Dissemination of NDM-producing *K. pneumoniae* and *E. coli* high-risk clones in Catalan healthcare institutions

Marta MARÍ-ALMIRALL[1a], Clara COSGAYA[1a], Cristina PITART[2a], Joaquim VIÑES[3,4], Laura MUÑOZ[1], Irene CAMPO[2], Anna CUSCÓ[4], Laura RODRÍGUEZ-SERNA[5], Gemina SANTANA[5], Ana DEL RÍO[6], Olga FRANCINO[3], Pilar CIRUELA[7,8], Isabel PUJOL[9,10], Frederic BALLESTER[11], Francesc MARCO[1,2], José Antonio MARTÍNEZ[6], Álex SORIANO[6], Jordi VILA[1,2] and Ignasi ROCA[1]*, on behalf of the MERCyCAT Study Group[†].

[1]Laboratory of Antimicrobial Resistance, ISGlobal, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.

[2]Department of Clinical Microbiology, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.

[3]SVGM, Molecular Genetics Veterinary Service, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.

[4]Vetgenomics, PRUAB, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.

[5]Department of Epidemiology and Preventive Medicine, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.

[6]Department of Infectious Diseases, Hospital Clínic - Institut d'investigacions Biomèdiques August Pi i Sunyer, Universitat de Barcelona, Barcelona, Spain.

[7]Public Health Agency of Catalonia (ASPCAT), Generalitat de Catalunya, Barcelona, Spain.

[8]CIBER de Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain.

[9]Laboratori de Microbiologia, Hospital Universitari Sant Joan de Reus, Reus, Spain

[10]Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Spain.

[11]Hospital Universitari Sant Joan de Reus-Laboratori de Referència del Camp de Tarragona i de les Terres de l'Ebre, Reus, Spain.

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#Address correspondence to:

Ignasi Roca, Carrer Rosselló 149-153, 1st floor (CEK building), E-08036, Barcelona, Spain. Tel.: +34 93 2275400 (Office: 4143; Lab: 2361); Fax: +34 93 3129410.

E-mail address: Ignasi.roca@isglobal.org.

[a]These authors equally contributed to this work.

[†]Other members of the MERCyCAT Study Group are listed in the Acknowledgements section.

Synopsis

Objectives

To characterise the clonal spread of carbapenem-resistant *K. pneumoniae* and *E. coli* isolates between different healthcare institutions in Catalonia, Spain.

Methods

Antimicrobial susceptibility was tested by disc-diffusion. MICs were determined by gradient diffusion or broth microdilution. Carbapenemase production was confirmed by lateral flow. PCR and Sanger sequencing were used to identify the allelic variants of resistance genes. Clonality studies were performed by PFGE and MLST. Plasmid typing, conjugation assays, S1-PFGE plus Southern blotting and MinION Oxford Nanopore sequencing were used to characterise resistance plasmids.

Results

Twenty-nine carbapenem resistant isolates recovered from three healthcare institutions between January-November 2016 were included: 14 *K. pneumoniae* isolates from a tertiary hospital in the south of Catalonia (hospital A); 2 *K. pneumoniae* isolates from a nearby healthcare centre; and 12 *K. pneumoniae* isolates and 1 *E. coli* isolate from a tertiary hospital in Barcelona (hospital B). The majority of isolates were resistant to all antimicrobial agents but colistin and all were NDM-producers. PFGE identified a major *K. pneumoniae* clone (n=27) belonging to ST147 and co-producing NDM-1 and CTX-M-15, with a few isolates also harbouring $bla_{\text{OXA-48}}$. Two sporadic isolates of *K. pneumoniae* ST307 and *E. coli* ST167 producing NDM-7 were also identified. $bla_{\text{NDM-1}}$ was carried in two related IncR plasmid populations and $bla_{\text{NDM-7}}$ in a conjugative 50 kb IncX3 plasmid.

Conclusions

We report the interhospital dissemination of XDR high-risk clones of *K. pneumoniae* and *E. coli* associated with the carriage of small, transferable plasmids harbouring bla_{NDM} genes.

Introduction

Carbapenem-resistance has been rising very rapidly during the last decades, hence reducing the available treatment options to tackle infections caused by main Gram-negative nosocomial pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and members of *Enterobacterales*, all of which are top priority pathogens according to the global priority list of antibiotic-resistant bacteria from WHO.[1] The main mechanism of carbapenem-resistance among these organisms is the production of carbapenem-hydrolysing β-lactamases, such as KPC, GES, OXA enzymes and metallo-β-lactamases (IMP, VIM or NDM among others).[2] In particular, there is great concern regarding the dissemination of NDM-producing Gram-negative bacteria as carriage of the bla_{NDM} gene is usually associated with resistance to all β-lactam antibiotics but monobactams plus co-resistance to additional antibiotic families, such as quinolones and aminoglycosides.[3,4] Since the initial identification of NDM-1 in an Swedish patient of Indian origin in 2008,[5] NDM-producing Gram-negative bacteria have been reported worldwide and up to 29 different NDM allelic variants are recorded at the NCBI reference gene catalogue (last accessed June 9th, 2020).[3,4] Among *Enterobacterales*, NDM has been described in several species but *Escherichia coli* and *Klebsiella pneumoniae* seem to be the most frequent hosts. In Spain, NDM was first described in 2011 in *E. coli* and only a few sporadic cases and outbreaks have been reported since.[6,7] Here we have examined and characterised the clonal spread of NDM-producing *K. pneumoniae* between different healthcare institutions in Catalonia, Spain.

Materials and methods

Bacterial samples

Twenty-nine carbapenem-resistant isolates from three healthcare institutions were included: 14 *K. pneumoniae* isolates recovered from January through October 2016 from a tertiary hospital in the south of Catalonia (hospital A); 12 *K. pneumoniae* isolates and 1 *E. coli* isolate recovered from July through November 2016 at a tertiary hospital in Barcelona (hospital B); and 2 *K. pneumoniae* isolates recovered in September and October 2016, respectively, from a primary healthcare centre also in the south of Catalonia. Isolates were from surveillance and clinical samples. Identification of species was performed by MALDI-TOF/MS. The clinical and microbiological data from all isolates and patients are provided in Table S1.

Susceptibility testing and resistance

Antimicrobial susceptibility was determined by disc diffusion in agar plates following EUCAST guidelines. Carbapenemase-producing *Enterobacterales* were selected according to EUCAST screening cut-off values to carbapenems.[8] Production of KPC, OXA-48-like, VIM, IMP or NDM carbapenemases was detected with the NG-Test®CARBA5 (NG-Biotech, France). MICs were determined by gradient diffusion (AB-bioMérieux, Sweden) except for susceptibility to colistin, that was determined by broth microdilution.[9] Results were interpreted according to EUCAST guidelines.[10] Isolates were categorised as MDR, XDR, or PDR according to *ad hoc* definitions.[11] *E. coli* ATCC25922 was used for quality control.

The presence of genes encoding carbapenemases,[7,12] ESBLs,[13] or 16S rRNA methyltransferases *(armA* and *rmtA-rmtH*),[14] was investigated by PCR and Sanger sequencing. Alleles were determined through sequence alignment against the NCBI reference gene catalogue (PRJNA313047, last accessed June 9, 2020).

Epidemiology and molecular typing

Clonality was studied by PFGE using *XbaI* genomic digestions and a CHEFF-DRIII system (Bio-Rad, Spain).[15] Molecular patterns were analysed with InfoQuest™FP-v.5.4 (Bio-Rad) and the unweighted pair group method with arithmetic mean to create dendrograms based on Dice's similarity coefficient, using bandwidth tolerance and optimisation values set at 1.5 and 1.2%, respectively. Isolates were considered within the same PFGE cluster (pulsotype) if their Dice similarity index was >85%.

MLST was performed according to the Pasteur scheme for *K. pneumoniae* and the Achtman scheme for *E. coli*.[16,17]

Plasmid analysis

Transferability of bla_{NDM} was studied by biparental conjugation in broth medium using azide-resistant (AzideR) *E. coli* J53 as recipient. Transconjugant strains (TC) were selected on LB agar plates containing 1 mg/L of imipenem and 100 mg/L of sodium azide. Plasmid profiling was performed by S1-nuclease digestion followed by PFGE and Southern hybridisation with digoxigenin-labelled probes against bla_{NDM}, $bla_{\text{OXA-48}}$ and $bla_{\text{CTX-M}}$.

Plasmid incompatibility groups were identified using the PBRT-2.0 kit (Diatheva, Italy).[18] Classification of IncR plasmids into IncR1 or IncR2 arbitrary groups was performed by PCR using the following primers: IS*26*_Rev, 5'-ggcactgttgcaaagttagcg-3'; IS*Aba125*_Rev, 5'-caaacatgaggtgcgacag-3'; Tn*5403*Int_Fwd, 5'-ggtttgcgtgacatcacttcg-3'; and Tn*5403*Int_Rev, 5'-ccgtgagtgtggctttagag-3'. Plasmids belonged to the IncR1 group if the PCR reaction was negative upon using primers IS*26*_Rev with IS*Aba125*_Rev, but positive when combining IS*26*_Rev with Tn*5403*Int_Rev and Tn*5403*Int_Fwd with IS*Aba125*_Rev (3 Kb and 4.3 Kb, respectively). Plasmids belonged

to the IncR2 group if positive to the IS*26*_Rev and IS*Aba125*_Rev primer combination (1.7 Kb) but negative for the other two primer pairs.

Genomic DNA extracted using the Wizard Genomic DNA purification kit (Promega, Spain) was sequenced on the MinION (Oxford Nanopore, UK) according to the manufacturer. Basecalling was done with Guppy-v3.0.3 and demultiplexing with qcat-v1.1.0 (https://github.com/nanoporetech/qcat). FASTQ files were mapped using Minimap2-v2.17 against plasmids from *Enterobacterales*.[19] Mapping reads were assembled with Flye-v2.5 (https://github.com/fenderglass/Flye). Annotation was done with Prokka-v.1.12 combined with BLASTP/BLASTN searches against the UniProtKB/Swiss-Prot and RefSeq databases.[20]

ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/), PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and ISFinder (https://www-is.biotoul.fr/) were used to identify antimicrobial resistance genes, mobile elements and plasmid replicons. Gene organisation diagrams were drawn using SnapGene®Viewer-v5.1.2 (https://www.snapgene.com/) and CGViewAdvanced-v.0.0.1.[21] Sequence comparisons were graphically displayed using Kablammo.[22]

FASTQ files of isolates HA-2, HA-3, HA-4, HB-377 and HB-536 have been deposited into the NCBI Sequence Read Archive (SRA) under accession numbers: SRR11828896, SRR11828895, SRR11828894, SRR18228893, and SRR11828892, respectively, BioProject PRJNA6346391. Plasmid annotated sequences are provided as supplementary material.

Results

Bacterial isolation and PFGE

Overall, 24 different patients were involved in the study, being either colonised (n=7) or infected (n=17), and typically presented multiple comorbidities (mostly hepatic,

pancreatic and cardiovascular diseases). Five of the infected patients died (29%). The most common treatment for infected patients was the administration of carbapenems together with tigecycline and/or colistin (Table S1).

In January 2016, a carbapenem-resistant NDM-producing *K. pneumoniae* isolate (HA-3) was recovered at a tertiary hospital (hospital A) in the province of Tarragona, Spain, from a urine sample of a patient admitted at the internal medicine ward who had just been transferred from a tertiary hospital in Barcelona (hospital D). From April through June 2016, four additional NDM-producing *K. pneumoniae* isolates were recovered from the surveillance and clinical samples of three different patients admitted at the internal medicine ward of hospital A.

At the beginning of July, an NDM-positive patient from hospital A was transferred to the liver ICU of a second university hospital in Barcelona (hospital B). The patient was isolated, and enhanced barrier precautions were implemented upon admission. Nevertheless, eleven additional NDM-producing *K. pneumoniae* isolates and one NDM-producing *E. coli* isolate were recovered from different wards from July through November.

During the same period, NDM-producing *K. pneumoniae* continued to disseminate at hospital A and eight new isolates were reported at both the internal medicine and surgery wards, two of them recovered from a newly admitted patient transferred again from hospital D. Furthermore, two additional NDM-producing *K. pneumoniae* isolates were also reported in September and October 2016, respectively, from a primary healthcare centre close to hospital A. Infection control measures and active screening of both carriers and environmental samples were intensified in all centres during this period and, at hospital B, hydrogen peroxide vaporizers were even used to decontaminate those wards

involved in the outbreak. No additional isolates were recovered after November 2016 (Figure 1).

The analysis of the 28 *K. pneumoniae* isolates by PFGE revealed the presence of two different pulsotypes, A and B (Figure 2). Most of the isolates were highly related and clustered together into pulsotype A, while pulsotype B contained a single strain recovered at hospital B (HB-536). Pulsotype A could be further subdivided into clusters A1 and A2 (20 and 7 isolates, respectively), differing by only one band and sharing 96% similarity (Figure 2). Isolates from pulsotype A1 were recovered from all three centres while those from the A2 pulsotype were exclusively from hospital A.

Antimicrobial resistance

Antimicrobial susceptibility testing showed that 25 out of the 27 pulsotype A *K. pneumoniae* isolates were XDR, only remaining susceptible to colistin. Interestingly, the two pulsotype A1 isolates recovered from the primary healthcare centre (HC-15 and HC-16) were also susceptible to all aminoglycosides and, therefore, considered MDR. The single *K. pneumoniae* isolate from pulsotype B (HB-536) as well as the NDM-producing *E. coli* isolate (HB-543) were also MDR, remaining susceptible to either amikacin, fosfomycin and colistin, or to gentamicin, amikacin, tobramycin, fosfomycin, tigecycline and colistin, respectively (Table S1).

PCR screening confirmed carriage of bla_{NDM} in all *K. pneumoniae* and *E. coli* isolates as well as $bla_{\text{CTX-M}}$-group 1 in all *K. pneumoniae* isolates. *K. pneumoniae* isolates from pulsotype A harboured the $bla_{\text{NDM-1}}$ allelic variant but both the *K. pneumoniae* isolate from pulsotype B (HB-536) and the single NDM-producing *E. coli* isolate (HB-543) carried $bla_{\text{NDM-7}}$. The β-lactamase gene $bla_{\text{TEM-1}}$ was also identified in these two isolates. DNA sequencing also confirmed the $bla_{\text{CTX-M-15}}$ allelic variant among *K. pneumoniae* isolates. Of

note, the *rmtF* gene was detected in the 25 XDR *K. pneumoniae* isolates from pulsotype A1.

All isolates were negative for bla_{KPC}, bla_{VIM} or bla_{IMP}, but four *K. pneumoniae* isolates from pulsotype A2 also carried the bla_{OXA-48} gene, encoding a class D carbapenem hydrolysing β-lactamase (Figure 2). Interestingly, isolates co-carrying OXA-48 and NDM were recovered at hospital A after the outbreak had been extended to hospital B, and the first patient at hospital A with NDM/OXA-48 (HA-4) represents a second referral from hospital D, the original source of the outbreak at hospital A (Figure 1).

Molecular typing and plasmid analysis

MLST studies identified isolates from pulsotype A as belonging to ST147, while the NDM-7-producing *K. pneumoniae* (pulsotype B) and *E. coli* isolates belonged to ST307 and ST167, respectively.

The transferability of putative plasmids harbouring bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{OXA-48} and bla_{NDM-7} was analysed by conjugation using *K. pneumoniae* producing NDM-1/OXA-48 or NDM-7 as donors. Four different types of transconjugant *E. coli* strains were obtained: TC1, carrying bla_{NDM-1}, $bla_{CTX-M-15}$, and bla_{OXA-48}; TC2, carrying bla_{NDM-1} and $bla_{CTX-M-15}$; TC3, carrying just bla_{OXA-48}; and TC4, that only acquired bla_{NDM-7}. None of the transconjugant strains acquired the *rmtF* gene. TC1 and TC2 transconjugants acquired resistance to all β-lactam antibiotics but not to aminoglycosides, while TC3 transconjugants only showed reduced susceptibility to β-lactams, and TC4 transconjugants acquired resistance to all β-lactams but aztreonam. The MIC values and molecular characteristics of representative isolates and transconjugant strains are shown in Table 1.

These results suggested that ST147 *K. pneumoniae* isolates carried bla_{NDM-1} and $bla_{CTX-M-15}$ in the same plasmid, while bla_{OXA-48} and *rmtF* were likely to be located in two

195 separate plasmids. In contrast, *bla*$_{\text{NDM-7}}$ and *bla*$_{\text{CTX-M-15}}$ were apparently located in
196 different plasmids in the ST307 *K. pneumoniae* isolate HB-536. To corroborate these
197 results, the location of the *bla*$_{\text{NDM-1}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-7}}$ genes was
198 investigated by S1-PFGE and Southern blotting. As shown in Figures 3a and 3b, DNA
199 probes specific for *bla*$_{\text{NDM}}$ and *bla*$_{\text{CTX-M-15}}$ hybridised with the same bands in NDM-1-
200 producing *K. pneumoniae* (ST147) isolates and the corresponding transconjugant strains
201 (TC1 and TC2), while the same probes hybridised with different bands in the NDM-7-
202 producing *K. pneumoniae* (HB-536) isolate and the corresponding transconjugant strain
203 (TC4) and only the *bla*$_{\text{NDM}}$ probe hybridised against the NDM-7-producing *E. coli* isolate
204 (HB-543). In addition, DNA probes against *bla*$_{\text{OXA-48}}$ hybridised with a smaller band in
205 an isolate coproducing NDM-1/OXA-48 (HA-4) and the same band was also identified
206 in the corresponding OXA-48 (TC3) or NDM-1/OXA-48 (TC1) transconjugant strains
207 (Figure 3c).
208 PBRT showed that *K. pneumoniae* isolates producing NDM-1 or NDM-1/OXA-48 were
209 positive for IncR and IncFII$_{\text{S}}$ or IncR, IncFII$_{\text{S}}$ and IncL plasmid replicons, respectively,
210 while the corresponding *E. coli* transconjugants presented the following plasmid
211 replicons: TC1 (NDM-1/OXA-48), positive for IncR and IncL; TC2 (NDM-1), positive
212 for IncR; and TC3 (OXA-48), positive for IncL. The *K. pneumoniae* isolate producing
213 NDM-7 (HB-536) presented IncX3, IncFIB-KN and IncFII$_{\text{k}}$ plasmid replicons but only
214 the IncX3 replicon was transferred to the *E. coli* transconjugant (TC4). Likewise, the
215 single *E. coli* clinical isolate producing NDM-7 also harboured an IncX3 plasmid replicon
216 (Table 1).
217 **Plasmid sequences**
218 *K. pneumoniae* isolates initially selected to characterise the plasmids harbouring *bla*$_{\text{NDM}}$
219 and *bla*$_{\text{OXA-48}}$ genes included: the first NDM-1-producing isolates at both hospital A and

hospital B (HA-3 and HB-377, respectively); the first NDM-1/OXA-48-producing isolate that was recovered at hospital A (HA-4), all of them belonging to ST147 (Figures 1 and 2); and the ST307 *K. pneumoniae* isolate producing NDM-7 (HB-536).

Genomic sequencing corroborated previous results showing that ST147 *K. pneumoniae* isolates harboured both the *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CTX-M-15}}$ genes in a single plasmid belonging to the IncR incompatibility group (Figures S1-S3). There were, however, some interesting differences. The HA-3 and HA-4 isolates, both recovered at hospital A from patients referred from hospital D, shared almost identical plasmids of circa 74 kb (pNDM-HA3 and pNDM-HA4, respectively) that carried *bla*$_{\text{NDM-1}}$ within a Tn*3000* transposon. In both plasmids the upstream IS*3000* element was partially replaced by a full length Tn*5403*, as previously described.[6] The Tn*3000* transposon was in turn flanked by two IS*26* elements in reverse orientation (Figure 4). The bulk of the remaining plasmid backbone shared high similarity (>99% average identity) with p48896_1 (CP024430), an IncR plasmid carrying *bla*$_{\text{CTX-M-15}}$ (but not *bla*$_{\text{NDM}}$) that was recently recovered from a ST147 *K. pneumoniae* isolate in Pakistan.[23]

Additional resistance genes included the aminoglycoside resistance gene *aph(3')-Ia* as well as *qnrB*, involved in low level quinolone resistance.[24] On the other hand, plasmid pNDM-HB377 (from the first isolate recovered at hospital B) presented a slightly smaller IncR plasmid of circa 67 kb, also containing both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CTX-M-15}}$. The genetic structures surrounding *bla*$_{\text{NDM-1}}$ in pNDM-HB377 were similar to those of pNDM-HA3 and pNDM-HA4, except for a 5,494 bp sequence containing Tn*5403* that was missing upstream of *bla*$_{\text{NDM-1}}$ (Figure 4). In addition, pNDM-HB377 presented an inversion of a 52 kb region containing *bla*$_{\text{CTX-M-15}}$, the IncR *repB* gene, and *mobC*, involved in plasmid mobilisation. The region containing the aminoglycoside modifying protein gene *aph(3')-Ia* was also missing (Figure 5).

These findings suggested a slightly different plasmid population between NDM-producing *K. pneumoniae* isolates from hospital A and hospital B. To corroborate such differences, specific PCR primers (see Materials&Methods) were designed to differentiate between pNDM-HA3/HA4-like plasmids (hereafter IncR1 group) and pNDM-HB377-like plasmids (hereafter IncR2 group). PCR screening showed that all NDM-1-producing *K. pneumoniae* isolates from hospital B and C carried $bla_{\text{NDM-1}}$ in a plasmid from the IncR2 group, while isolates from hospital A carried $bla_{\text{NDM-1}}$ in plasmids from either group (Figure 2). Sequence analysis of yet another IncR2 plasmid (pNDM-HA2) but from an isolate at hospital A, also yielded a plasmid of roughly 67 Kb almost identical to pNDM-HB377 (Figure S4). Interestingly, isolates carrying the IncR2 group plasmid belonged to pulsotype A1, while isolates harbouring the IncR1 group belonged to pulsotype A2 (Figure 2).

The immediate genetic structures downstream of $bla_{\text{NDM-1}}$ were identical in these plasmids and comprised genes commonly associated with bla_{NDM} genes in *Enterobacterales* and *Acinetobacter* spp. (Figure 4).[25,26]

In addition, the HA-4 isolate harboured a second plasmid of circa 64 Kb carrying $bla_{\text{OXA-48}}$ and belonging to IncL (pOXA48-HA4) that was highly similar (>99% average identity and 100% query coverage) to the IncL pUR17313-1 plasmid (KP061858) sequenced in Portugal from an *Enterobacter cloacae* clinical isolate (Figure S5).[27] $bla_{\text{OXA-48}}$ was located within Tn*1999.2* (Figure 4) and no additional antimicrobial resistance genes were identified in pOXA48-HA4.[28]

On the other hand, the ST307 *K. pneumoniae* isolate HB-536 carried $bla_{\text{NDM-7}}$ (but not $bla_{\text{CTX-M-15}}$) in an IncX3 plasmid of 50.5 Kb (pNDM-HB536) highly similar (>98% average identity and 99% query coverage) to the pAR_0162 plasmid recovered from *E. coli* (CP021682) (Figure S6). The sequence upstream of $bla_{\text{NDM-7}}$ comprised several

partial or complete insertion sequences and transposons (again Tn*5403* and IS*3000*) among which stood out the presence of an IS*Aba125* that was truncated by an IS*5* element. The region downstream of *bla*$_{\text{NDM-7}}$ also presented the canonical *ble*, *trpF* and *dsbC* genes, but the adjacent *cutA1* gene was partially deleted upon the insertion of an IS*26* element (Figure 4). The *bla*$_{\text{NDM-7}}$ gene was the only antimicrobial resistance gene present in plasmid pNDM-HB536.

Discussion

NDM was first reported in Spain in 2011 from an *E. coli* isolate in a Spanish traveller returning from India.[7] Since then, NDM-producing *Enterobacterales* have slowly crawled into the Spanish healthcare system, initially associated with imported sporadic cases, then autochthonous and, more recently, causing small hospital outbreaks.[25,29-35]

A recent nation-wide study comparing the genome sequences of NDM-producing *K. pneumoniae* and *E. coli* isolates in Spain also suggested the interhospital and interregional spread of a few clonal lineages.[6]

Here we report the interhospital dissemination of NDM-1-producing *K. pneumoniae* isolates between at least three healthcare settings in Catalonia from January through October 2016, some isolates also co-producing OXA-48. A single clone carrying both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{CTX-M-15}}$ in the same transferable IncR plasmid and belonging to the high-risk clone ST147 was responsible for such dissemination. Nevertheless, three distinct populations of ST147 isolates were identified according to the number and types of plasmids carried.

The first population clustered together into pulsotype A2, was associated with the isolate from the index case patient at hospital A, probably acquired from a previous hospital admission (hospital D), and carried *bla*$_{\text{NDM-1}}$ within an IncR1 plasmid of circa 74 kb characterised by the presence of a Tn*5403* insertion upstream of *bla*$_{\text{NDM-1}}$ (Figure 2). The

second population is a subset of the previous one, ST147 *K. pneumoniae* isolates with the IncR1 plasmid and belonging to pulsotype A2 but co-producing OXA-48 from an IncL plasmid. This population appeared at hospital A after a second patient transfer from hospital D. These two populations only disseminated among patients from hospital A (Figures 1 and 2). Interestingly, OXA-48-producing *K. pneumoniae* in Spain is commonly found in many hospitals and associated with several lineages, including ST405, ST15, and ST101 but to our knowledge, not ST147.[12,36] Most likely, the identification of OXA-48 associated with ST147 in this study reflects the acquisition of the IncL plasmid from OXA-48-producing strains previously circulating at hospital D, the original source of the outbreak.

The third population comprises ST147 *K. pneumoniae* isolates carrying $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M-15}}$ in an IncR2 plasmid of circa 67 Kb, highly similar to IncR1 except for a 52 Kb inversion and the loss of a region upstream of $bla_{\text{NDM-1}}$ (Figures 4 and 5).

IncR2 plasmids may have resulted from homologous recombination through *ltrA* sequences at an IncR1 plasmid that developed into this particular inversion, since *ltrA* genes were located flanking the inverted region. Isolates within this population belonged to pulsotype A1 and appeared first at hospital A but further spread to other centres through the referral of patients (Figures 1 and 2).

The *K. pneumoniae* ST147 is commonly associated with carbapenem resistance and is considered one of the main high-risk clones of MDR *K. pneumoniae* currently disseminating worldwide.[37,38] In Spain, NDM-1-producing *K. pneumoniae* ST147 was first reported in 2018 causing a small outbreak at a tertiary hospital in Tenerife.[34] More recently, a retrospective surveillance study has reported sporadic IncR NDM-1-producing ST147 *K. pneumoniae* isolates in the south of Catalonia during the summer of 2016 as well as causing another small outbreak in Galicia in 2015.[6] The genetic structures

surrounding *bla*$_{\text{NDM-1}}$ in those isolates matched that of IncR1 plasmids in the present study, although the plasmid was slightly larger (110 Kb) and lacked *bla*$_{\text{CTX-M-15}}$. The index case in our study was referred from another hospital within Catalonia and, hence, it is not clear whether these ST147 isolates are related to previous outbreaks in Spain.

In our study, two sporadic isolates of *K. pneumoniae* and *E. coli* producing NDM-7 and belonging to ST307 and ST167, respectively, were identified in one of the hospitals. They were recovered from two patients that overlapped at the same ward and both carried *bla*$_{\text{NDM-7}}$ in an IncX3 plasmid of circa 50 kb, thus suggesting horizontal transfer. *E. coli* and *K. pneumoniae* isolates harbouring *bla*$_{\text{NDM-7}}$ in similar IncX3 plasmids have also been described in Spain, mainly in Madrid, where they were associated with the dissemination of *K. pneumoniae* ST437 and *Enterobacter hormaechei* in 2013 and 2016, respectively.[6,35] In Catalonia, NDM-7 has been reported twice in sporadic ST679 (2013) or ST399 (2015) *E. coli* isolates associated with IncX4 or IncX3 plasmids, respectively, both of which were recovered from patients that had just returned from Pakistan.[29,39] In our study we could not relate any of these two patients to recent travel, although one of them was originally from Pakistan. Nevertheless, it is important to highlight that *K. pneumoniae* ST307 is also one of the main MDR *K. pneumoniae* high-risk clones and *E. coli* ST167 is considered an epidemic clone commonly associated with the worldwide dissemination of IncX3 plasmids harbouring *bla*$_{\text{NDM-5}}$ and *bla*$_{\text{NDM-7}}$.[37,40]

We acknowledge several limitations in our study. First, whole genome sequencing was performed using a single Nanopore approach, while the use of a hybrid approach would have allowed for additional and more accurate comparisons to isolates from previous studies. Also, only a handful of isolates were sequenced and sequence similarity was, therefore, assumed for the remaining isolates on the basis of PFGE, MLST and conventional PCR data. Unfortunately, further WGS studies were beyond our

345 possibilities. Finally, the index case and the spread of NDM-producing *K. pneumoniae* at
346 hospital D was not known, although we are aware that an investigation is currently on-
347 going at said hospital and will be made available in due time.
348 Overall, the results presented here report the dissemination of XDR and MDR high-risk
349 clones of *K. pneumoniae* and *E. coli* among different hospitals in Catalonia, whose
350 success in the clinical setting is likely related to the carriage of small, transferable
351 plasmids, harbouring bla_{NDM} genes that provide resistance to last-resort antimicrobials.
352 In Spain, such XDR clones are increasingly being reported in outbreak situations and
353 autochthonous dissemination, and our results reinforce previous findings suggesting the
354 national spread of NDM-producing *K. pneumoniae* associated with a few clonal lineages.
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361 **Other members of the MERCyCAT Study Group**
362 Pepa Pérez Jove, Emma Padilla and Mónica Ballestero-Téllez (Catlab, Centre analítiques
363 Terrassa AIE), Yuliya Zboromyrska, Miguel Ángel Benítez, Raquel Clivillé, Sabina
364 González and Iolanda Calvet (Consorci del Laboratori Intercomarcal de l'Alt Pendès,
365 l'Anoia i el Garraf), Carmen Gallés (Corporació de Salut del Maresme i la Selva), Goretti
366 Sauca (Hospital de Mataró), Carmina Martí-Sala and Mª Angeles Pulido (Hospital
367 General de Granollers), Anna Vilamala (Hospital General de Vic), Araceli González-
368 Cuevas, (Hospital General del Parc Sanitari Sant Joan de Déu), Amadeu Gené (Hospital
369 Sant Joan de Déu de Barcelona), Gloria Trujillo and Joan Lopez Madueño (Hospital Sant

Joan de Déu de Manresa), Xavier Raga (Hospital Sant Pau I Santa Tecla), Frederic Gómez, Ester Picó and Carolina Sarvisé (Hospital Universitari Joan XXIII de Tarragona), and Xesca Font (Hospital Universitari Sant Joan de Reus).

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Ethics Statement

Bacterial samples studied here were recovered from clinical samples used for microbiological diagnosis at clinical microbiology laboratories. Informed consent was, therefore, not required. The protocol for this study was approved by the Ethics Committee

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Transparency declarations

None to declare.

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513 **Figure Legends**

514 **Figure 1.** Temporal and spatial distribution of NDM-producing isolates recovered from
515 patients at three healthcare institutions in Catalonia from January through November
516 2016. Coloured boxes show the different wards hosting the patients at the time of
517 isolation. Bacterial isolates are represented by an inverted triangle (*K. pneumoniae*) or a
518 circle (*E. coli*). Colour codes indicate carriage of different combinations of β-lactamases
519 and IncR or IncX3 plasmids. Isolates recovered from patients referred from hospital D
520 are shown with an asterisk (*). The transfer of patients between the three healthcare
521 centres is represented with a dotted line. NS indicates isolates selected for Nanopore
522 sequencing. Superscript numbers indicate isolates recovered from the same patient (Table
523 S1).

524 **Figure 2.** PFGE dendrogram of NDM-producing *K. pneumoniae* isolates from three
525 healthcare centres in Catalonia (HA: hospital A; HB: hospital B; and HC: primary
526 healthcare centre). Other: Carriage of additional β-lactamases; PT: pulsotype. ST:
527 sequence type. PG: Plasmid incompatibility group of plasmids harbouring bla_{NDM}; NS:
528 Isolates selected for Nanopore sequencing. Braces indicate classification to the
529 corresponding PFGE cluster, or pulsotype. Isolates were included in the same pulsotype
530 if their Dice similarity index was ≥ 85%. Superscript numbers indicate isolates recovered
531 from the same patient (Table S1).

532 **Figure 3.** S1-PFGE and Southern hybridisation of *K. pneumoniae* or *E. coli* isolates
533 harbouring different combinations of β-lactamases. a) Hybridisation with the bla_{NDM} probe.
534 b) Hybridisation with the $bla_{CTX\text{-}M\text{-}15}$ probe. c) Hybridisation with the $bla_{OXA\text{-}48}$ probe. Braces
535 indicate molecular detection of different *bla* genes. Transconjugants are shown in blue.
536 Arrows indicate weak bands. Of note, mirroring double bands were attributed to

incomplete cleavage by the S1 nuclease so that other conformations of the plasmid rather than the linear one are also seen.

Figure 4. Schematic drawing showing the genetic elements surrounding the bla_{NDM} genes in pNDM-HA3, pNDM-HB377, and pNDM-HB536 as well as the bla_{OXA-48} gene in pOXA48-HA4. Arrows are proportional to the lengths of the genes and are oriented in the direction of transcription. Red arrows represent resistance genes, orange arrows represent full-length transposon-related genes and ISs, blue arrows represent partial or truncated transposon-related genes and ISs, and purple arrows are used for the remaining set of genes.

Figure 5. Graphical comparison of plasmids pNDM-HA3 and pNDM-HB377 in either: a) parallel orientation or b) antiparallel orientation (reverse complement sequence of pNDM-HB377). The shaded stripes show regions shared between the two plasmids. The location of the *ltrA* sequences flanking a 52 kb inversion is shown.

Tables

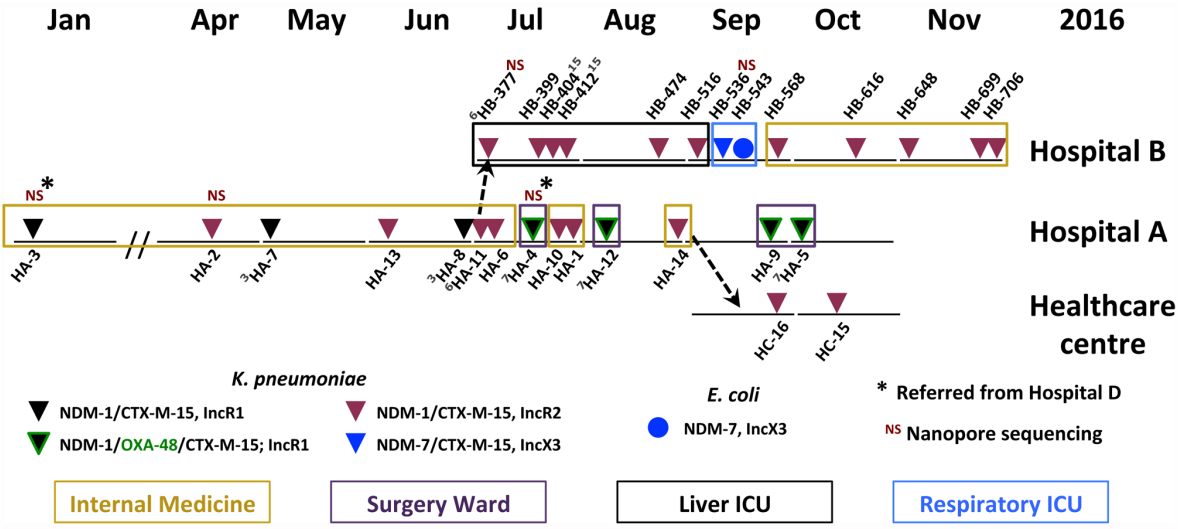
Table 1. Antimicrobial susceptibility and molecular characterisation of representative *K. pneumoniae* and *E. coli* isolates and their corresponding transconjugant *E. coli* strains (*E. coli* J53AzideR strain was used as recipient).

Strain	Sp.	MIC (mg/L)															PT	ST	NDM	Other	*rmtF*	PG
		IPM	MEM	FOX	FEP	CTX	CAZ	ATM	GEN	AMK	TOB	CIP	LVX	TGC	CST[a]	FOF						
HA-3	*Kp*	>32	>32	>256	>256	>32	>256	>256	>256	>256	>256	>32	>32	2	0.25	64	A2	ST147	1	CTX-M-15	+	R1
HA-2	*Kp*	>32	>32	>256	>256	>32	>256	>256	>256	>256	>256	>32	>32	2	0.125	48	A1	ST147	1	CTX-M-15	+	R2
HB-377	*Kp*	>32	>32	>256	>256	>32	>256	>256	>256	>256	>256	>32	>32	3	0.25	48	A1	ST147	1	CTX-M-15	+	R2
HA-4	*Kp*	>32	>32	>256	>256	>32	>256	>256	>256	>256	>256	>32	>32	2	0.125	64	A2	ST147	1	OXA-48 CTX-M-15	+	R1/L
HC-16	*Kp*	>32	>32	>256	>256	>32	>256	>256	2	2	6	>32	>32	4	0.25	48	A1	ST147	1	CTX-M-15	-	R2
HB-536	*Kp*	>32	>32	>256	256	>32	>256	48	24	4	8	>32	>32	4	0.25	12	B	ST307	7	TEM-1 CTX-M-15	-	X3
HB-543	*Ec*	>32	>32	>256	>256	>32	>256	>256	0.25	1	0.38	>32	>32	0.75	0.25	0.38	NA	ST167	7	TEM-1	-	X3
TC1	*Ec*	16	4	>256	32	>32	>256	32	0.125	0.5	0.38	0.125	0.25	0.25	0.125	1	NA	NA	1	OXA-48 CTX-M-15	-	R1/L
TC2	*Ec*	12	2	>256	16	>32	>256	12	0.25	0.5	0.25	0.125	0.38	0.38	0.125	1	NA	NA	1	CTX-M-15	-	R1
TC3	*Ec*	0.5	0.19	4	0.64	0.5	0.19	0.094	0.38	1.5	0.25	0.08	0.023	0.19	0.125	1	NA	NA	-	OXA-48	-	L
TC4	*Ec*	>32	>32	>256	8	>32	>256	0.125	0.38	1.5	0.25	0.012	0.016	0.125	0.125	1	NA	NA	7	-	-	X3
J53Az^{R}	*Ec*	0.19	0.016	4	0.023	0.016	0.047	0.125	0.064	0.75	0.094	0.008	0.016	0.094	0.125	0.094	NA	NA	-	-	-	NA

Sp.: Bacterial species; *Kp: K. pneumoniae*; *Ec: E. coli*; MIC: minimum inhibitory concentration; IPM: imipenem; MEM: meropenem; FOX: cefoxitin; FEP: cefepime; CTX: cefotaxime; CAZ: ceftazidime; ATM: aztreonam; GEN: gentamicin; AMK: amikacin; TOB: tobramycin; CIP:

ciprofloxacin; LVX: levofloxacin; TGC: tigecycline; CST: colistin; FOF: fosfomycin; PT: pulsotype; ST: sequence type; NDM: presence of bla_{NDM-1} or bla_{NDM-7}; Other: presence of additional *bla* genes; *rmtF*: presence of the *rmtF* gene; PG: plasmid incompatibility group of plasmids carrying bla_{NDM} and bla_{OXA-48}; [a] Colistin MIC was determined by broth microdilution. NA: not applicable.

561 **Figure 1**



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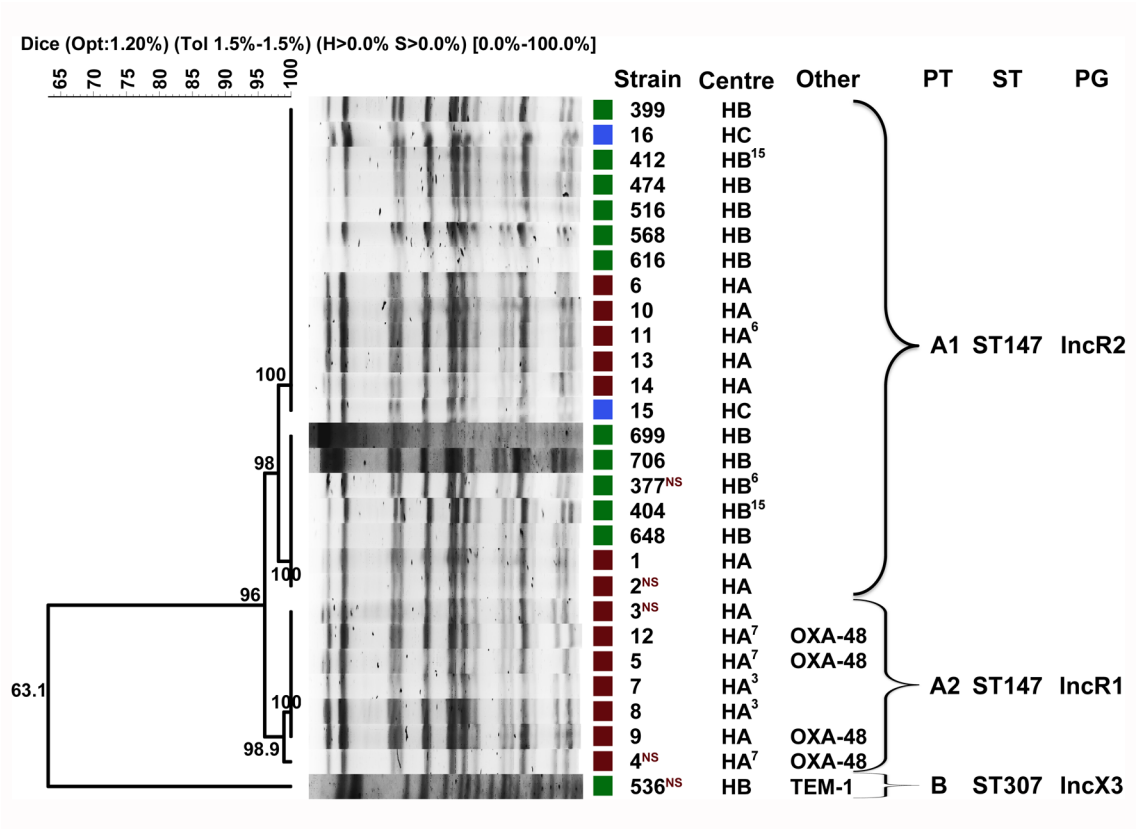
563 **Figure 2**



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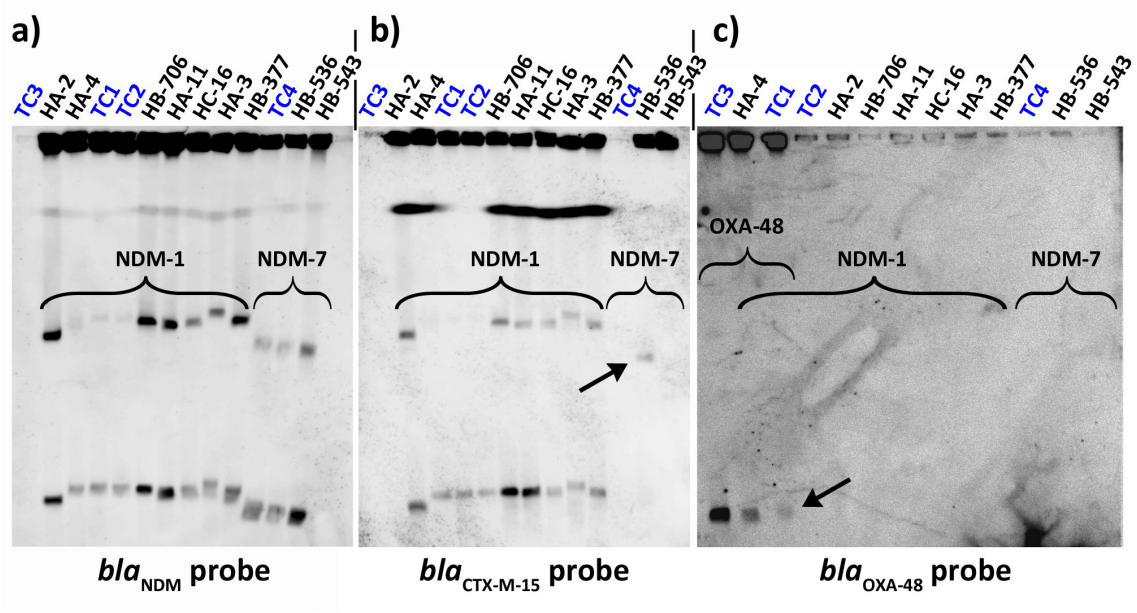
565

566 **Figure 3**



567

568 **Figure 4**

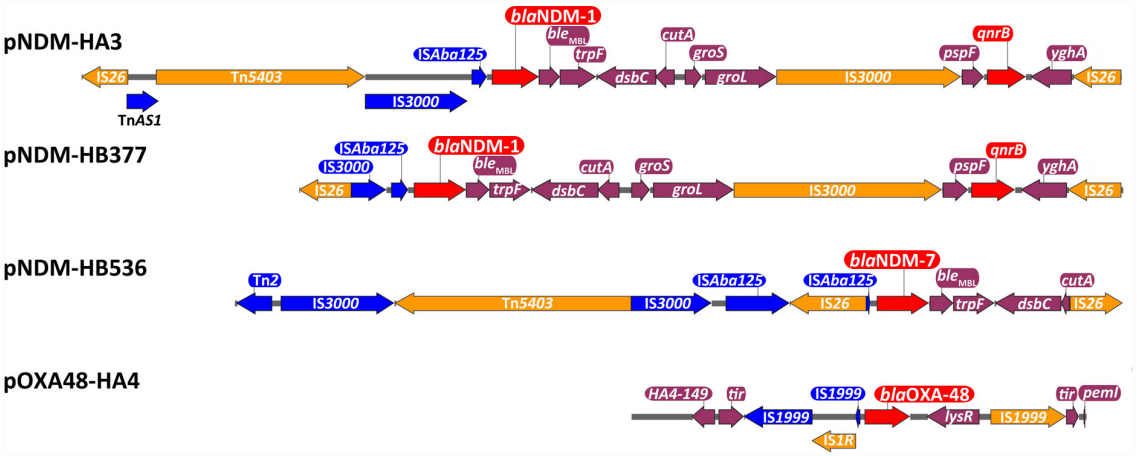


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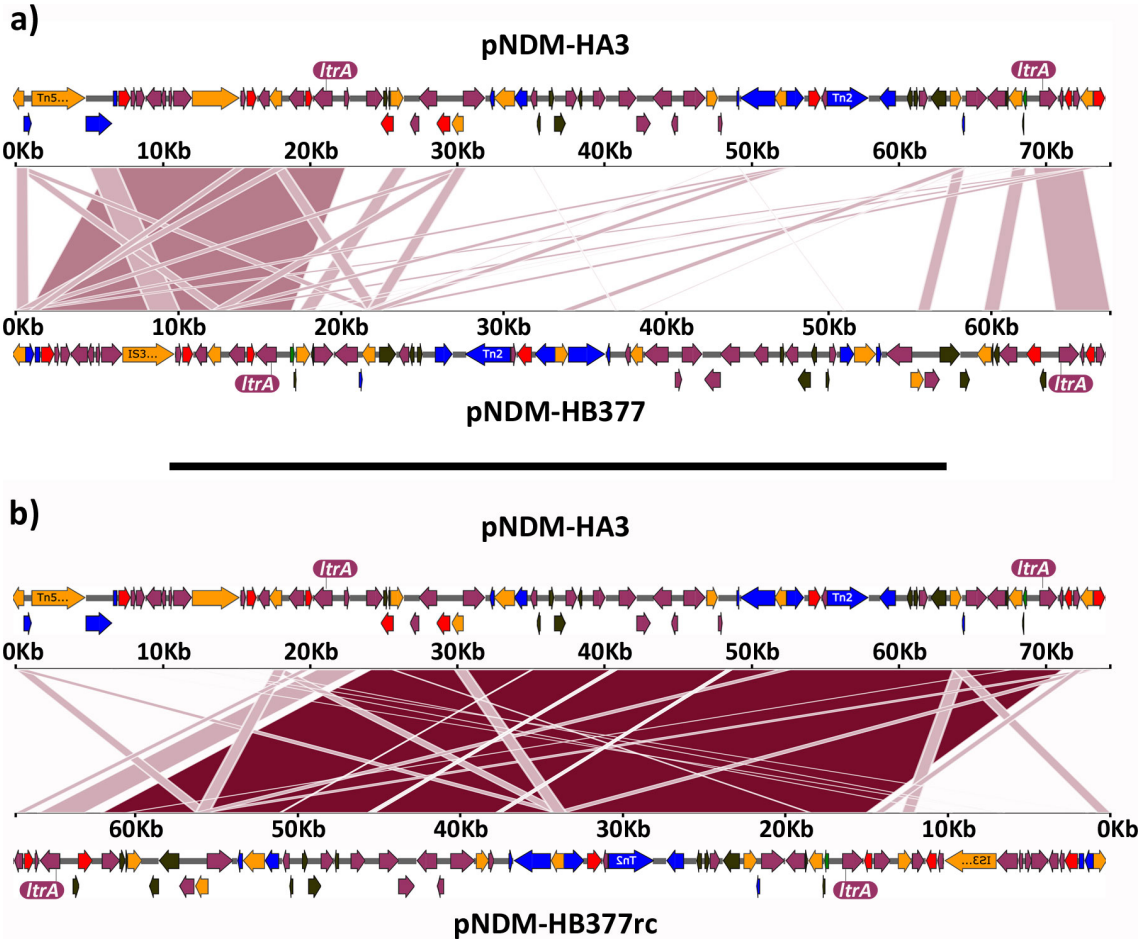
Figure 5


a)
pNDM-HA3
ltrA
Tn5...
Tn2
0Kb
10Kb
20Kb
30Kb
40Kb
50Kb
60Kb
70Kb
IS3...
pNDM-HB377
b)
pNDM-HA3
pNDM-HB377rc