healthcare settings (1). Carbapenemases are the most powerful β-lactamases, being able to hydrolyze almost all β-lactams. Of all the carbapenemases, the OXA-48 carbapenemase is currently the one that is spreading the most rapidly in many European countries (1). In France, the first OXA-48-producing isolate was a *Klebsiella pneumoniae* identified in Paris in 2009 from the sputum of a Tunisian patient (2). Subsequently, OXA-48-producing *Enterobacteriaceae* isolates were found in patients transferred from countries around the Mediterranean sea (1), causing large hospital outbreaks in western-European countries (1).

Otherwise, isolates containing New Delhi metallo-β-lactamase (NDM-1) were circulating in India as early as 2006, two years before the first European case was identified (3). Since 2008, there has been repeated import of NDM-1-positive bacteria from the Indian subcontinent to Europe, in addition to being endemic in the Middle East, Northern Africa, and the Balkans (3). The first identification of NDM-1 in France was in 2009, corresponding to an imported *Escherichia coli* isolate from India (4). Two years later, the first reported case of community acquired NDM-1 was identified in southern France, highlighting the risk of autochthonous acquisition (5).

We report here the case of a patient who traveled for medical care from Bulgaria to France. This patient had an osteitis caused by a carbapenemase-producing *K. pneumoniae* harboring both bla_{NDM} and bla_{OXA-48} genes. He also had a stool carriage of a *K. pneumoniae* that was also carrying the two genes. Genome of this extremely-drug-resistant isolate was sequenced and analyzed. The management of the infection included surgical and antibiotic treatment and a fecal microbiota transplantation which are reported here.

2. Material and methods

2.1. Case report

At the end of 2015, a 43-year-old man was admitted to our hospital in the south of France suffering from septic pseudarthrosis of his left arm and left femur. Three years before, in Romania, the patient had had a car accident that resulted in both left humeral and open left femoral fractures. He underwent a humeral plate osteosynthesis and external fixation of his femur. Fifteen days later, the patient was transferred to a tertiary hospital in Bulgaria for fever and suppuration of the leg wound (Figure 1). He then underwent a second surgical intervention for debridement and external fixation replacement. Two months before his admission in France, due to an infection of the pin site, a second external fixation replacement was performed. Despite several lines of empirical antibiotic therapy, the infection persisted, and the patient decided to travel to France for medical care. In our hospital, all osteosynthesis material was removed and bone samples were taken and cultured in the laboratory with standard procedures.

2.2. Microbiological procedures

Samples were inoculated on blood agar medium (Biomérieux, Marcy l'Etoile, France) and chocolate polyvitex agar under aerobic atmosphere at 37°C for 48h. Additionally, one blood agar plate was inoculated under anaerobic conditions for 10 days at 37°C. Screening for stool carriage was done using the ChromeID CARBA SMART medium (Biomérieux, France). Bacterial identification was performed by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) as previously described (6). Antimicrobial susceptibility testing testing was performed according the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendation. Minimum Inhibitory Concentration (MIC) of tigecycline and minocycline, doxycycline and Imipenem were determined using E-test (Biomérieux, France) while colistin MIC was obtained using the UMIC microdilution method

(Biocentric, Bandol, France). Real-time PCR of the carbapenemase genes (*bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{KPC}$) was performed on every strain. Conjugation test was performed in an azide-resistant *E. coli* J53 strain. Transconjuguants selection was done on Luria Bertani agar (Beckton Dickinson, Le Pont de Claix, France) supplemented with 120µg/mL sodium azide and 4µg/mL ertapenem. Carbapenemases PCR performed on transconjuguants confirmed the presence of the two carbapenemase genes.

2.3. Genome sequencing and analysis

The genome of one multi-drug-resistant (MDR) *K. pneumoniae* KP_DC isolated from the articular liquid (GenBank accession no. NJGM00000000) was sequenced by Miseq technology (Illumina Inc, San Diego, CA, USA) with a paired-end strategy. Genome was assembled with A5 software (7), aligned with Mauve (8) to the reference strain ATCC43816 KPRR (genbank accession number CP009208.1) and annotation was performed using Prokka (9) and Arg-annot (10) for the research of antibiotic resistance genes. Plasmid were found using PlasmidSeeker (11) and then reconstitute by mapping the reads of our genome with the reference sequence found with PlasmidSeeker using CLC Genomics Workbench version 7.5 (Qiagen, Hilden, Germany). The FAB formula of the IncF plasmid was determined using the Center for Genomic Epidemiology platform (https://cge.cbs.dtu.dk/).

3. RESULTS

Both bone biopsies from the humerus and the femur were positive for a carbapenemase-producing *K. pneumoniae*. Screening for stool carriage also found a carbapenemase-producing *K. pneumoniae.* All isolates remained susceptible to fosfomycin, nitrofurantoin, tigecycline, minocycline and colistin (Table 1). All these isolates harbored both NDM and OXA-48 genes, confirmed by PCR (Table 1). We started a treatment combining intravenous colistin (9 MUI, then 4.5 MUI 2 times/day), intravenous fosfomycin (4 g 3 times/day) and

oral doxycycline (100 mg 2 times/day) for 3 months (Figure 1). No adverse effects were observed during the treatment. The kidney and liver functions, which were normal before treatment, remained unchanged. Fecal microbiota transplantation (FMT) was performed on day 10 of hospitalization in our ward. In brief, as previously described (12), an anonymous, fully screened, stool donor was used for FMT. The patient was administered a bowel lavage followed by four doses of oral gentamicin (100 mg) and colistin (2.5 MIU) over 24 h prior to FMT. Fifty grams of donor stool was homogenized and diluted in 0.9% NaCl, and 400 mL was administered by nasogastric tube. No adverse events were observed. The patient was placed under contact precaution until three consecutive weekly collected stool samples were negative for carbapenemase-producing isolate. Control stool samples were still negative 12 months later. The patient recovered with bone consolidation and wound healing after a 12-months follow-up.

The genome of the *K. pneumoniae* KP_DC isolate was assembled into 71 contigs with lengths ranging from 919 to 733,430 bp and a GC content of 57.4 %. 92.3% was found to be genomic DNA but 7.4% of the contig did not map with the reference strain *K. pneumoniae* MGH78578 (NC_009648.1). *In silico* Multi Locus Sequence Typing (MLST) showed that this strain belonged to the ST307 type. MLST sequencing of the four other isolates with no available genome shows they also belonged to ST307. An IncFII and an IncL/M plasmids were found using PlasmidSeeker (11). Conjugation tests were positive for the two carbapenemase genes as well as for $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}28}$ genes.

The IncL/M conjugative plasmid that harbored the $bla_{OXA\text{-}48}$ gene was 61,682 bp length, with an average G+C content of 51.02% (p2G1140) (Figure 2a). Comparison with the reference plasmid pOXA-48 (accession number JN626286) found 98% identity and 98% query coverage. The genetic environment of the $bla_{OXA\text{-}48}$ gene includes a *Tn1999*-like transposon inserted within a *tir* gene, flanked on both sides by a direct repeat sequence of 9 bp

143 (CGTTCAGCA). In the 3'-5' direction from the *bla*$_{OXA-48}$ gene, we found the usual *mucB-*
144 *mucA-pemK-pemI* gene pattern. The *Tn1999*-like transposon was flanked on either side by
145 two imperfect insertion sequences (IS): two copies of IS10A on the left and both IS10A and
146 IS1 on the right (Figure 2a).
147 The conjugative IncFII plasmid (p1G1140) was a IncFII Y4:A-:B36 carrying a *bla*$_{NDM-1}$ gene
148 (Figure 2b). This plasmid shared 99% cover and 99% identity with the reference plasmid
149 (pRJF866) for a 110,787 bp length, with an average G+C content of 54.72%. The genetic
150 environment of *bla*$_{NDM-1}$ gene was made by two IS5 genes with several insertion sequences on
151 each extremity. This transposon also carried a dihydropteroate synthase (Sul1) involved in
152 sulfonamide resistance, a 16S rRNA methyltransferase (RmtC) responsible for
153 aminoglycoside resistance and a *ble*$_{MBL}$ gene leading to bleomycin resistance (Table 1).

154 **4. DISCUSSION**

155 Prior surgery and extended hospital stays in countries with high levels of antimicrobial
156 resistance, as well as the presence of wounds, are recognized as risk factors for MDR
157 organisms acquisition (1,13). Antibiotic selection pressure may be an additional factor that
158 influences colonization with these organisms. In a recent case-control study, prior use of
159 piperacillin-tazobactam, a carbapenem, a quinolone, or metronidazole was significantly
160 associated with infections caused by carbapenemase-producing enterobacteria (14). However,
161 the cumulative number of prior antibiotic exposures appears to be more critical than the use of
162 a specific class of antibiotics (13). This highlights the importance of screening the digestive
163 colonization by MDR organisms directly upon admission to hospitals for high risk patients,
164 especially in patients who have received healthcare in endemic countries or epidemic facilities
165 (13).
166 Old antibiotics (*i.e.*, colistin, fosfomycin, tetracyclines, mecillinam, temocillin, thiamphenicol,
167 pristinamycin...) have been increasingly reused in the last few years, with rising numbers of

clinical studies evaluating their efficacy for the treatment of multidrug-resistant bacterial infections, and pharmacokinetic/pharmacodynamic studies reassessing their optimal dosing (15). But despite the evidence that these old antibiotics are still effective, mostly available as generics, they are not universally marketed (16). Our patient was successfully treated with Fosfomycin despite the presence of a fosfomycin resistance gene *fosA* in the genome of the sequenced isolate. The *fosA* gene is widely distributed in the *Klebsiella pneumoniae* species and conferred a Fosfomycin MIC to approximatively 24 mg/L, allowing its use in clinical practice (MIC cut-off according to EUCAST guidelines = 32 mg/L)

It has been shown that stool carriage of CPE could be extended for as long as 40 months (17). Fecal microbiota transplantation has been proposed as an efficient way of reducing the duration of colonization by CPE, as it has emerged as therapy for MDR bacterial decolonization (12). It was successfully used in our case, since fecal samples were still negative one year later.

The ST307 appeared in literature in 2013 from strains isolated from clinical samples between 2007 and 2010 in Texas but recent analyzes showed that this clone emerged in the mid-1990s and spread worldwide (18). This clone has been frequently associated with ESBL and carbapenemase genes (18) and is becoming prevalent in hospital environment.
Double carbapenemases-producing bacteria have been increasingly reported in the world (19). This acquisition of more than one carbapenemase seems to increase MIC of imipenem (19), limiting its use even in synergistic association with another antibiotic. However, the presence of a double carbapenemase can have no impact on Imipenem MIC (19), as it depends on the expression level of the carbapenemases. This “arm race” is worried to concern and must be detected by microbiological screening.

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Declarations

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Competing Interests: None

Ethical Approval: Not required

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TABLES AND FIGURES

Number of tables: 1

Table 1. Antibiotic susceptibility testing of the five strains of *Klebsiella pneumoniae* isolated in the per-operatory samples and in stool.

Number of figures: 2

Figure 1. Timeline of osteoarticular infection in our patient.

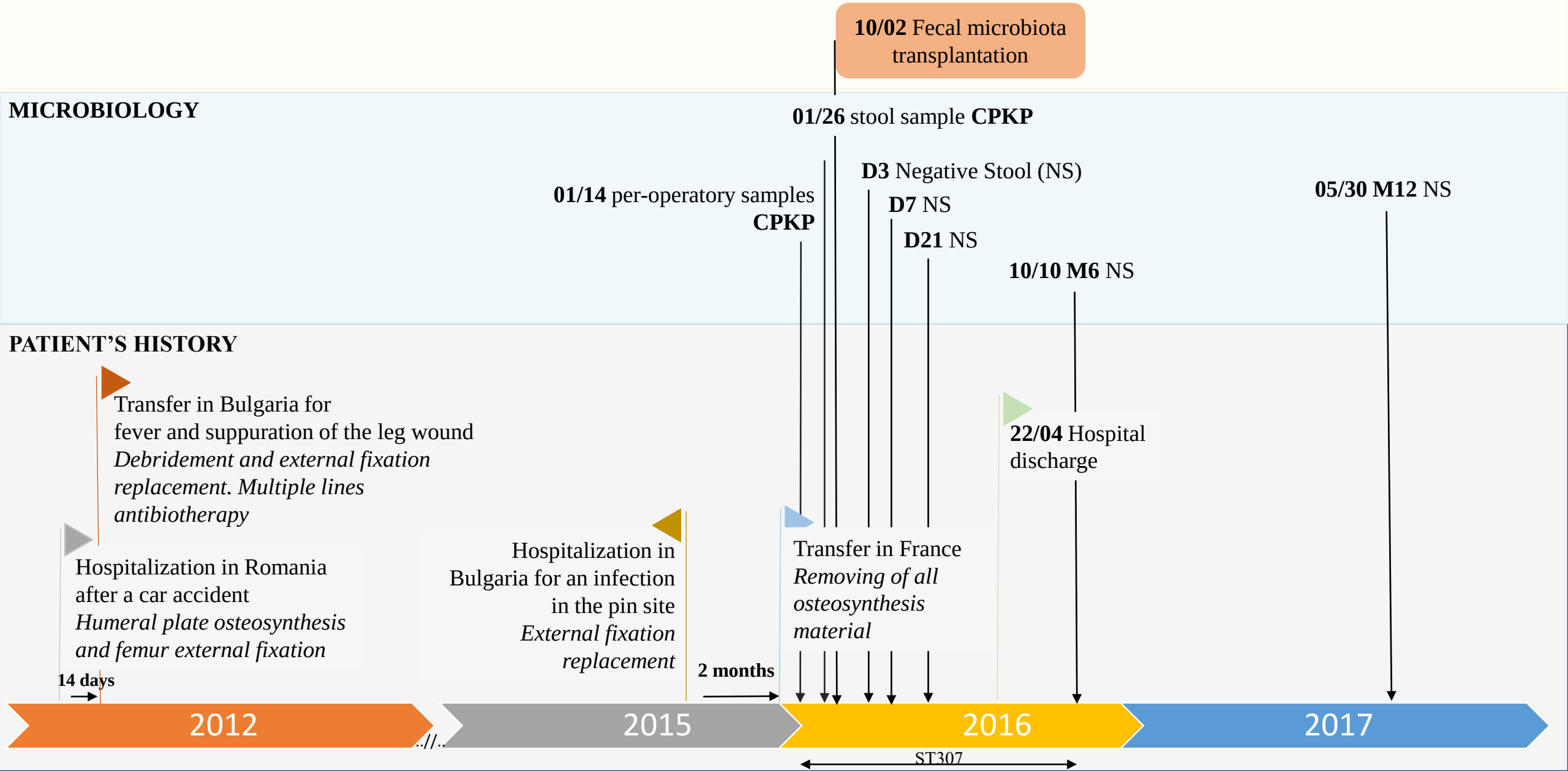
Figure 2. Representation of plasmids carrying carbapenemase genes compared to plasmids of reference. a**.** Genetic environment of the plasmid-mediated $bla_{oxa\text{-}48}$ gene and comparison with the reference plasmid pOXA-48 (accession number JN626286). b. Genetic environment of the plasmid-mediated $bla_{NDM\text{-}1}$ gene and comparison with the reference plasmid pRJF866 (accession number KF732966).

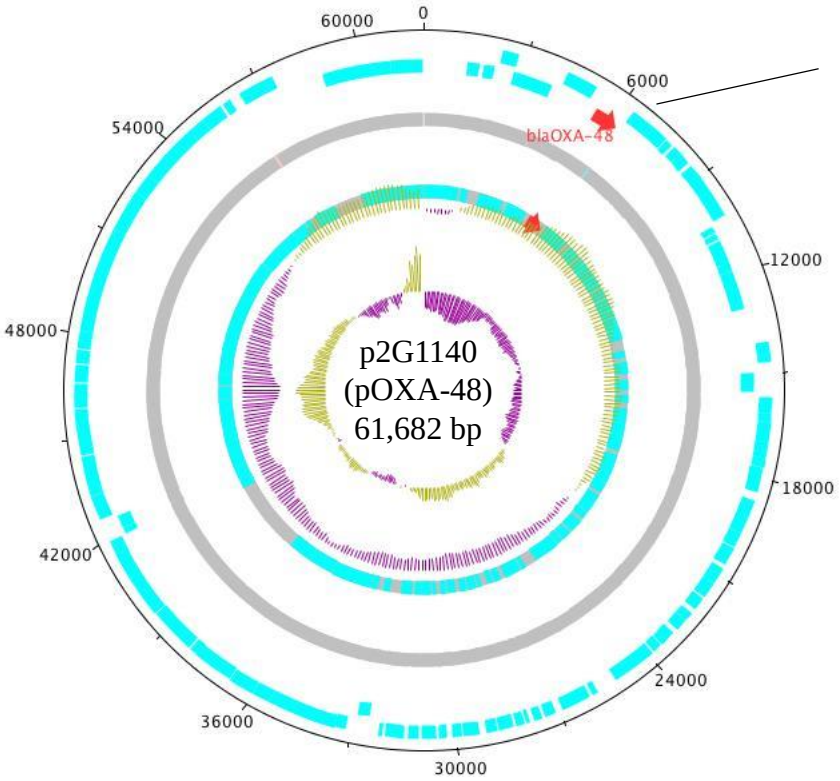
Table 1. Antibiotic susceptibility testing, resistance genes and MLST results of the five strains of *Klebsiella pneumoniae* isolated in the per-operatory samples and in stool.

Isolate number	**Strain 1**		**Strain 2**		**Strain 3 (genome)**		**Strain 4**		**Strain 5**	
Date of isolation	14/01/2016		14/01/2016		14/01/2016		14/01/2016		26/01/2016	
Nature of sample	Humeral bone biopsy 1		Humeral bone biopsy 2		Humeral bone biopsy 3		Femoral bone biopsy		Stool	
Antibiotic	**Ø (mm)**	**S/I/R**	**Ø (mm)**	**S/I/R**	**Ø (mm)**	**S/I/R**	**Ø (mm)**	**S/I/R**	**Ø (mm)**	**S/I/R**
Amoxicillin	0	R	0	R	0	R	0	R	0	R
Amoxicillin/Clavulanic acid	0	R	0	R	0	R	0	R	0	R
Piperacillin/Tazobactam	0	R	0	R	0	R	0	R	0	R
Ceftriaxone	0	R	0	R	0	R	0	R	0	R
Cefepim	9.9	R	9.9	R	9.9	R	10.4	R	10.8	R
Ertapenem	13.3	R	12.3	R	12.3	R	13.2	R	13.6	R
Imipenem	18	I	17.4	I	18	I	18.8	I	17.9	I
Gentamicin	13.6	R	0	R	0	R	13.8	R	0	R
Amikacin	0	R	0	R	0	R	12.7	R	0	R
Ciprofloxacin	10.3	R	16	R	15.9	R	0	R	16.5	R
Trimethoprime/Sulfamethoxazole	0	R	0	R	28.1	R	0	R	0	R
Fosfomycin	21.8	S	19.2	S	20.2	S	20.9	S	22.2	S
Nitrofurantoin	18.8	S	17.8	S	17.5	S	18.1	S	19.3	S
MIC Antibiotic (µg/mL)										
Colistin (microdilution method)	0.25	S	0.25	S	0.25	S	0.25	S	0.25	S
Imipenem (E-test)	8	I	8	I	6	I	1.5	S	8	I
Minocycline (E-test)	2	S	1.5	S	1.5	S	2	S	1	S
Tigecycline (E-test)	1.5	S	1	S	1	S	1.5	S	1	S
Doxycycline (E-test)	16	-	2	-	2	-	16	-	2	-
Resistome										
Carbapenems	*blaNDM-1* *blaOXA-48*		*blaNDM-1* *blaOXA-48*		*blaNDM-1* *blaOXA-48*		*blaNDM-1* *blaOXA-48*		*blaNDM-1* *blaOXA-48*	
Other ß-lactams	ND		ND		*bla*$_{CTX-M-15}$ *bla*$_{CTX-M-132}$ *bla*$_{SHV-28}$ *ampH*		ND		ND	
Aminoglycosides	ND		ND		*rmtC* *strB*		ND		ND	
Fluoroquinolone	ND		ND		*oqxA/ oqxB*		ND		ND	
Sulfamides	ND		ND		*Sul1*		ND		ND	
Fosfomycine	ND		ND		*fosA*		ND		ND	
Sequence Type[1]	307		307		307		307		307	

[1] According to the http://bigsdb.pasteur.fr/klebsiella/klebsiella.html database

TREATMENT **3 months** Colistin IV 9 MUI, 4.5 MUI x2/day + Fosfomycin IV 4 g x3/day + Doxycycline PO 100 mg x2/day

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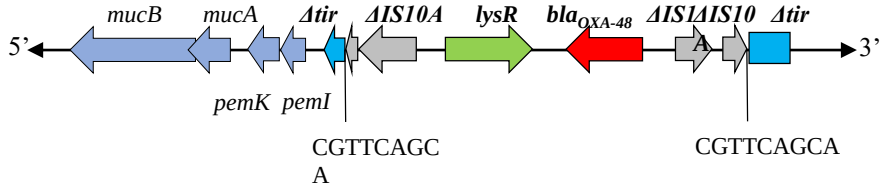
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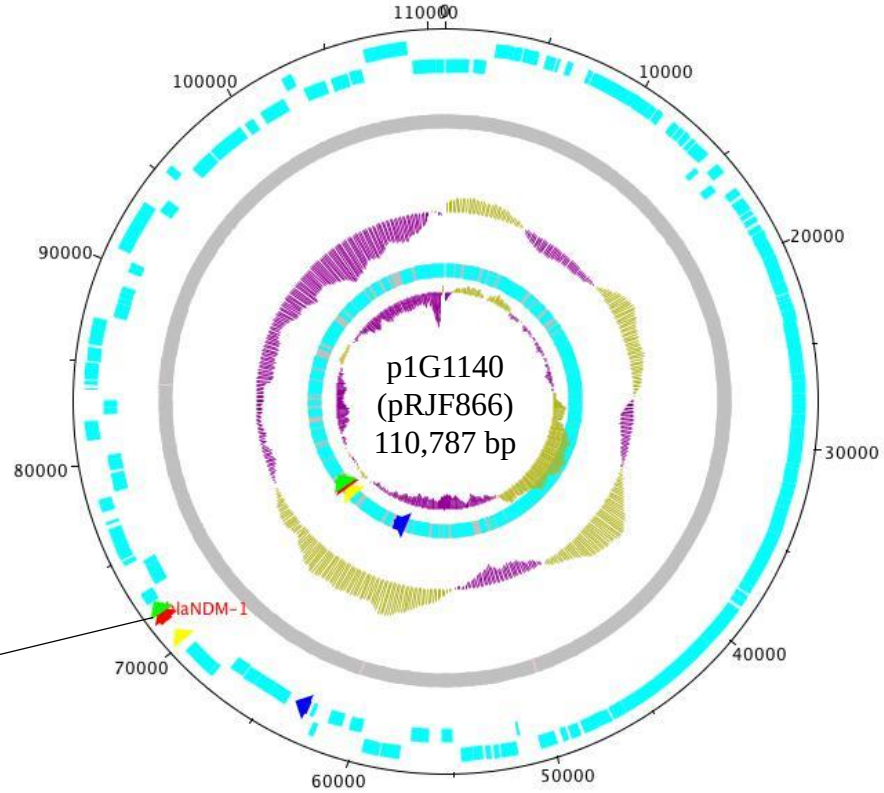
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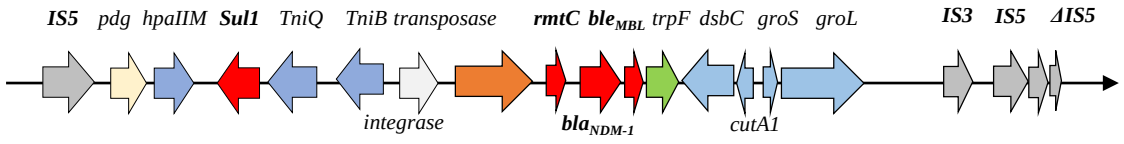
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IS5
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